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Gcn4 Is Required for the Response to Peroxide Stress in the Yeast *Saccharomyces cerevisiae*

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An oxidative stress occurs when reactive oxygen species overwhelm the cellular antioxidant defenses. We have examined the regulation of protein synthesis in *Saccharomyces cerevisiae* in response to oxidative stress induced by exposure to hydroperoxides (hydrogen peroxide, and cumene hydroperoxide), a thiol oxidant (diamide), and a heavy metal (cadmium). Examination of translational activity indicates that these oxidants inhibit translation at the initiation and postinitiation phases. Inhibition of translation initiation in response to hydroperoxides is entirely dependent on phosphorylation of the α subunit of eukaryotic initiation factor (eIF)2 by the Gcn2 kinase. Activation of Gcn2 is mediated by uncharged tRNA because mutation of its HisRS domain abolishes regulation in response to hydroperoxides. Furthermore, Gcn4 is translationally up-regulated in response to H_2O_2, and it is required for hydroperoxide resistance. We used transcriptional profiling to identify a wide range of genes that mediate this response as part of the Gcn4-dependent H_2O_2-regulon. In contrast to hydroperoxides, regulation of translation initiation in response to cadmium and diamide depends on both Gcn2 and the eIF4E binding protein Eap1. Thus, the response to oxidative stress is mediated by oxidant-specific regulation of translation initiation, and we suggest that this is an important mechanism underlying the ability of cells to adapt to different oxidants.

INTRODUCTION

Organisms are exposed to reactive oxygen species (ROS) during the course of normal aerobic metabolism or after exposure to radical-generating compounds. ROS cause wide-ranging damage to macromolecules, eventually leading to cell death (Halliwell and Gutteridge, 1989; Gutteridge, 1994). To protect against oxidant damage, cells contain effective defense mechanisms, including antioxidant enzymes and free radical scavengers (Temple *et al*., 2005). It is now well-established that yeast cells can adapt to an oxidative stress by altering global transcription patterns, including genes encoding antioxidants and other metabolic enzymes (Gasch *et al*., 2000; Causton *et al*., 2001). Additionally, they respond by invoking complex regulatory mechanisms that include global inhibition of translation. For example, we have shown that exposure of yeast cells to hydrogen peroxide results in a rapid and reversible inhibition of protein synthesis (Shenton *et al*., 2006). This reduction in protein synthesis may allow time for the turnover of existing mRNAs and proteins while gene expression is reprogrammed to deal with the stress.

The initiation phase of protein synthesis is the main target of regulation, and it represents a key control point for eukaryotic gene expression (Hinnebusch, 2000). Phosphorylation of eukaryotic initiation factor (eIF) 2 is important for this control in response to diverse stress conditions. eIF2 is a guanine nucleotide binding factor, which in its GTP-bound form interacts with the initiator methionyl-tRNA (Met-$tRNA_i^{Met}$) to form a ternary complex that is competent for translation initiation. After each round of initiation, eIF2 is released from the ribosome as a binary complex with guanosine diphosphate (GDP). GDP is replaced by GTP in a guanine-nucleotide exchange reaction catalyzed by eIF2B. Met-$tRNA_i^{met}$ can only bind the eIF2/GTP complex, so translational control can be regulated by the activity of eIF2B. In both yeast and mammals, this is achieved by phosphorylation of the α subunit of eIF2 at a conserved serine (Ser51) residue (Pavitt *et al*., 1998; Harding *et al*., 2000). Phosphorylation converts eIF2 from a substrate to a competitive inhibitor of the guanine nucleotide exchange factor eIF2B and the resulting decrease in eIF2B activity leads to reduced ternary complex levels, which inhibits translation initiation (Pavitt *et al*., 1998).

Four mammalian kinases have been identified that inhibit translation initiation by phosphorylating eIF2α. These eIF2α kinases are regulated independently in response to various different cellular stresses (Dever, 2002; Proud, 2005). For example, PKR-like endoplasmic reticulum eIF2α kinase (PERK) has been found in all multicellular eukaryotes, and it is a component of the unfolded protein response. Consistent with its central role in the endoplasmic reticulum (ER) stress response, cells lacking PERK fail to phosphorylate eIF2α, and they do not down-regulate protein synthesis during ER stress conditions (Bertolotti *et al*., 2000). Attenuating protein synthesis may act to reduce the burden of newly synthesized ER client proteins on the ER folding machinery. Additionally, eIF2α phosphorylation induces translation of specific mRNAs, such as that encoding the metazoan activating transcription factor 4 (*ATF4*) (Lu *et al*., 2004; Vattem and Wek, 2004). *ATF4* mediates the "integrated stress response" whose targets include genes encoding proteins involved in

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amino acid metabolism and resistance to oxidative stress, ultimately protecting against the deleterious consequences of ER oxidation (Harding *et al*., 2003).

In the yeast *Saccharomyces cerevisiae*, Gcn2 is the sole eIF2α kinase (Dever, 2002). It is activated in response to a variety of conditions, including nutrient starvation (amino acids, purines, and glucose) and exposure to sodium chloride, rapamycin, and volatile anesthetics (Hinnebusch, 2005; Palmer *et al*., 2005). Depletion of amino acids leads to an accumulation of uncharged tRNA, which activates the Gcn2 protein kinase via its HisRS-related domain. It is likely that other stress conditions ultimately affect the levels of uncharged tRNA in the cell. For example, volatile anesthetics inhibit amino acid uptake (Palmer *et al*., 2005), and Gcn2 is activated by glucose starvation partly through an effect on vacuolar amino acid pools (Yang *et al*., 2000). Phosphorylation of eIF2α by Gcn2 reduces global protein synthesis, but it also enhances translation of the *GCN4* mRNA. Translation of the *GCN4* mRNA is activated in response to low ternary complex levels in a mechanism involving four short upstream open reading frames (Hinnebusch, 2005). Gcn4 is itself a transcription factor that activates gene expression of many targets, including amino acid biosynthetic genes (Natarajan *et al*., 2001). Thus, analogous to the mammalian integrated stress response, activation of Gcn4 serves to overcome the imposed amino starvation, which initially led to the translational induction of *GCN4* expression.

We have previously analyzed the regulation of protein synthesis in response to oxidative stress induced by exposure to hydrogen peroxide (Shenton *et al*., 2006). H_2O_2 induces a dose-dependent inhibition of protein synthesis. This inhibition primarily occurs at the level of translation initiation, and it is regulated by Gcn2-mediated phosphorylation of eIF2α. In this current study, we have extended this analysis to include reagents that induce the formation of different ROS. This is important because an oxidative stress can be caused by many different ROS with differing reactivities. We induced oxidative stress by exposing cells to cumene hydroperoxide (CHP), cadmium sulfate (hereafter referred to as cadmium), and diamide. Our data show that phosphorylation of eIF2α is a common response that inhibits translation initiation in response to oxidative stress caused by diverse ROS, but there are additional oxidant-specific regulatory mechanisms. Furthermore, Gcn4 is specifically upregulated in response to H_2O_2 and the Gcn4-dependent H_2O_2 regulon is required for resistance to hydroperoxides, but not to other oxidants.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

The *S. cerevisiae* strains used in this study are listed in Table 1. Strains were grown in rich YEPD medium (2%, wt/vol, glucose; 2%, wt/vol, bactopeptone; and 1%, wt/vol, yeast extract) or minimal SD medium (0.17%, wt/vol, yeast nitrogen base without amino acids; 5%, wt/vol, ammonium sulfate; and 2%, wt/vol, glucose) supplemented with appropriate amino acids and bases (Sherman *et al*., 1974) at 30°C and 180 rpm. Media were solidified by the addition of 2% (wt/vol) agar. Stress sensitivity was determined by growing cells to stationary phase and spotting onto agar plates containing various concentrations of oxidants.

Plasmids

Plasmids containing *GCN2*, *gcn2-m2*, and *gcn2-S577A* were kindly provided by Dr. A. G. Hinnebusch (National Institutes of Health, Bethesda, MD), and they have been described previously (Cherkasova and Hinnebusch, 2003). Plasmids containing *EAP1* and *eap1^{m3}* have been described in Ibrahimo *et al*. (2006).

Table 1. Yeast strains used in this study

Strain	Genotype	Reference/ source
CY4	*MATa ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11 can1-100*	Grant *et al.* (1996)
CY1138	As in strain CY4 but *gcn2::URA3*	Shenton *et al.* (2006)
CY1124	As in strain CY4 but *gcn4::KanMX4*	This study
CY1388	As in strain CY4 but *eap1::KanMX4*	This study
CY1389	As in strain CY4 but *eap1::KanMX4 gcn2::URA3*	This study
SCY51	*MATa ura3-52 gcn2::KanMX4*	Rohde *et al.* (2004)
GP3007	*MATa ura3-52 leu2-3 trp1Δ63 sui2Δ p[SUI2, LEU2]*	G. Pavitt (University of Manchester)
GP3002	*MATa ura3-52 leu2-3 trp1Δ63 sui2Δ p[SUI2-S51A, LEU2]*	G. Pavitt
RJD2125	*MATa ura3-52 leu2-3 his3-11 GCN4::MYC9*	Lipford *et al.* (2005)
CY1536	As in strain RJD2125 but *gcn2::URA3*	This study

Western Blot Analysis

Protein extracts were electrophoresed under reducing conditions on SDS-polyacrylamide gel electrophoresis minigels and electroblotted onto polyvinylidene difluoride membrane (GE Healthcare, Chalfont St. Giles, United Kingdom). Blots were probed using eIF2α and phosphospecific eIF2α antibodies as described previously (Holmes *et al*., 2004). Myc-tagged proteins were detected using anti-myc antibodies (Roche Applied Science, Indianapolis, IN).

Analysis of Protein Synthesis

The rate of protein synthesis was measured in exponential phase cells treated with various concentrations of cumene hydroperoxide, diamide, or cadmium sulfate. Cells were treated with oxidants for 15 min and pulse-labeled for the last 5 min of the treatment with 85 μM L-[^{35}S]cysteine/methionine as described previously (Shenton and Grant, 2003). For the analysis of ribosome distribution on sucrose density gradients, yeast cultures were grown to exponential phase and treated with oxidants for 15 min. Extracts were prepared in 100 μg of cycloheximide/ml and layered onto 15–50% sucrose gradients. The gradients were sedimented via centrifugation at 40,000 rpm in a Beckman ultracentrifuge for 2.5 h, and the A_{254} measured continuously to give the traces shown, as described previously (Ashe *et al*., 2000). Monosome and polysome peaks were quantified using the National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/).

Microarray Hybridizations and Data Analysis

Yeast cells were grown in triplicate to midexponential phase in minimal SD media. The preparation of RNA, probes, and hybridization to whole yeast genome microarrays (Yeast Genome 2.0 array) was performed as described previously (Wishart *et al*., 2005). The complete data sets are publicly available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress; accession number E-MEXP-998). Arrays that passed outlier data-quality assessment using dChip software (Li and Wong, 2001) were normalized with robust multichip average (Bolstad *et al*., 2003). Statistical tests were performed with limma by using the lmFit and eBayes functions (Smyth, 2004). Gene lists of differentially expressed genes were controlled for false discovery rate (fdr) errors by using the method of QVALUE using default settings (Storey and Tibshirani, 2003). Microarray statistical procedures were performed with Bioconductor (Gentleman *et al*., 2004). Principal components analysis was performed using a covariance dispersion matrix with Partek Genomics Suite, version 6.3 (Partek, St. Charles, MO). Clustering was performed using a k-means clustering algorithm ("Slope" similarity metric "Super Grouper" plugin of maxdView software (available from http://bioinf.man.ac.uk/microarray/maxd/). Clustering was performed on the means of each sample group (log 2) that had been z-transformed (for each probe set the mean set to zero, SD to 1). For each cluster, functional enrichment was determined using FunSpec (Robinson *et al*., 2002). Data were verified by real-time reverse transcription-polymerase chain reaction (RT-PCR) by using the MyIQ single-color real-time PCR detection system and iScript SYBR Green Supermix (Bio-Rad Laboratories).

We compared our data set of genes that were differentially expressed in response to H_2O_2 in wild-type versus the *gcn4* deletion mutant with amino

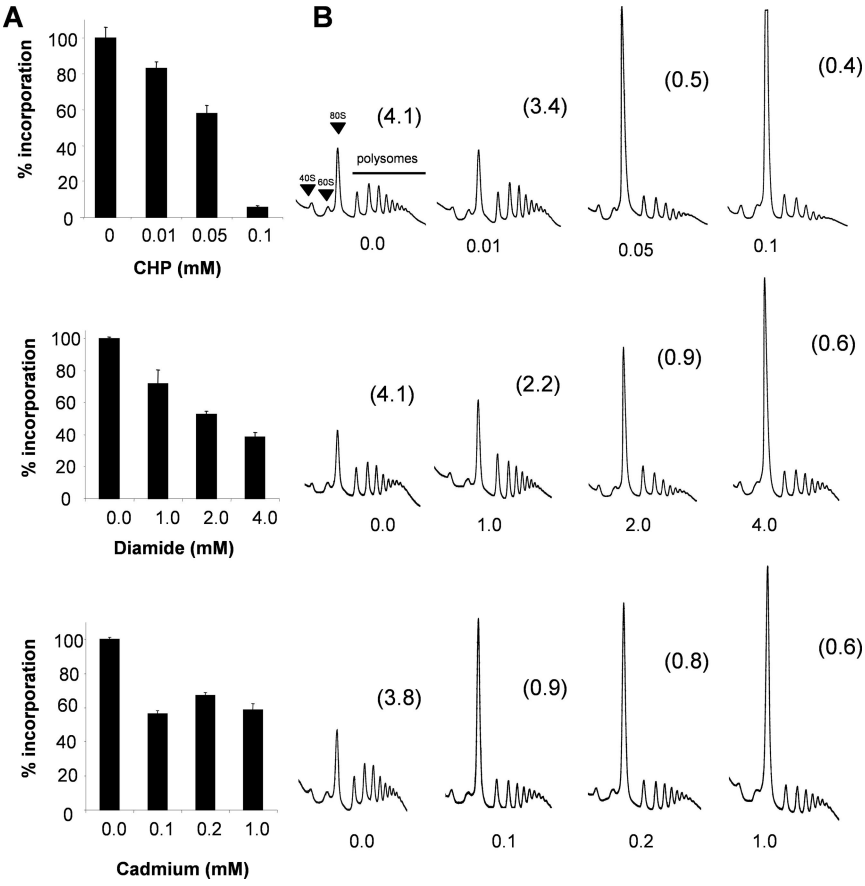

Figure 1. Inhibition of protein synthesis is a common response to oxidative stress. (A) Wild-type cells were grown to exponential phase in minimal SD media, and protein synthesis was measured by pulse labeling with [^{35}S]cysteine/methionine for 5 min. Data are shown for untreated cultures (100%) and after treatments with CHP, diamide, or cadmium for 15 min. (B) Oxidative stress inhibits translation initiation. Polyribosome traces are shown for the wild-type strain treated with the indicated concentrations of oxidants for 15 min. The peaks that contain the small ribosomal subunit (40S), the large ribosomal subunit (60S), and both subunits (80S) are indicated by arrows. The polysome peaks generated by 2, 3, 4, 5, etc., 80S ribosomes on a single mRNA are marked with a line. Numbers in brackets are the p:m ratio determined by the ratio between the area under the monosome to the polysome peaks. Representative data are shown from repeat experiments.

acid starvation microarray data published by Natarajan *et al*. (2001). Because the input for QVALUE is simply a list of p values, it was possible to calculate q values for the microarray data of Natarajan *et al*. (2001) by using their own p values. Given the array platform differences, we only considered data where the gene is present on both array platforms.

RESULTS

Inhibition of Translation Initiation Is a Common Response to Oxidants

Protein synthesis was examined to determine whether translation inhibition is a common response to oxidative stress induced by different oxidants. Oxidative stress was induced by exposure to CHP, cadmium, or diamide. Cells were treated with various concentrations of oxidants for 15 min, and the rate of protein synthesis was measured during the final 5 min by the incorporation of [^{35}S]cysteine/methionine. Similar to H_2O_2, the aromatic hydroperoxide CHP caused a dose-dependent inhibition of protein synthesis at concentrations up to 0.1 mM (Figure 1A). Inhibition was observed at relatively low concentrations compared with H_2O_2, which maximally inhibited protein synthesis at concentrations of ~1 mM (Shenton *et al*., 2006). Exposure to diamide caused a dose-dependent inhibition of protein synthesis at concentrations between 1 and 4 mM (Figure 1A). Exposure to the heavy metal cadmium also inhibited protein synthesis with ~50% inhibition observed at concentrations between 0.1 and 1.0 mM (Figure 1A). In contrast to the other oxidants, inhibition with cadmium was not dose dependent. The reason for this is unclear, but it may reflect differences in the way in which cadmium is taken up by cells or is detoxified, compared with other oxidants. For example, treatments with different doses of cadmium may result in similar intracellular concentrations of cadmium because cadmium requires active uptake, compared with other oxidants such as hydrogen peroxide, which are freely diffusible.

Translational activity was further analyzed by examining the distribution of polysomes in response to oxidants. Polysomes are ribosomes that are actively translating mRNAs; they can be separated on sucrose density gradients and quantified by measuring absorbance at 254 nm. Extracts prepared from the untreated strain exhibited normal profiles, including peaks corresponding to 40S and 60S ribosomal subunits, monosomes (80S ribosomes), and polysomes. There was a dramatic shift of ribosomes from the polysomal region into the monosome or 80S peak after treatment with all three oxidants, which is indicative of decreased translation initiation (Figure 1B). Interestingly, inhibition of translation initiation did not increase in response to CHP at concentrations between 0.05 and 0.1 mM, despite a greatly reduced rate of protein synthesis as measured by radiolabeling (Figure 1A). These data provide that first indication that similar to hydrogen peroxide, CHP inhibits translation at the postinitiation phase. The addition of cycloheximide to the cultures during the stress treatments maintained the polysomes, indicating that inhibition is not due to mRNA degradation (data not shown). Together, these data indicate that inhibition of translation initiation is a common response to oxidative stress induced by exposure to diverse oxidants.

Oxidative Stress Induces Gcn2-dependent eIF2α Phosphorylation

Oxidative stress caused by exposure to H_2O_2 induces Gcn2-mediated phosphorylation of eIF2α (Shenton *et al*., 2006). To

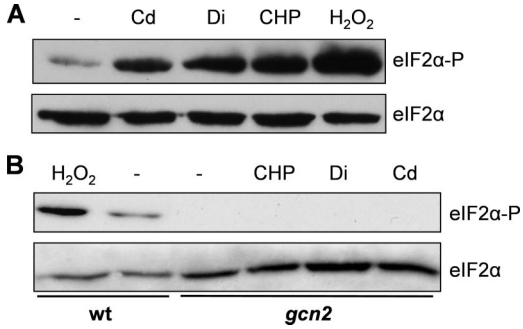

Figure 2. Phosphorylation of eIF2α in response to oxidative stress. (A) Western blot analysis of eIF2α and eIF2α-P. The wild-type strain was grown to exponential phase in minimal SD media and treated with 0.5 mM H_2O_2, 0.1 mM CHP, 0.2 mM cadmium, or 4.0 mM diamide for 15 min. (B) Phosphorylation is dependent on the presence of *GCN2*. A *gcn2* mutant was exposed to the same oxidant treatments as described above. Representative data are shown from repeat experiments.

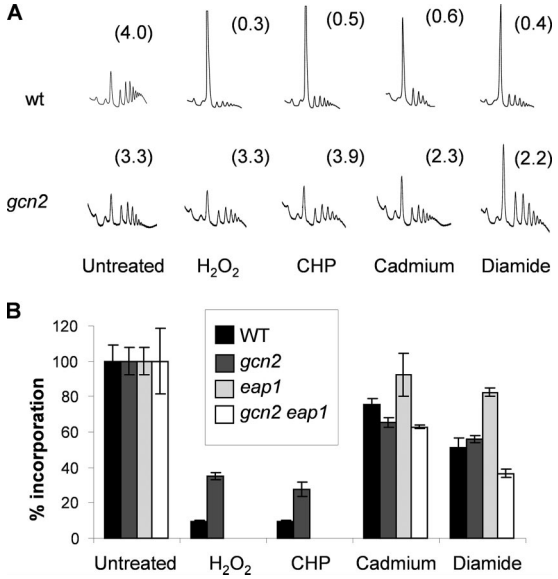

Figure 3. Gcn2 mediates an inhibition of translation initiation in response to different oxidants. (A) Polysome traces are shown for the wild-type and *gcn2* mutant strain after treatments with oxidants as described for Figure 2A. Numbers in brackets are the p:m ratio. (B) Wild-type and *gcn2* mutant cells were grown to exponential phase, treated with oxidants as described for Figure 2A, and protein synthesis measured as described for Figure 1A. Representative data are shown from repeat experiments.

test whether the translational inhibition observed in response to other oxidants relies upon this pathway, we examined eIF2α phosphorylation by immunoblot analysis. Oxidant concentrations were chosen that caused a substantial redistribution of polysome profiles (0.1 mM CHP, 0.2 mM cadmium, and 4.0 mM diamide). An increase in phosphorylation was observed in response to all three oxidants compared with the untreated control (Figure 2A). No phosphorylation of eIF2α was observed in response to any of the oxidants in a *gcn2* mutant (Figure 2B), confirming that Gcn2-mediated phosphorylation of eIF2α is a common response to oxidative stress.

Polysome analysis revealed that Gcn2 mediates the inhibition of translation initiation in response to hydroperoxides because no inhibition was observed in the *gcn2* mutant with H_2O_2 or CHP (Figure 3A; compare polysome:monosome ratios). Additionally, less inhibition of protein synthesis was observed in the *gcn2* mutant after treatments with H_2O_2 or CHP (Figure 3B). The rate of protein synthesis was increased from ~10% in the wild-type strain to ~35% in the *gcn2* mutant after treatments with H_2O_2 or CHP. Protein synthesis was, however, still inhibited in the *gcn2* mutant, despite their being no inhibition of translation initiation detected by polysome analysis. This is consistent with our previous observation that H_2O_2 inhibits translation at both the initiation and postinitiation (elongation or termination) phases of protein synthesis (Shenton *et al*., 2006). These data indicate that Gcn2 is required for the inhibition of translation initiation in response to peroxides, but translation is still inhibited at the postinitiation phase in a *gcn2* mutant.

The inhibition of translation initiation after treatments with cadmium or diamide was also largely abrogated in the *gcn2* mutant (Figure 3A). However, in contrast to the response to peroxides, a shift of ribosomes from the polysomal region into the monosome or 80S peak was still observed in the *gcn2* mutant (Figure 3A). Quantification of polysome traces revealed that the ratio of polysomes:monsomes (p:m) was decreased by ~90% in the wild-type strain compared with an approximate 33% reduction in the *gcn2* mutant in response to cadmium or diamide (Figure 3A). These data indicate that Gcn2 is the major factor that inhibits translation initiation in response to cadmium or diamide, but translation initiation can still be inhibited in response to these oxidants in a *gcn2* mutant. Furthermore, little or no restoration of protein synthesis was observed in the *gcn2* mutant treated with cadmium or diamide compared with the untreated condition (Figure 3B). These data indicate that in contrast to hydroperoxides, cadmium and diamide inhibit translation initiation via both a Gcn2-dependent and Gcn2-independent mechanism.

Activation of Gcn2 in Response to Oxidative Stress Conditions

Gcn2 can be activated in response to diverse stress conditions. Depletion of amino acids leads to an accumulation of uncharged tRNA, which activates the Gcn2 protein kinase via its HisRS-related domain. Alternatively, rapamycin stimulates eIF2α phosphorylation by Gcn2 via dephosphorylation of Ser577 in Gcn2 (Hinnebusch, 2005). To determine whether oxidants activate Gcn2 via these same pathways, we analyzed translational activity in strains containing a mutation in the HisRS domain that abolishes tRNA binding (*gcn2-m2*; Garcia-Barrio *et al*., 2002) or containing a mutation in Ser577 (*gcn2-S577A*; Cherkasova and Hinnebusch, 2003). The Ser577 mutant has been shown to dampen the effects of rapamycin on eIF2α phosphorylation, suggesting that Gcn2 activation by rapamycin involves Ser577 dephosphorylation. Translation inhibition in response to H_2O_2 or CHP was unaffected by mutation of Ser577 (Figure 4). In contrast, no inhibition was observed in response to hydroperoxides in the *gcn2-m2* mutant, indicating that hydroperoxides activate Gcn2 via binding of uncharged tRNA. Similarly, immunoblot analysis revealed that the phosphorylation of eIF2α observed in response to hydroperoxides is dependent on the *m2* motif but is unaffected by mutation of Ser577 (Figure 5A). The experiments shown in Figures 4 and 5A were performed using a prototrophic strain (SCY51), ruling out any amino acid starvation arising as a result of inhibiting the uptake of an auoxtrophic amino acid.

Analysis of the *gcn2-m2* and *gcn2-S577A* mutants provided further evidence that cadmium and diamide can in-

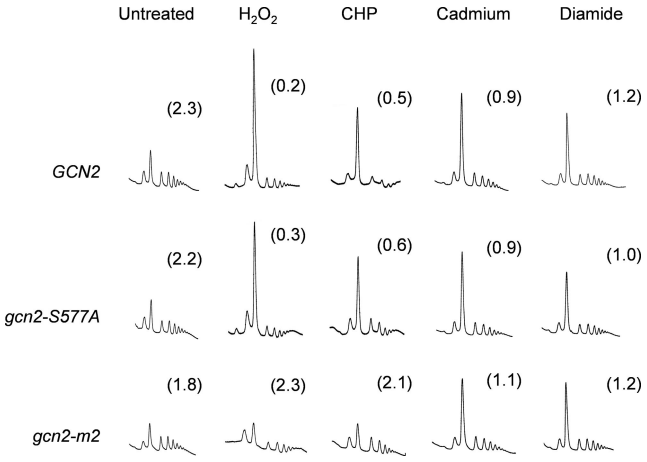

Figure 4. Oxidant-mediated inhibition of translation initiation requires the HisRS domain of Gcn2. Polysome traces are shown for a prototrophic *gcn2* mutant strain (SCY51) containing wild-type, *gcn2-S577A*, or *gcn2-m2* alleles of *GCN2*. Strains were treated with 0.5 mM H_2O_2, 0.1 mM CHP, 1.0 mM cadmium, or 10.0 mM diamide for 15 min. Numbers in brackets are the p:m ratio. Representative data are shown from repeat experiments.

hibit translation initiation independently of Gcn2. In contrast to hydroperoxides, cadmium and diamide inhibited translation initiation in both the *gcn2-m2* and *gcn2-S577A* mutants (Figure 4). It should be noted the SCY51 (σ background) strain used for these experiments is generally more resistant to oxidative stress than the W303 strain used for previous experiments; hence, higher concentrations of cadmium (1.0 mM) and diamide (10 mM) have been used. Quantification of polysome traces revealed that cadmium and diamide decreased the ratio of polysomes:monsomes (p:m) in the *gcn2-m2* mutant by ~33%, similar to the reduction in the *gcn2* deletion mutant (Figure 2A). Phosphorylation of eIF2α was observed in the *gcn2-S577A* mutant, but not in the *gcn2-m2* mutant, confirming that cadmium and diamide inhibit translation initiation independently of eIF2α phosphorylation (Figure 5B). Furthermore, inhibition of translation initiation was observed in response to cadmium

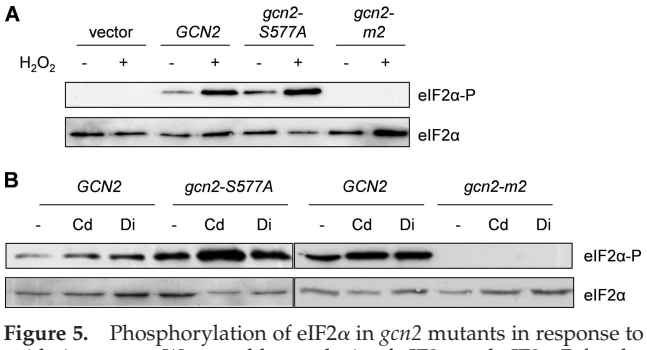

Figure 5. Phosphorylation of eIF2α in *gcn2* mutants in response to oxidative stress. Western blot analysis of eIF2α and eIF2α-P for the prototrophic *gcn2* mutant strain (SCY51) containing an empty vector, wild-type, *gcn2-S577A*, or *gcn2-m2* alleles of *GCN2*. Strains was grown to exponential phase in minimal SD media and treated with H_2O_2 (A) or cadmium and diamide (B) as described for Figure 4. Note that the panel on the right of B has been overexposed relative to the panel on the left to confirm that no eIF2α is detected in a *gcn2-m2* mutant. Representative data are shown from repeat experiments.

or diamide in a mutant containing a nonphosphorylatable allele of eIF2α (*sui2-S51A*), whereas, no inhibition was observed in response to H_2O_2 (data not shown). These data further indicate that cadmium and diamide can inhibit translation initiation via a mechanism that does not involve Gcn2-mediated phosphorylation of eIF2α.

EAP1 Is Required for Inhibition of Translation Initiation in Response to Cadmium and Diamide

Translation initiation can be controlled by regulating the availability of the cap-binding factor eIF4E. We therefore examined whether cadmium and diamide inhibit translation initiation through a mechanism that depends on the eIF4E-binding proteins (4EBPs) Caf20 and Eap1. No requirement was found for Caf20 to mediate translation in response to cadmium or diamide (data not shown). In contrast, Eap1 is partially required for the inhibition of translation initiation in response to both oxidants. The inhibition of translation initiation in response to H_2O_2 was unaffected in the *eap1* mutant (data not shown). Loss of *EAP1* or *GCN2* partially abrogated the inhibition of translation initiation in response to cadmium or diamide (Figure 6A). Furthermore, inhibition was abolished in a *gcn2 eap1* double mutant, indicating that Gcn2 and Eap1 mediate the inhibition of translation initiation in response to both cadmium and diamide (Figure 6A). Protein synthesis was still inhibited in the *eap1 gcn2* mutant (Figure 3B), further confirming that oxidants inhibit translation at both the initiation and postinitiation (elongation or termination) phases of protein synthesis (Shenton *et al*., 2006).

To confirm that the translation function of Eap1 is responsible for inhibition, we examined translational activity in an *eap1*^{m3} mutant, *eap1::Y109A; L114A*, that cannot bind eIF4E (Ibrahimo *et al*., 2006). A plasmid containing this allele was transformed into an *eap1* null and translational activity examined after treatment with cadmium (Figure 6B). Inhibition of translation initiation was similar in the *eap1*^{m3}mutant to the vector control (see Figure 6B p:m ratios) consistent with the idea that Eap1-eIF4E binding is required for the control of translation initiation in response to cadmium. To further confirm this finding we examined a *gcn2 eap1*^{m3}double mutant (Figure 6B). Inhibition of translation initiation was largely abrogated in response to cadmium stress, comparable with the *gcn2 eap1* null mutant (Figure 6A).

Translational Induction of GCN4 Is Not a General Response to Oxidative Stress

To further characterize the role of translational control in oxidative stress, we examined the sensitivity of general control mutants to oxidants. Mutants lacking *GCN4* were sensitive to H_2O_2 and CHP, indicating that Gcn4 is required for tolerance to hydroperoxides (Figure 7A). The sensitivity of the *gcn4* mutant to peroxides is comparable with that of other yeast antioxidant mutants in this same strain background such as those lacking the *YAP1* transcription factor or unable to synthesize glutathione (Grant *et al*., 1996). In contrast, the *gcn4* mutant was unaffected in resistance to cadmium or diamide. The *gcn2* mutant was no more sensitive than the wild type to ROS in spot tests.

Given that Gcn4 is required for resistance to hydroperoxides, we examined whether different oxidants induce the synthesis of Gcn4. We have previously shown that low concentrations of H_2O_2 causes a modest (<2-fold) induction of *GCN4* expression by using a *GCN4-lacZ* reporter construct (Shenton *et al*., 2006). This result is complicated, because the reporter construct only contains the first 56 codons of *GCN4*

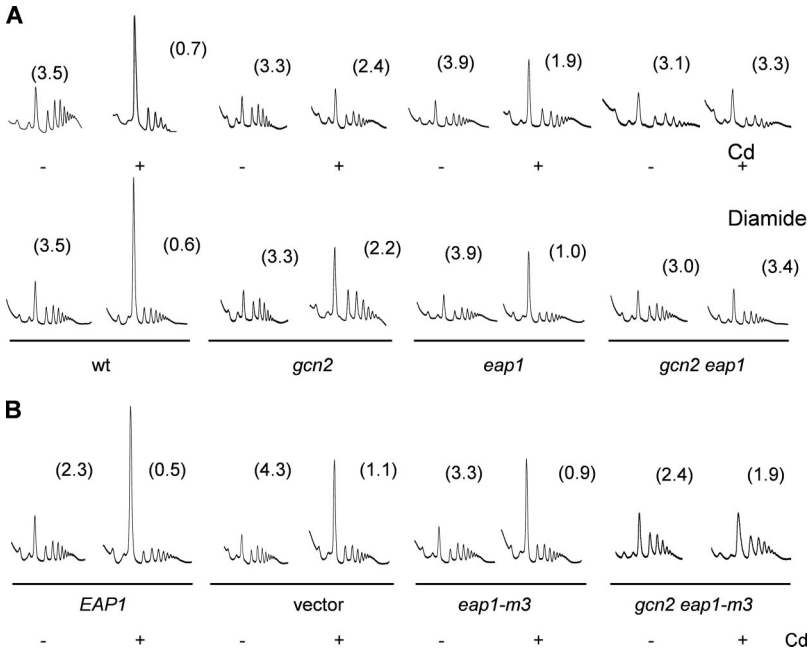

Figure 6. Eap1 is required for the inhibition translation initiation in response to cadmium or diamide. (A) Polysome traces are shown for wild-type, *gcn2*, *eap1*, and *gcn2 eap1* mutant strains treated with cadmium or diamide. Translation initiation is less inhibited in the absence of *EAP1* or *GCN2*, and no inhibition is observed in the *gcn2 eap1* double mutant. (B) Polysome analysis of the $EAP1^{m3}$ binding mutant shows that cadmium induced inhibition of translation initiation is dependent on the eIF4E binding site. Numbers in brackets are the p:m ratio. Representative data are shown from repeat experiments.

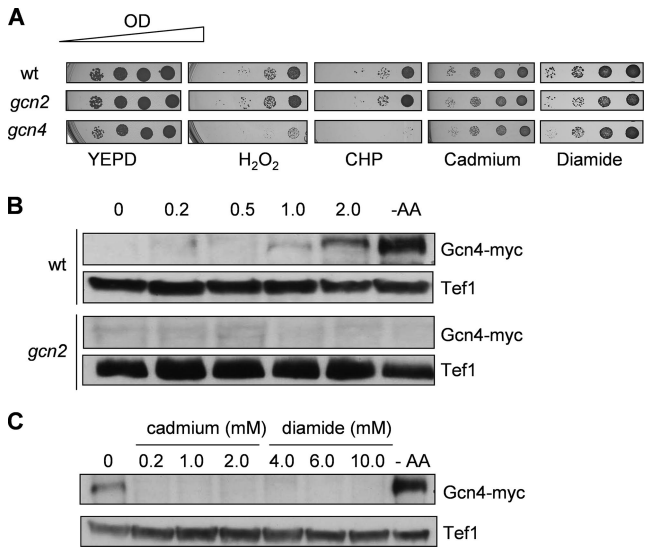

Figure 7. Gcn4 is required for resistance to hydrogen peroxide. (A) Sensitivity to oxidative stress was determined by spotting strains onto YEPD plates containing various concentrations of H_2O_2, cadmium or diamide. Cultures of wild-type, *gcn2*, and *gcn4* mutant strains were grown to stationary phase, and the A_{600} was adjusted to 1, 0.1, 0.01, or 0.001 before spotting onto plates containing various concentrations of oxidants. Growth was monitored after 3-d incubation at 30°C. Results are shown for plates containing no oxidant (YEPD), 4.0 mM H_2O_2, 0.1 mM CHP, 10 μM cadmium, and 2.5 mM diamide. No growth of any of the strains was observed at higher concentrations of cadmium or diamide. Western blots are shown probed for Gcn4-myc in a wild-type and *gcn2* mutant in response to H_2O_2 (B) and for the wild-type strain in response to cadmium and diamide (C). Induction of Gcn4 in response to an amino acid starvation is shown as a control (−AA). An amino starvation was induced by shifting cells to minimal media lacking any amino acids for 2 h. The same blots were probed with an antibody against Tef1 (eEF1α) as a loading control. Representative data are shown from repeat experiments.

fused to a large heterologous reporter gene (*lacZ*). *GCN4-lacZ* therefore may not accurately reflect translational control of *GCN4*, particularly for oxidants, which inhibits translation at a postinitiation phase (Shenton *et al*., 2006). To overcome this problem, we measured *GCN4* expression by using an epitope-tagged version of *GCN4*, which contains the entire coding region of *GCN4*-fused to a small Myc-tag (Lipford *et al*., 2005). Cells were treated with various concentrations of H_2O_2, cadmium and diamide for 2 h and Gcn4 levels determined by immunoblot analysis. Elevated *GCN4* expression was observed in response to H_2O_2 at concentrations of 1.0 and 2.0 mM (Figure 7B). Induction in response to amino acid starvation is included as a positive control, and it indicates that Gcn4 is induced to higher levels than for peroxide treatments (Figure 7B). To confirm that the induction of *GCN4* expression seen in response to hydrogen peroxide is a translational control response, expression was examined in a mutant lacking *GCN2* (Figure 7B). No induction was observed in the *gcn2* mutant in response to amino starvation or hydrogen peroxide indicating that phosphorylation of eIF2 is required for this response. In contrast to peroxide, no induction was observed in response to cadmium or diamide and Gcn4 seemed to be somewhat lower compared with the untreated control after treatment with these oxidants (Figure 7C). Lowered levels of Gcn4-myc protein are unlikely to arise due to degradation in the cell extracts isolated from oxidant-treated cells because trichloroacetic acid extraction was used to denature proteases before cell breakage. Together, these data indicate that Gcn4 is specifically required for the response to hydroperoxides.

Identification of the Gcn4-dependent H_2O_2 Regulon

To further understand the role of the Gcn4 pathway in the response to oxidative stress, we analyzed the global transcriptional response to H_2O_2. Wild-type, *gcn2*, and *gcn4* mutant cells were analyzed after treatment with 0.5 mM H_2O_2 for 15 min. This concentration of H_2O_2 was chosen because it causes maximal phosphorylation of eIF2α after a 15-min treatment (Shenton *et al*., 2006). Microarray analysis revealed that this H_2O_2 treatment caused a significant effect

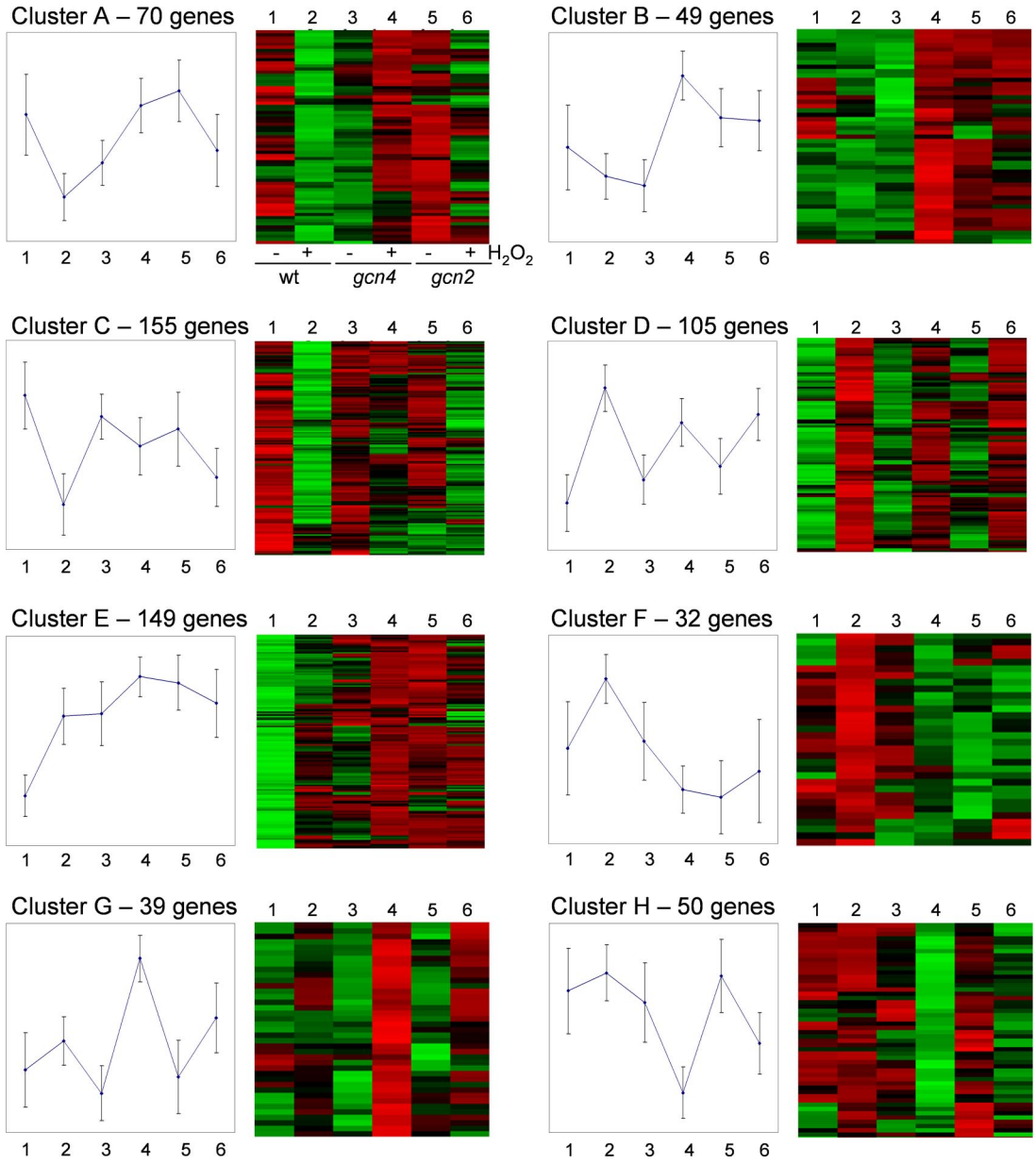

Figure 8. Identification of the Gcn4-dependent H_2O_2 regulon. Genes were identified which displayed significantly different responses to hydrogen peroxide in the *gcn2* or *gcn4* mutants and assigned to one of eight clusters by using a k-means clustering algorithm (left). The data for each cluster are represented as a profile of the z-transformed (for each probe set, the mean set to 0 and SD to 1 using maxdView), log 2 values for the mean of each condition. Error bars indicate the maximum and minimum values within each group. The number of genes in each cluster is specified. Displayed on the right is the same z-transformed data shown as an Eisen color plot. Red and green indicate positive and negative change from zero, respectively, with color intensity indicating the degree of deviation.

on the transcriptome, with 476 genes up-regulated greater than twofold and 161 genes down-regulated greater than twofold in the wild-type strain. We identified which genes show *gcn2*- or *gcn4*-dependent H_2O_2 expression by measuring the interaction term in an analysis of variance model. This was done in two separate tests for wild-type versus *gcn2* mutant and for wild-type versus *gcn4* mutant. Differentially expressed genes picked for subsequent analysis met the criterion of q value (a fdr-corrected p value) <0.05 for either the interaction of wild-type versus *gcn2* or wild-type versus *gcn4* (see *Materials and Methods*). This produced a data set of 376 genes whose expression was significantly different in the *gcn4* mutant, and 357 genes whose expression was significantly different in the *gcn2* mutant, compared with the wild-type, respectively. This enriched data set was segregated into eight clusters based on similarity of expression profile across the data set using a k-means clustering algorithm (Figure 8). The microarray data were validated for a range of genes by using real-time RT-PCR analysis (Figure 9). Representative genes are shown from cluster B (*YOR1*), cluster D (*GCY1*), cluster E (*ATP3*, *SDH1*, *COR1*), and cluster G (*FRE1*). The RT-PCR analysis confirmed the general trends that were detected from the microarray analysis. For each of the clusters shown in Figure 8, significant overrepresentation of genes belonging to a particular classification was determined using FunSpec (Table 2).

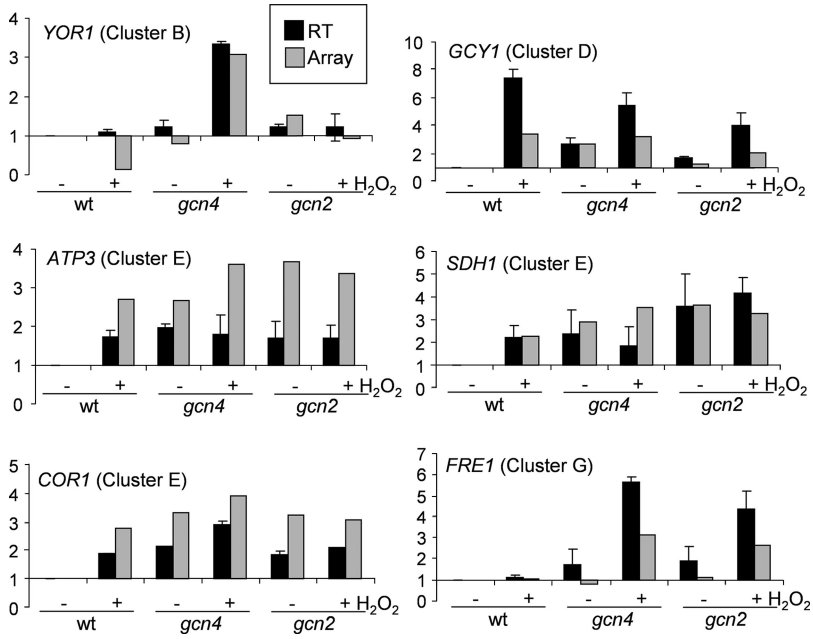

Figure 9. Validation of microarray data. The microarray data were confirmed by RT-PCR analysis for representative genes. The numbers shown are –fold induction in response to H_2O_2 in WT, *gcn4*, and *gcn2* mutant cells from the RT-PCR and microarray analyses. Black bars denote data from the RT-PCR, and gray bars denote data from the microarray analysis.

Genes Up-Regulated by Hydrogen Peroxide in the Wild Type

Several genes were up-regulated in response to H_2O_2 in the wild-type strain, but this regulation was altered in *gcn* mutants (clusters D–F). Cluster D included genes in which the up-regulation was dampened in both the *gcn2* and *gcn4* mutants, which would be expected for genes regulated via translational control of *GCN4* expression. This cluster encompasses many metabolic genes, including genes affecting amino acid, lipid, carbon, and nitrogen metabolism consistent with the idea that extensive metabolic reconfiguration is required after oxidative stress (Godon *et al*., 1998). Several genes encoding stress defenses or antioxidants were identified in this cluster. Most prominently, a catalase gene (*CTT1*) was up-regulated that functions to detoxify hydrogen peroxide (Izawa *et al*., 1996). Several osmotic stress (*RVS161*, *GPD1*, *HOR2*, *CAP1*, and *GCY1*), heat stress (*SSE2* and *HSP30*), and stationary phase (*YGP1*)-related genes were identified, emphasizing the physiological link between diverse environmental stress conditions (Gasch *et al*., 2000). Cluster E included genes in which expression was constitutively elevated in *gcn2* and *gcn4* mutants, indicating that Gcn4 normally acts to repress these genes. This cluster encompassed a significant number of mitochondrial genes affecting energy generation and respiration in agreement with previous reports suggesting that mitochondrial function is particularly important for resistance to H_2O_2 (Grant *et al*., 1996; Thorpe *et al*., 2004). Cluster F-included genes where expression was moderately down-regulated in response to peroxide in the *gcn4* mutant and moderately up-regulated in the *gcn2* mutant, but no significantly overrepresented functional classes were identified in this cluster (Table 2).

Genes Down-Regulated by Hydrogen Peroxide in the Wild Type

Clusters A and C included genes that are down-regulated in response to H_2O_2 exposure. Cluster C was the largest cluster identified, and it included genes in which down-regulation was dampened in both the *gcn2* and *gcn4* mutants consistent with regulation via translational control of *GCN4* expression. This cluster included a large number of gene involved in transcription and RNA metabolism. This presumably indicates a requirement to down-regulate energy-consuming processes during stress conditions as noted previously during the environmental stress response (Gasch *et al*., 2000). Similarly, several genes affecting the cell cycle were down-regulated, consistent with previous observations indicating that the cell cycle is arrested as part of the defense response against H_2O_2 stress (Flattery-O'Brien and Dawes, 1998). Surprisingly, the *SKN7* transcription factor was identified as a component of cluster C. Skn7 is a two-component system response regulator that cooperates with the Yap1 transcription factor for the induction of many oxidative stress genes (Ikner and Shiozaki, 2005). Regulation of Yap1 activity via down-regulation of Skn7 may therefore represent a previously unrecognized response to hydrogen peroxide. Cluster A included genes that were down-regulated in both the wild-type and *gcn2* mutants, but they were moderately up-regulated in the *gcn4* mutant. The main functional classes identified in this cluster predominantly affected lipid metabolism.

Genes That Are Unaffected in the Wild Type but Show Altered Expression in Response to Hydrogen Peroxide in gcn Mutants

Many genes were identified that showed little change in gene expression in the wild-type strain, but they were altered in response to hydrogen peroxide in *gcn* mutants. Gene expression was strongly up-regulated in the *gcn4* mutant in both clusters B and G. The *gcn2* mutant was similar to the wild-type strain for cluster G, whereas expression was constitutively elevated in the *gcn2* mutant for cluster B. Significantly, genes affecting heavy metal uptake and use were overrepresented in both clusters. This included several genes involved in iron (*FRE1*, *FRE2*, *FRE3*, *FRE4*, *FRE5 SIT1*, *ARN1*, *ARN2*, *SMF3*), copper (*CCC2*), and zinc (*COT1*) transport. Increased iron uptake during oxidative stress is somewhat surprising given that it can potentially lead to the generation of the hydroxyl radical ($^{\bullet}OH$) via the Fenton reaction. However, we have previously noted that a number

Table 2. Functions overrepresented in clusters A–H

Cluster	Classification	p	Genes	Source[a]
A	Lipid, fatty acid, and isoprenoid use	2.5E-03	*FAT1 SEC59 COX10*	1
	Farnesyltranstransferase	3.4E-04	*BTS1 COX10*	2
	Membrane fraction	2.2E-03	*FAT1 MAL31 COG3 LCB4*	4
B	Channel/pore class transporters	8.5E-04	*TOK1 AQY2*	1
	Drug transporters	2.3E-03	*YCL073C YOR1 PKR1*	1
	Regulation of/interaction with cellular environment	3.7E-03	*PTC1 ALR2 TOK1 FRE2 SSK1 FRE5*	1
	Ferric-chelate reductase	1.2E-03	*FRE2 FRE5*	2
	Mitochondrion inheritance	4.2E-03	*PTC1 MDM12*	3
	tRNA splicing	4.9E-03	*PTC1 SEN54*	3
	Membrane	1.4E-03	*RFT1 YCL073C ALR2 YOR1 TOK1 FRE2 PEX13 ATP10 ASI1 GOT1 COS10 MDM12 SEN54*	4
C	Transcription	4.6E-04	*POP8 SRD1 NHP2 YDR026C NGG1 PCF11 ADA2 SNU13 RPB9 STE12 TRA1 SKN7 GIN1 SSL2 RPA34 SET2 CBP1 RRN3 TPK3 CNB1 LOS1 BDF1 LEU3 PRP39 YML081W SOK2 MSU1 UBP10 SSU72 HAL9 MRS2 SWI1 NOP4 RPO26*	1
	Intracellular signaling	2.2E-03	*GPI1 SKN7 TOR1 TPK3 SOK2 RGS2*	1
	Cell communication	8.8E-04	*SKT5 STE7 LRG1 RAD24 WSC4 STE12 SKN7 SYG1 TOR1 CNB1 GIS4 UFO1 ATX1 HAL9 WSC3 ROD1 RGS2 PDE2 MAK3*	3
	RNA metabolism	4.1E-03	*POP8 SRD1 NHP2 CWC2 PCF11 SNU13 CGR1 MPT5 UTP9 CBP1 LOS1 REX3 PRP39 MSU1 MRS2 NOP4*	3
	Regulation of cell cycle	4.3E-03	*CLN3 CDC27 RAD24 MPT5 CDC55 ULP2 TOR1 IBD2*	3
	Nucleus	2.1E-03	*CLN3 POP8 CDC27 HMT1 SRD1 MSH3 NHP2 CWC2 NGG1 PCF11 ADA2 SNU13 RAD24 CGR1 RPB9 BRR6 STE12 TRA1 THP2 UTP9 SKN7 ULP2 SSL2 DPB11 RPA34 SET2 RRN3 LOS1 BDF1 LEU3 PRP39 UBP8 IBD2 UBP10 SSU72 HAL9 SWI1 NOP4 RPO26*	4
D	Amino acid metabolism	5.2E-04	*UGA2 LEU2 STP4 YFL030W YFR055W YIL042C SRY1 MCM1 GAD1 HIS3 GLN1*	1
	Metabolism	8.0E-04	*YAL061W ACH1 UGA2 LEU2 CIT2 GPD1 STP4 UGA4 CEM1 HOR2 YFL030W HXK1 YFR055W YGL039W PDE1 YGR043C SKN1 HXT5 YIL042C PIG2 TDH1 YJR096W SRY1 MCM1 ISF1 GAD1 PDR16 GCY1 HIS3 PYK2 GLN1*	1
	Stress response	2.4E-03	*YRO2 SSE2 HSP30 GPD1 HOR2 CTT1 MGA1 MCM1 YGP1*	1
	Response to stress	4.2E-04	*UGA2 RVS161 HSP30 GPD1 HOR2 CTT1 CAP1 SIP5 YGP1 GCY1 OXR1*	3
	Response to external stimulus	4.8E-03	*UGA2 RVS161 GPD1 HOR2 CAP1 SIP5 MDG1 PDR16 GCY1 OXR1*	3
E	Respiration	6.8E-07	*COR1 ATP3 ATP16 SDH4 RIP1 COX4 CBP4 QCR10 YKR016W YKR046C NDI1 QCR2*	1
	Energy	1.8E-05	*COR1 ATP3 ATP16 KGD2 SDH4 RIP1 COX4 ADH4 CBP4 PDX1 QCR10 SDH1 YKR016W YKR046C SDH2 NDI1 GPH1 QCR2*	1
	Mitochondrion	7.1E-04	*MRPL16 COR1 ATP3 ATP16 KGD2 SDH4 RIP1 RML2 MET13 COX4 CBP4 PDX1 QCR10 RSM7 SDH1 SDH2 MEF1 NDI1 QCR2*	1
	Cytoplasm	4.1E-03	*CCR4 MRPL16 COR1 ATP3 YBR071W HSP26 VID24 CDC28 CVT17 ATP16 YDL072C YDR116C SAC6 KGD2 RSM24 SDH4 MHR1 RIP1 RML2 PRB1 MET13 COX4 CBP4 PDX1 APM2 QCR10 ERG9 YIL006W RSM25 URA8 RSM7 SDH1 YKR046C COX19 UBI4 SDH2 SHM2 MEF1 NYV1 CKI1 VRP1 PPZ1 TUB1 NDI1 YPK2 SPC24 SCS7 VPS27 PFK27 CRC1 DED1 HST2 GRE1 PRE2 ANT1 GPH1 QCR2*	4
F	None			
G	Homeostasis of cations	7.0E-05	*CCC2 SMF3 FRE1 FRE4 COT1 FRE3*	1
	Detoxification	3.8E-04	*SIT1 ARN1 ARN2 YHR048W YLR046C*	1
	Transport facilitation	5.5E-04	*CCC2 SIT1 YHL035C ARN1 ARN2 YHR048W SHE4 COT1*	1
	Heavy metal ion transporter	4.8E-09	*CCC2 SIT1 ARN1 ARN2 SMF3 COT1*	2
	Oxidoreductase	1.9E-03	*AAD3 TRR1 POX1 FRE1 FRE4 FRE3*	2
	Iron-siderochrome transport	4.3E-11	*SIT1 ARN1 ARN2 FRE4 FRE3*	3
	Heavy metal ion homeostasis	9.8E-10	*SIT1 ARN2 SMF3 MAC1 ISU2 COT1 FRE3*	3
	Endosome	1.3E-03	*SIT1 ARN1 ARN2*	4
H	Amino acid biosynthesis	2.1E-08	*LYS2 LYS21 LYS20 LYS4 TRP4 LYS12 LYS1 ILV3 LYS9 ARG1*	1
	Metabolism	9.4E-04	*LYS2 CTP1 PSA1 LYS21 LYS20 LYS4 TRP4 ALG2 PCT1 BIO2 LYS12 LYS1 ILV3 YSR3 GAT3 LYS9 ARG1 ODC2*	1

[a] Source refers to classification source: 1, MIPS Functional Classification; 2, GO Molecular Function; 3, GO Biological Process; and 4, GO Cellular Component. Classifications with $p < 0.005$ are shown.

of iron transport genes are translationally up-regulated in response to oxidative stress and suggested that this may indicate a requirement to restore iron homeostasis after ROS exposure (Shenton *et al*., 2006). Cellular iron is found largely complexed in cells, for example in iron–sulfur (Fe/S) clusters. Oxidation of these clusters causes release of the iron, resulting in enzyme inactivation; hence, there may be a requirement to replace lost iron. Cluster H included genes that were unaffected in the wild type, but they were down-regulated in response to H_2O_2 stress in both the *gcn2* and *gcn4* mutants. This cluster included several metabolic genes, including those affecting amino acid (lysine, arginine, isoleucine, and tryptophan) and vitamin (*BIO2*) biosynthesis. These genes have been identified previously as genes that are regulated by Gcn4 in response to amino acid starvation (Natarajan *et al*., 2001). It is unclear why these genes are down-regulated in *gcn* mutants, but it may indicate that Gcn4regulated genes may be disrupted in response to oxidative stress in strains lacking general control.

Comparison with the Gcn4-dependent Response to Amino Acid Starvation

Given that Gcn4 has best been characterized as a transcriptional regulator that responds to amino acid starvation conditions, we compared our expression data with previous amino acid starvation expression data. Many genes have been identified that are induced or repressed in response to starvation for histidine by treatment with 3-aminotriazole (3-AT) (Natarajan *et al*., 2001). The scatter plots in Figure 10 show a comparison of changes in gene expression due to H_2O_2 exposure and 3-AT treatment in wild-type and *gcn4* mutant strains. Although there is not a strong correlation between peroxide and amino acid starvation-regulated genes on a genome-wide scale, many genes were similarly induced or repressed in response to both treatments. Furthermore, loss of *GCN4* dampened this effect, and we were able to identify 64 genes that show Gcn4-dependent expression in both data sets (Figure 10, genes marked with a square symbol; Supplemental Table 2). The 44 up-regulated genes included significant overrepresentation of genes affecting metabolism (carbon, amino acid, and energy) and the stress response. The 22 down-regulated genes included significant overrepresentation of genes affecting transcription.

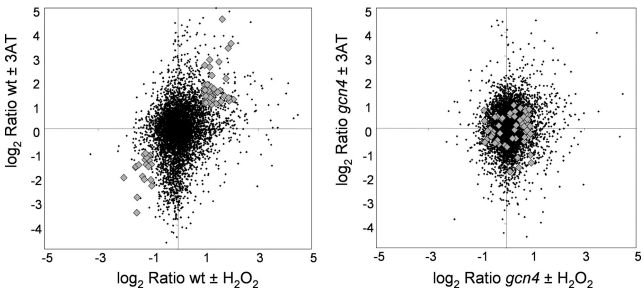

Figure 10. Comparison of peroxide-regulated genes with the Gcn4-dependent response to amino acid starvation. The peroxide expression data were compared with previous amino acid starvation expression data (Natarajan *et al*., 2001). Scatter plots show a comparison of changes in gene expression due to H_2O_2 exposure and 3-AT treatment in wild-type (left) and *gcn4* mutant (right) strains. Genes denoted with a square symbol indicate 64 genes whose expression was altered by greater than twofold in the wild-type strain, but not in the *gcn4* mutant.

DISCUSSION

Global regulation of protein synthesis is a well-known response to several different types of stress. We have previously shown that exposure of yeast cells to hydrogen peroxide results in a rapid and reversible inhibition of protein synthesis (Shenton *et al*., 2006). This seems to be an evolutionarily conserved response that inhibits global translation while promoting continued translation of defense genes that protect against and/or repair the resulting oxidant damage (Harding *et al*., 2003; Shenton *et al*., 2006). H_2O_2 inhibits translation initiation via a mechanism that depends on the Gcn2 protein kinase, which phosphorylates the α subunit of eIF2 (Shenton *et al*., 2006). In this current study, we have shown that eIF2α is also phosphorylated in response to diverse oxidants. This is somewhat surprising given the differing mechanisms of toxicity mediated by these oxidants, and it suggests that translation inhibition mediated via phosphorylation of eIF2α is a general response to oxidative stress. However, diamide and cadmium also inhibit translation initiation via a Gcn2-independent mechanism, which may provide further oxidant-specific regulation of gene expression.

Hydrogen peroxide is a ubiquitous molecule formed as a by-product of aerobic respiration and after exposure to diverse biological and environmental factors. It can damage cells by promoting oxidative stress, but it also plays important roles as a signaling molecule in the regulation of many biological processes (reviewed in Veal *et al*., 2007). As well as being both freely diffusible and reactive, H_2O_2 also must also be removed from cells to avoid Fenton and Haber–Weiss reactions leading to the formation of highly reactive hydroxyl radicals (reviewed in Temple *et al*., 2005). Cumene hydroperoxide is a lipid-soluble hydroperoxide that is widely used as an intracellular source of ROS (Thorpe *et al*., 2004). It can generate highly reactive free radicals such as the alkoxy radical, resulting in high mutagenicity and toxicity (Simic *et al*., 1989). Cadmium is a highly toxic metal and a well-established human carcinogen. It is capable of entering cells via the same transport systems used by the essential heavy metals. Once inside the cell, the main mechanism for toxicity is through the depletion of glutathione and binding to sulfydryl groups (Valko *et al*., 2005). It can also displace iron and copper from various cytoplasmic and membrane proteins, increasing the levels of unbound free or chelated copper and iron ions contributing to oxidative stress via Fenton reactions (Valko *et al*., 2006). Diamide is a membrane-permeable, thiol-specific oxidant, which promotes the formation of disulfides (Kosower and Kosower, 1995). It reacts rapidly and specifically with glutathione; thus, it causes oxidative stress by inhibiting antioxidant defenses. Hence, these diverse oxidants can generate ROS through both direct and indirect mechanisms.

Hydroperoxides, diamide, and cadmium all seem to activate Gcn2 via a similar mechanism. Mutations in the *m2* motif of the HisRS-like domain (Y1119L and R1120L) that abolish tRNA binding by Gcn2 (Cherkasova and Hinnebusch, 2003) abrogate (hydroperoxides) or reduce (cadmium and diamide) inhibition. These results suggest that ROS activate Gcn2 via a mechanism that elevates uncharged tRNA levels. However, it should be noted that we cannot rule out that some as yet undefined signal requires this same domain for Gcn2 activation. Gcn2 is known to be activated in response to a variety of conditions, including nutrient starvation (amino acids, purines, and glucose) and exposure to sodium chloride, rapamycin, ethanol and volatile anesthetics (reviewed in Hinnebusch, 2005; Palmer *et al*., 2005).

Depletion of amino acids leads to an accumulation of uncharged tRNA, which activates the Gcn2 protein kinase via its HisRS-related domain. It is likely that other stress conditions ultimately affect the levels of uncharged tRNA in the cell. For example, volatile anesthetics inhibit amino acid uptake (Palmer *et al*., 2005), and Gcn2 is activated by glucose starvation partly through an effect on vacuolar amino acid pools (Yang *et al*., 2000). Our data indicate that ROS activate Gcn2 in a prototrophic strain (a strain that does not require exogenous amino acids from the growth media), ruling out any effects on amino acid uptake. This raises the question as to how ROS cause an amino acid starvation, effect cellular uncharged tRNA levels, or both. Oxidative stress may affect the transport and storage of amino acids within cells, similar to the effect of glucose starvation. Additionally, oxidative stress may conceivably cause an accumulation of uncharged tRNA through a variety of mechanisms. Free amino acids and amino acids in proteins are highly susceptible to oxidation by ROS, which may cause an imbalance in amino acid pool sizes (Stadtman and Levine, 2003). Alternatively, the proteins and nucleic acids which are required for tRNA-aminoacylation may be susceptible to oxidation, resulting in an accumulation of uncharged tRNA and activation of Gcn2.

The signals activating Gcn2 in response to rapamycin and NaCl are not well understood. Rapamycin seems to work by blocking Tor-mediated phosphorylation of Gcn2 at Ser577 (Cherkasova and Hinnebusch, 2003). However, activation of Gcn2 by rapamycin and NaCl still requires the HisRS-related domain of Gcn2, as well as Gcn1 and Gcn20, which are thought to mediate the activation of Gcn2 by uncharged tRNA (Narasimhan *et al*., 2004). ROS act differently to rapamycin or NaCl because a Gcn2-S577A mutant can still be activated to phosphorylate eIF2α. Heterologous protein production also activates Gcn2 through an effect on uncharged tRNA levels, although it is unclear how heterologous expression affects amino acid levels (Steffensen and Pedersen, 2006). This is particularly interesting because heterologous protein production has previously been shown to induce an oxidative stress (Bannister and Wittrup, 2000); hence, Gcn2 may be activated via a ROS-dependent mechanism during heterologous expression. It is tempting to speculate that Gcn2 activation is a defense mechanism that is invoked in response to any condition that elevates the cellular concentrations of ROS.

A key control point for regulating eukaryotic translation initiation is via binding of initiation factors to the mRNA cap (Richter and Sonenberg, 2005). The initiation factor eIF4E recognizes the mRNA cap structure and also interacts with the "multi-adaptor" protein eIF4G. This process is critical for the recruitment of ribosomes to the 5′ end of mRNAs and translation initiation can be regulated by the competitive binding of eIF4E-binding proteins (4E-BPs) to eIF4E. Two such proteins have been identified in yeast, Caf20 and Eap1 (Altmann *et al*., 1997; Cosentino *et al*., 2000). These 4E-BPs have broad functions in cell growth, proliferation, and development (Ibrahimo *et al*., 2006; Park *et al*., 2006). Disruption of *EAP1* (but not *CAF20*) was found to impair cadmium- and diamide-induced regulation of translation initiation. In contrast, peroxide-induced inhibition was unaffected in the *eap1* mutant, highlighting the different translational control mechanisms invoked by these different oxidants. Simultaneous loss of *EAP1* and *GCN2* abrogated the translation initiation inhibition mediated by cadmium and diamide. Similarly, yeast cells respond to a membrane stress by attenuating translation initiation via a mechanism that is mediated by Gcn2 and Eap1 (Deloche *et al*., 2004). This response may serve to prevent the mislocalization of proteins after disruption of vesicular transport pathways. Interestingly, diamide readily oxidizes the ER and mutants defective in vacuolar protein-sorting functions are particularly sensitive to diamide (Cuozzo and Kaiser, 1999; Thorpe *et al*., 2004). Furthermore, diamide-stress induces the expression of many cell wall biosynthesis genes and genes involved in protein secretion and processing in the ER, suggesting that diamide causes defects in secretion (Gasch *et al*., 2000). Diamide may therefore, invoke a membrane transport defect that promotes Gcn2- and Eap1-mediated attenuation of translation initiation. Cadmium toxicity is less well understood, and it is unclear at present whether cadmium particularly affects protein secretion or trafficking. Unlike diamide, cadmium does not directly oxidize glutathione, but it may affect the redox state of secretory processes via an indirect effect on protein and low- molecular-weight thiols.

Gcn4 is a transcriptional activator of amino acid biosynthetic genes. It is translationally up-regulated in response to phosphorylation of eIF2α (Hinnebusch, 2005). However, despite the finding that phosphorylation of eIF2α is a general response to oxidative stress, not all oxidants induce translational expression of *GCN4*. Gcn4 is up-regulated in response to H_2O_2, and it is specifically required for hydroperoxide resistance. We have proposed that translation of the *GCN4* mRNA is specifically able to continue during peroxide stress, a condition where global translation is inhibited at both the initiation and postinitiation phases (Shenton *et al*., 2006). *GCN4* is induced by the well-known Gcn2-dependent, eIF2α phosphorylation mechanism, but its translation must also be resistant to the postinitiation block in protein synthesis. In contrast, Gcn4 protein levels are somewhat reduced after diamide or cadmium stress. One possibility to explain these findings is that *GCN4* expression is inhibited in response to cadmium and diamide by Eap1-mediated inhibition of translation initiation. However, additional translational regulatory mechanisms must exist because loss of both *GCN2* and *EAP1* does not restore the rate of protein synthesis to uninhibited levels after diamide or cadmium stress. Presumably, *GCN4* expression is inhibited by other translational control mechanisms which act at the postinitiation phase in response to these oxidants. Loss of both *GCN2* and *EAP1* abrogates the inhibition of translation initiation in response to cadmium and diamide, but it does not affect this postinitiation block. Gcn2 and Eap1 do not affect the postinitiation block; hence, any inhibition of ribosomal transit induced by diamide or cadmium should be comparable in wild-type and *gcn2 eap1* mutant cells inhibiting protein synthesis. Induction of *GCN4* expression correlates with the finding that Gcn4 is required for resistance to peroxides but not to diamide and cadmium. The sensitivity of the *gcn4* mutant was determined using spots tests, which measure the tolerance of strains to a lethal dose of oxidant. This sensitivity therefore suggests that basal levels of expression of Gcn4-traget genes are required for peroxide-tolerance. In contrast, the *gcn2* mutant was unaffected in sensitivity to peroxides in this assay. The requirement for Gcn2 is apparent during adaptive conditions when it is required to inhibit translation initiation and to induce the expression of *GCN4* in response to low concentrations of hydrogen peroxide stress in actively growing cells.

Previous transcriptional profiling studies have shown that ~10% of the yeast genome is regulated by Gcn4 in response to amino acid starvation (Natarajan *et al*., 2001). The broad transcriptional response controlled by Gcn4 suggests that Gcn4 acts as a master regulator of gene expression. Our analysis of a *gcn4* mutant indicates that Gcn4 is required for a more limited set of genes in response to H_2O_2-stress.

Comparison of peroxide and amino acid starvation-regulated genes revealed that there is not a strong correlation at the genome-wide level. Nevertheless, several genes are similarly regulated by both stress conditions, including 64 genes (altered by greater than twofold), which require Gcn4 for regulation in response to both stress conditions. Hydrogen peroxide stress damages many intracellular targets and affects diverse cellular processes. Our data indicate that part of the response to peroxide is mediated through an amino acid starvation. However, additional regulatory inputs must control Gcn4-target gene expression in response to ROS, as seen previously for glucose and heterologous protein production (Yang *et al*., 2000; Steffensen and Pedersen, 2006). Similarly other transcription factors including the yeast coactivator protein Mbf1, and Hac1, which regulates the unfolded protein response, are known to mediate Gcn4 transcriptional activity (Takemaru *et al*., 1998; Patil *et al*., 2004).

Cluster analysis revealed a complex pattern of transcriptional regulation in *gcn* mutants. The requirement for translational regulation of *GCN4* expression in response to hydrogen peroxide stress was most clearly shown by the identification of genes that are up-regulated (clusters D–F) or down-regulated (cluster C) dependent on the presence of both *GCN2* and *GCN4*. It is also apparent from our data that loss of *GCN4* alters the peroxide-regulated expression of many genes independent of Gcn2. These include genes that are similarly regulated in the wild-type and *gcn2* mutant, but that are up-regulated (clusters A, B, and G) or down-regulated (cluster H) in the *gcn4* mutant. The sensitivity of the *gcn4* mutant to hydrogen peroxide may mean that regulation of these genes is somehow preadapted such that they strongly respond to stress conditions in the absence of normal Gcn4-mediated control. It is now well established that the response to oxidative stress requires extensive reprogramming of transcription and translation. Our data indicate that translational control of *GCN4* expression and transcriptional control of Gcn4 target genes are key components of this adaptive response.

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