Disruption of Yeast Forkhead-associated Cell Cycle Transcription by Oxidative Stress⬛

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The effects of oxidative stress on yeast cell cycle depend on the stress-exerting agent. We studied the effects of two oxidative stress agents, hydrogen peroxide (HP) and the superoxide-generating agent menadione (MD). We found that two small coexpressed groups of genes regulated by the Mcm1-Fkh2-Ndd1 transcription regulatory complex are sufficient to account for the difference in the effects of HP and MD on the progress of the cell cycle, namely, G1 arrest with MD and an S phase delay followed by a G2/M arrest with HP. Support for this hypothesis is provided by *fkh1fkh2* double mutants, which are affected by MD as we find HP affects wild-type cells. The apparent involvement of a forkhead protein in HP-induced cell cycle arrest, similar to that reported for *Caenorhabditis elegans* and human, describes a potentially novel stress response pathway in yeast.

INTRODUCTION

Reactive oxygen species (ROS) are by-products of aerobic metabolism, but are also attributes of the extracellular environment. They pose a threat to organisms by damaging a variety of cellular macromolecules, including DNA, membrane lipids, and proteins and are implicated with carcinogenesis, aging, and numerous degenerative diseases (Finkel and Holbrook, 2000; Neumann *et al.*, 2003). The major ROS derived from oxygen are superoxide ions, hydrogen peroxide (HP), and hydroxy radicals, ordered here by increasing reactivity (reviewed in Shackelford *et al.*, 2000).

Oxidative stress in yeast has been studied by exposing cells to agents that are already reactive, typically HP, or drugs that cause the intracellular accumulation of reactive oxygen species (Godon *et al.*, 1998; Dumond *et al.*, 2000; Gasch *et al.*, 2000). Menadione (MD) is such a drug, generating reactive superoxide ions, which can be further oxidized to give HP (Monks *et al.*, 1992; Shackelford *et al.*, 2000). It has been previously reported that exposure to HP or MD results in a cell cycle arrest (Flattery-O'Brien and Dawes, 1998; Leroy *et al.*, 2001). However, the arrest points are not similar for the two agents. MD was reported to arrest cells at the G1 phase of the cell cycle, whereas HP was suggested to cause a G2 arrest by an alternate mechanism from that affected by MD (Flattery-O'Brien and Dawes, 1998); others reported that HP exposure causes a delay in the S phase (Leroy *et al.*, 2001).

Hundreds of genes are regulated so that they are expressed at specific times in the cell cycle and not at others (Cho *et al.*, 1998; Spellman *et al.*, 1998). The major part of this coordination is achieved by the sequential activation of a small number of transcription regulators (reviewed in Mendenhall and Hodge, 1998): MBF (a complex of Mbp1p and Swi6p) and SBF (a complex of Swi4p and Swi6p) are responsible for activating G1 transcription (Koch *et al.*, 1993); Fkh1p and a complex formed by the cooperative promoter binding of Mcm1p and Fkh2p and a later recruitment of Ndd1p are responsible for activating G2/M transcription (Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Hollenhorst *et al.*, 2001); Swi5p and Ace2p, which are themselves expressed during G2/M, are the transcription factors responsible for activating the greater part of the next wave of M/G1 transcription (Dohrmann *et al.*, 1992; Toyn *et al.*, 1997); and Mcm1p, this time alone, is additionally responsible for activating transcription of several other M/G1 genes (McInerny *et al.*, 1997).

The highest degree of conservation (sequence-wise) is displayed by the transcription factors responsible for G2/M transcription, Mcm1p, and the two forkhead proteins Fkh1p and Fkh2p. In *Saccharomyces cerevisiae*, only these latter two (of four forkhead homologues) are implicated in cell cycle regulation. In mammals, within which this family has expanded to include ~40 members (Carlsson and Mahlapuu, 2002), there are at least two subfamilies, FOXO (including four members) and FOXM (including only FOXM1), that are involved in cell cycle regulation (Medema *et al.*, 2000; Alvarez *et al.*, 2001; Tran *et al.*, 2002; Wang *et al.*, 2002). The *FOXO* proteins, much like their *Caenorhabditis elegans* homologue Daf-16, are known to respond to oxidative stress and are crucial for cellular protection from oxidative stress (Kops *et al.*, 2002; Nemoto and Finkel, 2002; Murphy *et al.*, 2003; Brunet *et al.*, 2004).

In this study, we attempted to gain better understanding and higher resolution of yeast cell cycle responses to oxidative stress by following cell cycle progression and genome-scale transcriptional responses under oxidative stress caused by MD and HP. Our results help to shed more light on

Table 1. Strains used in this study

Name	Genotype	Source or reference
DBY8724	MATa ura3Δ(EcoRV-StuI) bar1::URA3	Spellman *et al.* (1998)
DBY8781 (DY150)	MATa ade2-1 trp-1 leu2-3112 his3-11,15 ura3 can1-100	Bhoite and Stillman (1998)
DBY8834	MATa ade2-1 trp-1 leu2-3112 his3-11,15 ura3 can1-100 bar1Δ fkh1Δ fkh2Δ	This laboratory

possible mechanisms mediating cell cycle effects of oxidative stress. Most importantly, they suggest the participation of a transcription regulatory complex containing a yeast forkhead protein in mediating these effects. This describes a potentially novel oxidative stress mechanism in yeast and expands the known functional conservation of such responses.

MATERIALS AND METHODS

Strains

S. cerevisiae strains used in this study are listed in Table 1. All share the S288C genetic background, except DBY8834 and DBY8781, that have the W303 background. Unless mentioned otherwise, experiments were carried out using strain DBY8724.

Growth Conditions

Cells were grown with shaking (295 rpm) in YPD at 30°C. Early exponential phase cultures (OD_{600} of 0.2–0.4 or $6–12 \times 10^6$ cells/ml) were synchronized by an α factor-induced G1 arrest and a subsequent release in prewarmed fresh YPD medium, to a density of 1×10^7 cells/ml. Culture volumes were 600–800 ml for microarray experiments or 150 ml for all other experiments. The α factor (Sigma-Aldrich, St. Louis, MO) final concentration was 7 nM for DBY8724 and DBY8834, or 0.8 μM for all other strains; incubation time was 90 min.

Menadione or hydrogen peroxide (Sigma-Aldrich) were added at the designated times after release. We maintained the concentration of HP essentially constant by dripping-in fresh medium containing 9 mM HP at a rate of ~0.1 ml/min (by using a peristaltic pump [Bio-Rad, Hercules, CA] equipped with masterflex tubing #13), while monitoring HP concentrations as described previously (Green and Hill, 1984). Stopping the drip-in of HP-containing medium resulted in a fall of its concentration by one-half within 15 min.

Cell Cycle Phase Determination

Cells in samples were counted under the microscope, 200–400 cells per time point. Unbudded cells were considered G1 cells; small-budded cells, with buds <50% of the mother cell size, S phase cells; and large-budded cells, G2 or M phase cells. The percentage of each of these variants was calculated out of the total cells counted.

DNA Content Determination

Flow cytometric analyses were performed as described previously (Haase and Lew, 1997) by using an FACScan workstation (Beckman Coulter, Fullerton, CA). Briefly, cells were fixed in 70% ethanol, washed once with water, and incubated for 1–2 h, at 37°C, in 2 mg/ml boiled RNase A (Sigma-Aldrich) in 50 mM Tris-HCl, pH 7.5. Cells were then spun down and resuspended in a 5 mg/ml pepsin (Sigma-Aldrich), 55 mM HCl solution for 30–60 min at 37°C. This was followed by a 1-h incubation at room temperature with 75 mM propidium iodide (PI; Sigma-Aldrich) dissolved in 180 mM NaCl, 70 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5 ($1\times$ PI solution). After PI staining, $\sim 1 \times 10^6$ cells were placed in a 6-ml polystyrene tube (BD Biosciences, San Jose CA) containing $0.1\times$ PI solution diluted in 50 mM Tris, pH 7.5, lightly sonicated, and analyzed.

HP and MD Concentrations

We probed a range of concentrations for both agents to select the minimal doses sufficient for affecting cell cycle (0.2 mM for HP, 2 mM for MD). Reducing HP concentration to 0.1 mM resulted in growth rates close to normal. Increasing MD concentration to 4 mM produced similar cell cycle effects but seemed to compromise cell viability.

Sampling Cultures for Microarray Analyses

For all microarray experiments, aliquots of 25–35 ml were drawn out for extracting total RNA; cells were vacuum-collected onto a 45-μm filter (Os-

monics, Minnetonka, MN), snap-frozen in liquid nitrogen, and kept at −80°C until use.

Sampling times were as follows: HP1 ([HP] = 0.23 ± 0.04 mM): 0, 14, 20, 30, 40, 55, 63, 70, 77, 85, 105, 112, 120, 140, 155, 170, 190, 230, 250 and 275 min after release from G1 arrest; HP2 ([HP] = 0.28 ± 0.05 mM) : 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 100, 120, 140, 160, 180 min after release; MD1: 0, 14, 20, 30, 40, 55, 70, 77, 85, 100, 120, 140, 170, 210 min after release; and MD2: 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 77, 85, 100, 120, 130, 140 min after release.

RNA Preparation and Hybridization

Total RNA was isolated using the hot acid phenol method, followed by ethanol precipitation (Spellman *et al.*, 1998). Poly(A)+ mRNA, used for all cDNA microarray analyses, was extracted from total RNA by using the Oligotex midi kit (QIAGEN, Valencia, CA). One to 2 μg was used for each labeling reaction. cDNA probes were labeled using a 3′-anchored (oligo)dT primer, essentially as described previously (Spellman *et al.*, 1998). Experimental samples were used to generate Cy5-labeled cDNA probes, whereas mRNA reference pools extracted from unsynchronized cultures grown to early log phase under normal conditions, were used to generate Cy3-labeled cDNA probes. Three different reference RNA preparations were used for hybridization: one for each of the time courses HP1 and MD1, and a third preparation for both HP2 and MD2. Cy5- and Cy3-labeled probes were hybridized together to microarrays printed with polymerase chain reaction-amplified fragments (DeRisi *et al.*, 1997), ultimately allowing us to follow expression (after application of data quality filters; see below) of 4772 *S. cerevisiae* genes.

Data Acquisition and Analysis

Image acquisition and analysis were performed using the GenePix 4000 microarray scanner (Axon Instruments, Union City, CA) and GenePix Pro 3.0, respectively. Data was subjected to quality control filters, normalized, and stored at the Stanford Microarray Database (Sherlock *et al.*, 2001) (http:// genome-www5.stanford.edu/MicroArray/SMD/). Raw data could be downloaded from this site. Subsequent analyses of data from this study only used spots representing successfully amplified genes, with fluorescent intensities that were either >1.2-fold greater than the local background in one channel and >1.05 in the other, or vice versa. For each individual time course, only open reading frames for which >80% of spots followed the rule above were selected. The genes from the four time courses were then grouped. Sequences represented only in HP2 and MD2 were filtered out to remove the many intergenic regions printed on these arrays and similarly were those represented only in one time course. This produced a final list of 4772 genes. Log-transformed (base 2) ratios were used for subsequent analysis (see Web Supplement).

Statistical Analyses

For each time course, we applied a nonparametric *t* test (Troyanskaya *et al.*, 2002) to compare time points before and after the appearance of a stress-induced response. The time points in the "before" and "after" groups were as follows: HP1, before: 14 min after release from G1 arrest, 20, 20/redo (subsequently averaged for presentation); after: 40, 49, 55, 63, 63/redo (subsequently averaged for presentation), 70, 77, 85, 105; HP2, before: 7,14, 21, 28; and after: 35, 42, 49, 56, 63, 70, 77; MD1, before: 14, 20, 30; after: 40, 55, 70, 77, 85, 100, 120, 140; and MD2, before: 14, 21, 28; after: 42, 49, 56, 63, 77, 85, 100, 120, 130, 140.

Genes with p < 0.05 from the *t* test were considered as responding. Because genes that responded weakly to stress sometimes showed p values as high as 0.15, we considered as not responding only those with p values >0.15. Genes considered as responding to one agent but not to the other were those obtained by applying the following rule to the designated gene lists: ((agent1_response1 ∪ agent1_response2) ∩ (agent2_noresponse1 ∩ agent2_noresponse2)).

Data Arrangement and Visualization

All manipulation of preclustering files was achieved using the PCL_Analysis program package (Murray *et al.*, 2004) (http://pcl-analysis.sourceforge.net/). After hierarchical clustering, data were visualized using Java treeview (Saldanha, 2004) (http://jtreeview.sourceforge.net/). Because our interest was in the pattern of expression rather than in absolute values, all figures show data

that were median centered for each time course separately. This facilitates comparison of time courses for which different reference RNA samples were used.

Promoter and Functional Annotations Analyses

For each subcluster, we searched for *cis*-regulatory motifs (represented as PSSMs), within 500-bp upstream of each gene in the subcluster (sequences were retrieved from SGD on July 2, 2002; Cherry *et al.*, 1998). We used a discriminative motif finder (Segal *et al.*, 2003), based on Bayesian networks, which attempts to discover motifs that are overrepresented in the upstream sequences of cluster genes compared with the upstream sequences of genes in all other clusters. During the motif finding phase, motifs are evaluated by their ability to discriminate the upstream sequences of clustered genes from upstream sequences of the other genes. Motifs are selected based on their discriminatory power. Overall, we discovered 11 potentially novel motifs by using this approach. We used the GeneXPress statistical analysis and visualization tool (Segal *et al.*, 2003) to assign p values for the enrichment of these motifs, as well as known fungi motifs from Version 6.2 of TRANSFAC (Wingender *et al.*, 2001), in subclusters of interest compared with the relevant cell cycle phase grouping. This p value represents the discriminatory power that each motif has.

Searches for GO annotations enriched in gene groups compared with the entire yeast genome were performed with GOTermFinder (http://db.yeast-genome.org/cgi-bin/SGD/GO/goTermFinder). Comparing those groups to subparts of the genome was achieved with GeneXPress by using version 1.311 of the GO annotations.

RESULTS

Cell Cycle Effects of HP and MD

To define better the differences in cell cycle arrest between oxidative stress caused by HP and MD, we exposed synchronized cultures to HP or MD at different points in the cell cycle. Cells were synchronized at G1 with α factor as described previously (Spellman *et al.*, 1998) and exposed to a constant concentration of HP (see *Materials and Methods*) for 110 min beginning at two times after release from arrest: 25 and 58 min (Figure 1). As can be seen by comparing Figure 1, A and C, the cells arrested differently depending upon the time of addition of HP. When HP was added at 25 min, after the onset of S phase (Figure 1A), cells accumulated as large-budded cells after a considerable delay in S phase (for a comparison with normal cell cycle progression of synchronized cultures, see Web Supplement Figure i). This is consistent with a block at the immediately subsequent G2 or M phases. Figure 1B shows the results of DNA content analysis, which documents the onset of S phase, the S phase delay,

and the G2/M arrest seen in Figure 1A. However, when HP was added at 58 min, after bud emergence (Figure 1C) and completion of DNA synthesis (Figure 1D), there was no arrest in the same cell cycle; instead, cells continued through to the next cycle. The G2/M arrest does not depend on the cessation of HP exposure; essentially the same results were obtained when HP exposure was continued (our unpublished data).

When synchronized cells were exposed to MD, a different result was obtained. Figure 2A shows just the unbudded (G1) cell counts for a time series of exposures beginning at 25, 40, 60, and 70 min after release from G1 arrest. It is clear from these results, as well as from DNA content measurements (Figure 2B), that in all of these cases MD induced a G1 arrest in the next cell cycle, regardless of the timing of its addition relative to the S phase.

The Oxidative Stress Response Cluster

To study the transcriptional responses underlying the cell cycle effects of HP and MD, we used DNA microarrays to follow changes in abundance of mRNAs for all the yeast genes. We performed duplicate time courses in which HP or MD were added at 25 min after release from G1 (i.e., before the completion of S phase) as described above. Figure 3 shows the robust oxidative stress response (Gasch *et al.*, 2000) for each of the experiments; no major difference between HP and MD was observed in this respect. The figure is the result of clustering of data for the 4772 genes that passed quality filters—only the stress response clusters (459 induced genes and 664 repressed genes) are shown (see Web supplement Figure ii for the entire data set).

We searched these clusters for enriched GO process annotations by using the GO Term Finder program as described in *Materials and Methods*. We found, for both HP and MD, the major features reported previously (e.g., induction of antioxidants and heat shock proteins and repression of the translation apparatus; Gasch *et al.*, 2000). We did not observe enrichment, under any type of oxidative stress, for genes involved in DNA repair or DNA metabolism, although DNA damage is believed to play a significant role in the deleterious effects of oxidative stress (Shackelford *et al.*, 2000; Cooke *et al.*, 2003); only a few DNA repair genes were
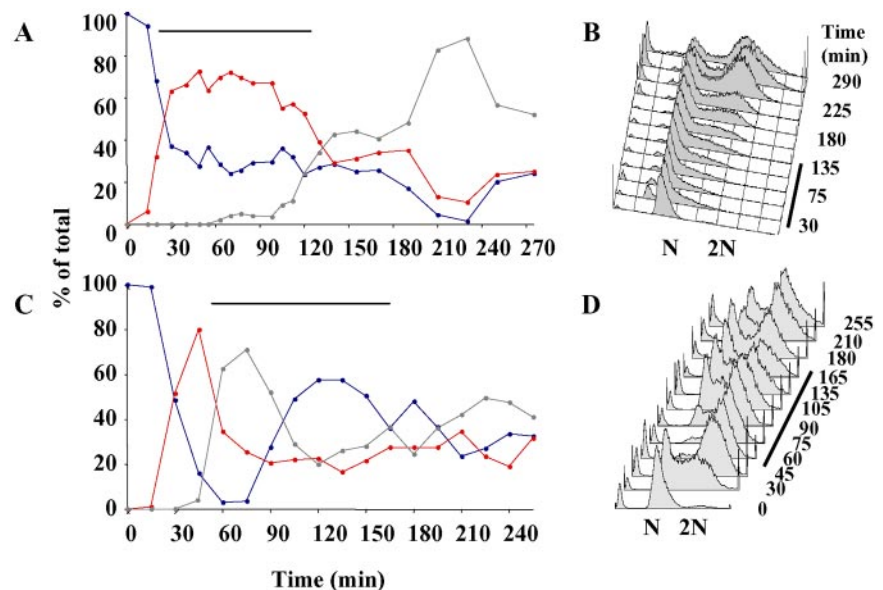
Figure 1. Hydrogen peroxide induces a biphasic cell cycle arrest. (A and C) Cell cycle time courses with percentages of G1 cells (blue line), S-phase cells (red line), and G2/M cells (gray line), out of the total number of cells counted. Synchronized cultures of DBY8724 were released from G1 arrest at time zero and subsequently treated with HP for the time designated by a horizontal line (25 min after release to 135 min in A; 58–165 in C). (B and D) FACS analysis using the DNA-binding dye propidium iodide to measure DNA content (designated as N for haploid, pre-replication, DNA content or 2N for diploid DNA content). Cell samples were taken from the same time courses shown in A and C. Side bars designate incubation times with HP.
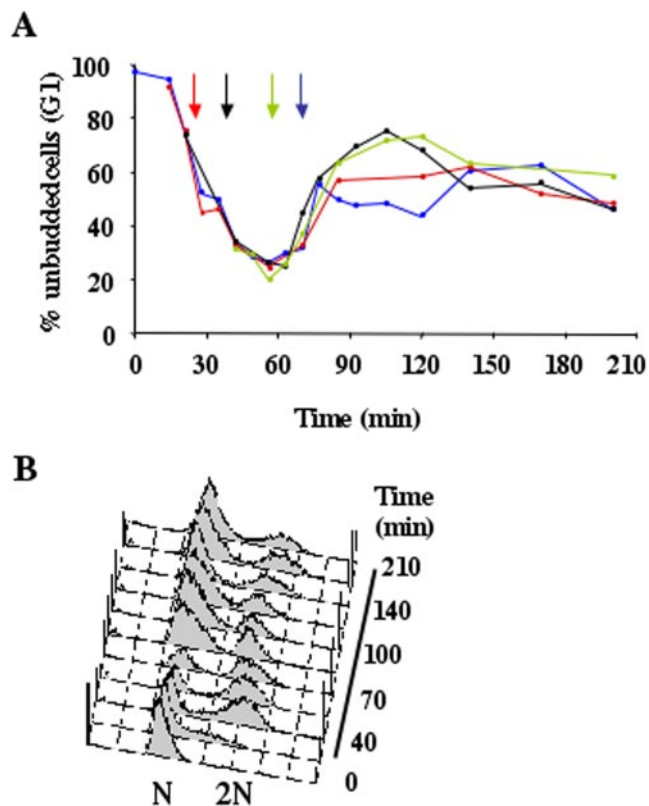
Figure 2. Menadione induces a G1 arrest independent of time of addition. (A) Results from four time courses showing percentages of G1 cells out of all counted cells per time point. MD was added at time points designated with arrows after release from a G1 arrest: red line, 25 min after release; black line, 40 min; green line, 60 min; and blue line, 70 min. (B) FACS analysis by using propidium iodide to measure DNA content of cell samples from a time course in which MD was added 25 min after release from a G1 arrest. Side bar designates the incubation time with MD.

significantly induced, namely, *DDR48*, *CAC2*, and *MAG1*. This is in agreement with previous studies of DNA damage responses in yeast showing a relatively minor transcriptional component (Gasch *et al.*, 2001; Chang *et al.*, 2002).

### Differential Cell Cycle Transcriptional Effects of HP and MD

In the absence of striking differences in the massive transcriptional oxidative stress responses to HP and MD, we looked specifically for differences in cell cycle-related transcription. To this end, we examined the expression of 674 genes with good data out of ~800 genes previously shown to be cell cycle regulated (Spellman *et al.*, 1998). After the initiation of treatment, most of these transcripts seemed to be plainly misregulated, showing basal level transcription, or fluctuations, rather than a clear periodic pattern (Figure 4). This is in contrast to a possible scenario in which HP-treated cells present an accumulation of S or G2 transcripts and MD-treated cells present accumulation of G1 transcripts.

Not all cell cycle expression was halted. Smaller, sometimes delayed, peaks were apparent under MD exposure for S/G2, G2/M, M/G1, and G1 genes (Figures 5 and 6). In addition, for all gene groups, small clusters existed that responded to the stress signal (indicated with arrowheads in
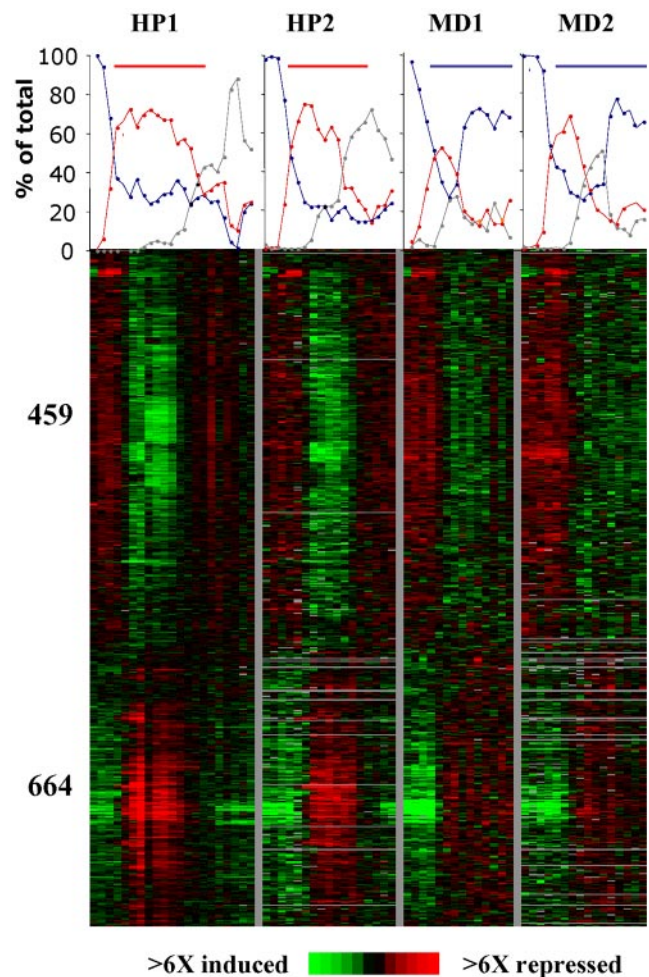
Figure 3. The oxidative stress cluster. Top, cell cycle progression of treated cultures used for expression analyses. Shown are percentages of G1 (blue), S (red), and G2/M (gray) cells out of the total counted. Bottom, overview of the shared oxidative stress transcriptional response. Genes included (see Web Supplement) are those chosen based on hierarchical clustering and visual inspection of the entire filtered data set that respond both to MD and to HP. For each gene in each time course, separately, expression values were median centered to bring out the expression patterns and to assist visual comparison between different time courses in which different reference mRNA batches were used. HP1 is the same time-course experiment presented in Figure 1A. The color scale used to represent variations in transcript abundance is shown in the key at the bottom of the figure. Gray represents missing values.

Figure 4). Figure 5 plots the average gene expression for the principal cell cycle subclusters (Figure 4, arrows). The G2/M cluster was composed of 13 genes that included the two mitotic cyclin genes, *CLB1* and *CLB2*, and the gene encoding the Swi5p transcription factor (Figure 5A). The M/G1 cluster was composed of 18 genes, including the Pho85 cyclin gene *PCL9*, the gene for the cyclin-dependent kinase inhibitor Sic1p, and several genes important for cell wall organization and cytokinesis (Figure 5B). The G1 cluster consisted of eight genes that included cytokinesis and cell wall organization genes. This latter group of genes showed expression patterns that greatly resembled those of the M/G1 cluster, with genes known to perform functions similar to the M/G1 genes, together suggesting that the two clusters are actually one (Figure 5, B and C). A full depiction of subclusters, gene
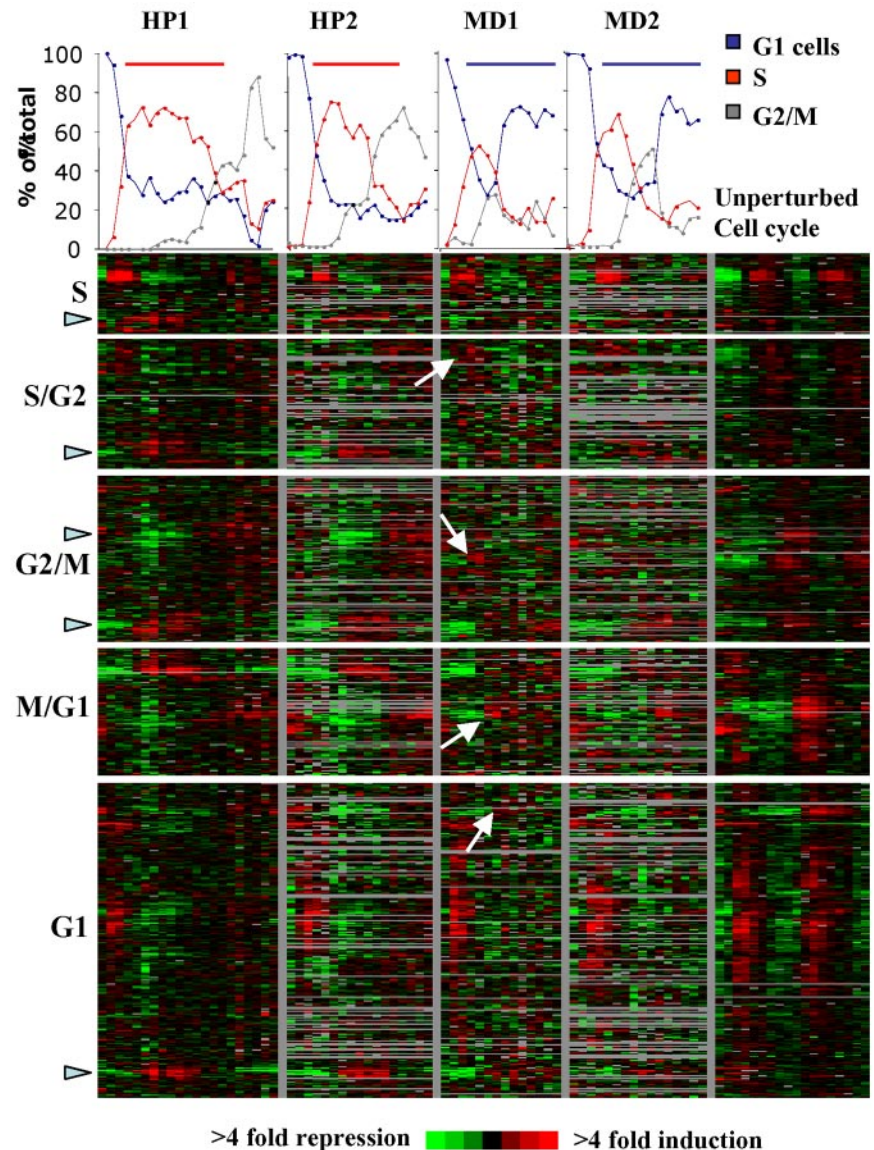
Figure 4. Cell cycle transcription is mostly misregulated under both types of oxidative stress. Shown is an overview of cell cycle-regulated transcription during both types of oxidative stress (four leftmost time courses), as well as under normal conditions (rightmost time course; Spellman *et al.*, 1998). Genes are grouped according to cell phase in which their transcription peaks (shown on the left) and clustered separately in each of these groups. The corresponding physiological time courses are presented on top. Arrowheads designate stress response subclusters. Arrows designate subclusters that maintained periodic transcription under MD exposure. The color scale used to represent variations in transcript abundance is shown in the key at the bottom of the figure. Gray represents missing values.

lists, and enriched promoter binding motifs is presented in Web Supplement Figure iii and its accompanying Table i.

The residual cell cycle clusters that maintained periodic expression under MD (but not HP) treatment included many genes previously associated with regulation by the yeast forkhead homologues Fkh1p and Fkh2p (Zhu *et al.*, 2000); also see Web Supplement Figure iv). The G2/M cluster corresponds to the Clb1 cluster, which is directly regulated by these proteins, whereas the M/G1 and G1 clusters correspond to the Sic1 cluster, which is regulated by a member of the Clb1 cluster, encoding the Swi5p transcription factor. To exhaustively identify similarly regulated genes, we sorted all the genes by Pearson correlation with the values for either *CLB1* or *PIR1*, genes which are at the heart of the G2/M and M/G1 clusters. This resulted in the identification of two additional genes in the G2/M cluster, not reported in Zhu *et al.* (2000) encoding a putative integral membrane protein, Sur7p, and an RNA-binding and localizing protein, Hek2p, suggesting that they may have roles in cell cycle FKH-regulated processes. *HEK2* was previously reported as FKH regulated (Pic *et al.*, 2000). Further examination of the

G1 cluster in Figure 5 similarly implicated a few additional genes: *GIC2*, encoding a small GTPase-interacting protein that localizes to the incipient bud site; *RME1*, encoding a transcription regulator that is regulated by Swi5p (Frenz *et al.*, 2001); *SHQ1*, a gene important for small nucleolar ribonucleoprotein particles stability; and YJL217W, encoding a hypothetical protein.

We also studied the upstream sequences of all the implicated genes for enriched motifs (Table 2) and found several associated with forkhead-protein–regulated transcription. Notable among these are the Mcm1p binding site in promoters of genes of the G2/M cluster and the Swi5p binding site in promoters of genes of the M/G1 and G1 clusters. Mcm1p is part of the active complex that includes Fkh2p, and Swi5p is the actual target of this complex and the regulator of the M/G1 cluster members. Fkh1p is not as likely as Fkh2p to be implicated in the difference between HP and MD response, because, as shown in Table 2, the majority of promoters of genes in the G2/M cluster (40–60%) were shown previously to be bound by components of the Mcm1p–Fkh2p–Ndd1p complex, whereas only 23% were shown to be bound by
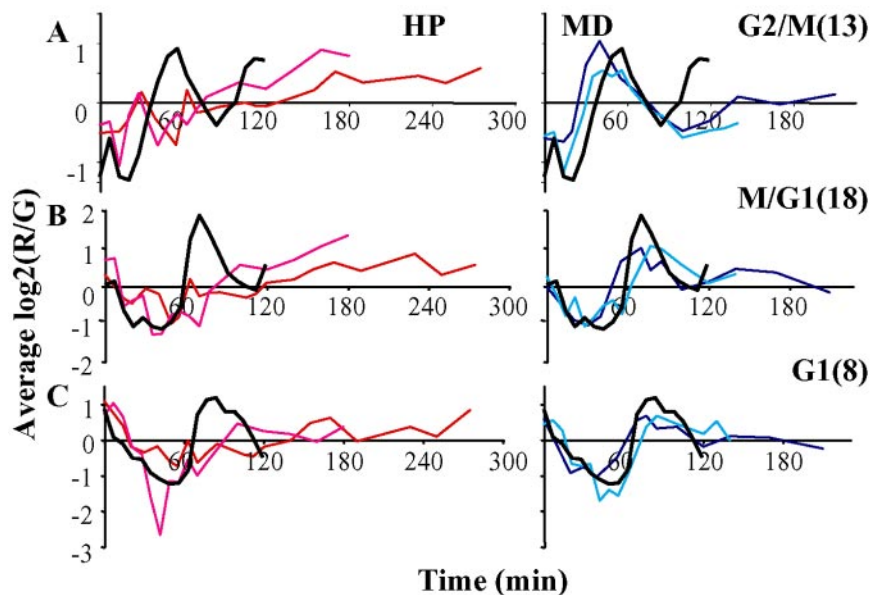
Figure 5. Minimal gene clusters continue cycling under MD exposure but not under HP exposure. Shown are average expression time courses for small clusters of genes presenting a close-to-normal expression patterns under MD treatment, but not HP. Black lines represent normal cell cycle expression (Spellman *et al.*, 1998); red and magenta lines, HP1 and HP2 time courses, respectively; blue and light blue lines, MD1 and MD2 time courses, respectively. (A) G2/M cluster, comprised of *SUR7, BUD3, YOR315W, ALK1, HST3, SWI5, CLB1, CLB2, YNL058C, YNL057W, IQG1, CHS2, SRC1,* and *HEK2*. (B) M/G1 cluster, *SIC1, YNL046W, CYK3, YLR194C, ASH1, AMN1, NIS1, TEC1, DSE4, YPL158C, PCL9, DSE3, EGT2, PST1, PIR3, PIR1, HSP150,* and *GPA1*. (C) G1 cluster, *YJL217W, GIC2, SHQ1, DSE2, DSE1, SCW11, CTS1,* and *RME1*.

Fkh1p (Simon *et al.*, 2001). The promoter binding data presented in Table 2 fortify the connection between the forkhead homologues, particularly Fkh2p (as part of the Mcm1p–Fkh2p–Ndd1p complex), and residual cell-cycle-regulated gene expression.

Testing the Role of Forkhead Homologues after MD Exposure

The experiments and analysis described above implicate the Mcm1p–Fkh2p–Ndd1p complex in maintaining the ability to continue the cell cycle after introduction of MD and the expression of a full-blown oxidative stress response. The experiments also raise the possibility that this function of the complex is inhibited in HP-treated cells. We reasoned that Mcm1p is probably not affected by HP, because several genes of the pheromone pathway that are known to be regulated by this protein alone, i.e., *MFA2, AGA1,* and *AGA2* (Simon *et al.*, 2001), were properly regulated in both MD- and HP-treated cells (see Web Supplement). This suggested that it is the activity of the assembled complex that may be affected by HP.

To test whether inactivation of the Mcm1–Fkh2–Ndd1 complex could affect the arrest point under oxidative stress, we treated cells mutant for both *FKH1* and *FKH2* with MD, such that the redundancy of the two forkhead proteins would not mask the effect of oxidative stress. Because *fkh1fkh2* double mutants do not go readily through cytokinesis, discrimination between G1 and G2/M cells is difficult. However, small-budded cells representing S phase cells are easily discernable and were therefore used as a marker of the S phase delay typical of a pre-S HP exposure. Figure 6 compares the responses of *fkh1fkh2* double mutants and a nearly isogenic wild-type strain to MD. *fkh1fkh2* cells exposed to MD after the onset of S phase proceeded through S just as untreated cells, albeit a little slower (Figure 6, F and G). This was similar to cell cycle progression in wild-type cells treated with MD (Figure 6, B and C). However, *fkh1fkh2* cells treated just before the onset of S (25 min after release) were delayed in S phase as wild-type cells exposed to HP (Figure 6E). This effect did not represent a general exacerbation of stress responses in these mutants, because re-

sponses to HP and to osmotic stress were comparable in mutant and wild-type strains (Web Supplement Figure v).

These results support the hypothesis that differential interference with the function of a forkhead transcription factor, probably Fkh2p, could be responsible for the differential cell cycle arrest caused by HP and MD.

Additional Differential Transcriptional Responses to HP and MD

To find, in an organized and relatively unbiased manner, any additional genes differentially expressed after HP or MD exposure during the cell cycle, we applied a nonparametric *t* test to compare expression of each gene, before and after addition of the oxidative stress agent (see *Materials and Methods*). Two lists were thus generated: one containing 867 genes responding to HP but not to MD and another containing 276 genes responding only to MD. Details can be found in the Web Supplement (Figure vi).

We found only a few, marginally significant, enriched annotations in these lists relative to all stress response genes. More significant enrichment was found for one set of genes involved in general regulation of mRNA transcription (p = 0.003; hypergeometric distribution): TAF6, TAF10, TAF12, TAF14, TOA1, RAD3, and TFB4 were significantly more repressed in HP-treated cells. This suggests a differential role of HP in reduction of mRNA transcription, as opposed to the general stress repression of genes associated with translation (e.g., ribosomal proteins).

Differences in the level of single genes also were detected. *GRX1, GRX3,* and *GRX5* were induced by HP but not by MD (Figure 7A). The products of these genes form the glutaredoxin system, functioning together with glutathione as electron donors in thiol-disulfide exchange reactions (Carmel-Harel and Storz, 2000). Such reactions are crucial for protecting protein thiol groups, particularly those of cysteine residues, from oxidation-induced formation of sulfur bridges and misfolding. *YCL033C*, a gene that encodes a methionine (R)-sulfoxide reductase also was coexpressed with these three. Likewise, *MXR1*, the gene encoding the similar enzyme responsible for reducing the (S)-enantiomer, and using the thioredoxin system for electron transfer (Le-
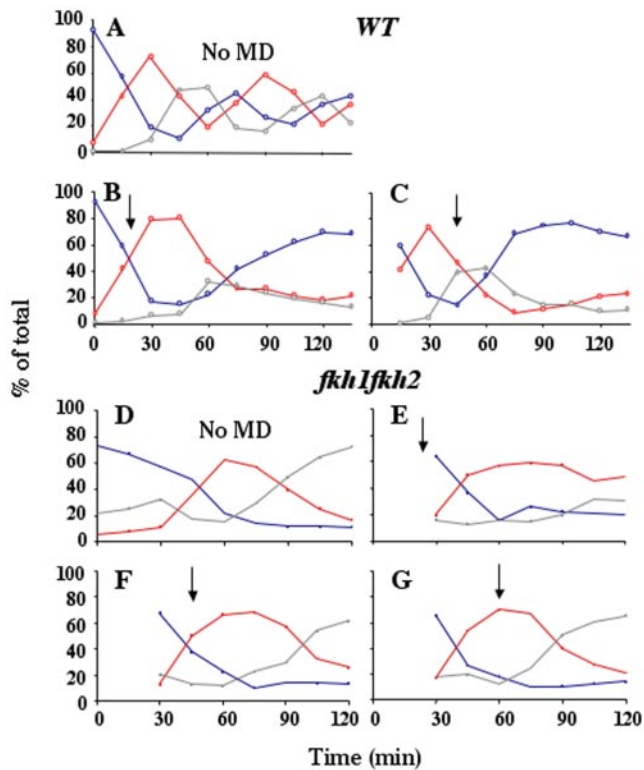
Figure 6. MD mimics HP-induced S-phase arrest in *fkh1fkh2* double mutants. Shown are cell cycle time courses, with percentages of G1 cells (blue line), S-phase cells (red line), and G2/M cells (gray line), out of the total number of cells counted, of the nearly isogenic strains DBY8781 (wild type; open circles) and DBY8834 (*fkh1fkh2bar1*). The only non-fkh difference in these strains (*bar1*) required the use of different concentrations of α factor for synchronization (see *Materials and Methods*). Cultures synchronized at G1 were divided into equal volumes, released from arrest at time zero, and treated with 2 mM MD at times designated with arrows. Wild-type cells were either not treated with MD (A), or exposed to MD starting at 21 min after release (before completion of S phase; B); or starting at 45 min after release (at the end of S phase; C). *fkh1fkh2* cells were either not treated (D), treated before initiation of S phase (25 min after release; E), or treated after initiation of S phase, starting at 40 min (F) or 60 min (G) after release.

vine *et al.*, 2000) was much more induced by HP than MD. The three thioredoxin genes *TRX1*, *TRX2*, and *TRX3* behaved similarly (Figure 7A). These differences suggest that the cellular responses to HP and MD are in some way distinguishable beyond the differences in cell cycle regulation.

As with HP-responding genes, additional responses specific to MD were observed at the single gene level. These could point at specific outcomes of MD exposure. However, in some cases it may be the HP-specific lack of response that is more interesting than the MD-specific response. Notable among these was the missing repression of *DUN1*, a kinase that is a major player in activation of the DNA damage checkpoint (Figure 7B, top). As a general theme, G1-peaking transcripts were repressed under both types of oxidative stress (Figure 4). However, *DUN1* was not repressed under HP exposure in contrast to a marked repression under MD exposure. Dun1p has been shown to regulate the transcription of genes involved in DNA damage repair (Zhou and Elledge, 1993). Among them is *RNR4*, one of two catalytic subunits of the ribonucleotide reductase complex (Huang

and Elledge, 1997) that was included among the HP-induced genes and showed a distinctly late induction. We pulled out genes with expression profiles that presented a high Pearson correlation coefficient (> 0.7) with that of this gene and found those to include the second catalytic-subunit-encoding gene, *RNR2*, as well as seven more genes encoding reductases, one dehydrogenase, flavohemoglobin, and three Ty retrotransposons (Figure 7B, bottom). The functional significance of these late induced genes is yet unclear, but induction of the *RNR* genes serves as a marker confirming the functional significance of the lack of *DUN1* repression under HP exposure, suggesting counter-balancing forces affecting *DUN1* expression, and also supports the idea of an underlying distinction in the responses to MD and HP.

DISCUSSION

The results described above shed light on the differential consequences of exposure of yeast cells to two different sources of reactive oxygen species: hydrogen peroxide, itself a reactive oxygen species, and Menadione, which produces such species internally. Following leads in the literature (Flattery-O'Brien and Dawes, 1998), we found a dramatic difference in the cell cycle response of synchronized cells to the two agents: HP caused a delay in S and ultimately a G2/M arrest, but only when administered before or during the S phase. Menadione resulted in a G1 arrest, regardless of when it was administered.

The differences in cell cycle response between MD and HP are probably not due to a difference in intracellular concentrations, because a decrease in HP concentration or an increase in MD concentration did not converge to one type of effects or the other. This result supports previous findings that preexposure to HP did not increase resistance to a subsequent challenge exposure to MD (Flattery-O'Brien *et al.*, 1993).

The differences we found in the global transcriptional response to the two agents were much less than dramatic: the overall response, which includes several hundred genes whose expression is increased and several hundred genes whose expression is decreased is grossly similar, as was observed previously (Gasch *et al.*, 2000). Additional, less conspicuous, agent-specific transcriptional responses also exist, but they mostly fail to reveal underlying functional differences.

Several approaches to a more detailed analysis did reveal some differences, the major one of which is that MD treatment does not cause complete misregulation of cell cycle-regulated transcription, whereas HP treatment causes an almost complete loss of the periodic transcription patterns reported previously (Cho *et al.*, 1998; Spellman *et al.*, 1998). Specifically, two small gene clusters, which are essential for cell cycle progression through G2 and M (Fitch *et al.*, 1992; Toyn *et al.*, 1997), maintain normal transcription patterns under MD treatment but not HP.

We were able to trace this difference to the activity of the yeast forkhead homologues by noting first that the forkhead transcription signature reported by Zhu *et al.* (2000) corresponded with the residual periodic transcription pattern left after MD treatment. This suggests that forkhead-protein–associated transcription is responsible for driving MD-treated cells through G2 and M. Supporting this hypothesis, we observed that *fkh1fkh2* double mutants, when treated with MD, now altered the cell cycle similar to wild-type HP-treated cells. Specifically, when MD was applied early in S, cells delayed considerably as small-budded cells, whereas when MD was applied later, cells progressed to become

Table 2. Promoter binding and enriched *cis*-regulatory motifs

| Cell cycle subcluster[a] | Promoter binding[b] | | | | | | Enriched motifs[c] | |
	Fkh1	Fkh2	Mcm1	Ndd1	Swi5	Ace2	(Name, #/total, P-value)	Genes with motif[d]
G2/M (13)	SUR7 YOR315W CLB2	SUR7 YOR315W ALK1 SWI5 CLB2 YNL058C	SUR7 YOR315W ALK1 SWI5 CLB2 YNL058C	SUR7 YOR315W HST3 SWI5 CLB2 YNL058C IQG1 CHS2		SUR7 YOR315W	G2/M_cell_cycle, 12/13, 6.7E-5	SUR7 BUD3 YOR315W ALK1 HST3 SWI5 CLB1 CLB2 YNL057W IQG1 CHS2 SRC1 (but not YNL058C)
							STRE, 8/13, 0.02	SUR7 HST3 CLB2 YNL058C YNL057W IQG1 CHS2 SRC1
							MCM1, 7/13, 0.03	ALK1 HST3 SWI5 CLB2 YNL058C CHS2 SRC1
M/G1 (18)	AMN1	AMN1	PIR3 PIR1		SIC1 YLR194C ASH1 AMN1 JIP1 TEC1 YPL158C PCL9 EGT2 PST1 PIR3 PIR1 HSP150	AMN1 NIS1 TEC1 DSE4 EGT2 HSP150	M/G1_cell_cycle (SWI5), 18/18, 2.5E-6	SIC1 YNL046W CYK3 YLR194C ASH1 AMN1 NIS1 TEC1 DSE4 YPL158C PCL9 DSE3 EGT2 PST1 PIR3 PIR1 HSP150 GPA1
G1 (8)	DSE2 CTS1	GIC2 DSE2 DSE1 CTS1			SCW11 CTS1	DSE2 DSE1 SCW11 CTS1	G1_cell_cycle, 8/8, 7.6E-7	YJL217W GIC2 SHQ1 DSE2 DSE1 SCW11 CTS1 RME1
							(SWI5), 7/8, 5E-5	YJL217W GIC2 SHQ1 DSE2 DSE1 SCW11 RME1

[a] Subclusters are designated by the cell cycle phase in which their expression normally peaks. Number of genes in each subcluster is shown in parentheses.

[b] Promoter binding by transcription factors, shown below (based on promoter localization data; Simon *et al.*, 2001). Genes shown are those for which binding by the designated transcription factor was considered statistically significant ($p < 0.002$).

[c] Motif logos. Shown are those that were shared and enriched in promoters of the clustered genes. Novel motifs are designated with the respective subcluster name; known motifs are designated with TRANSFAC identifiers. Motif name is followed by the number of genes with motif out of the total number of genes in the subcluster, and the p value describing the significance of motif enrichment in the subcluster compared with the entire cell cycle phase cluster.

[d] Genes containing the presented motifs in their promoter.

large-budded cells. This mimicked the arrest phenotype of HP-treated cells both with regards to the initial arrest point as well as to the timing requirements of the treatment.

Based on motif analysis and promoter binding data (Simon *et al.*, 2001), it is likely that Fkh2p is the key player in regulating the misregulated genes rather than Fkh1p. Mcm1p and Fkh2p cooperatively bind the promoters of target genes and subsequently recruit Ndd1p (Koranda *et al.*, 2000; Hollenhorst *et al.*, 2001). We were able to rule out inactivation of Mcm1p by HP, because the expression of several M/G1 genes, which are known targets of Mcm1p alone, showed normal expression. This suggests that HP specifically affects gene regulation associated with the assembled complex.

One feature that we could not fully explore, because of loss of synchrony over time, is the behavior of HP-treated wild-type cells (or MD-treated *fkh1fkh2* double mutants) in the subsequent cell cycles. We have preliminary indications that the arrest, in these cases, will be in the next G2/M, as opposed to the next G1 (Figure 1; also see Web Supplement Figure vii, which shows morphological details of arrested

cells). This is consistent with the idea that it is an S-phase function that is lost in HP- but not MD-treated cells and causes the G2/M arrest.

The putative S-phase function could be the assembly of the Mcm1p–Fkh2p–Ndd1p complex subunits on promoters of target genes. This assembly is completed during S phase (Koranda *et al.*, 2000; Simon *et al.*, 2001). We have shown that misregulation of the complex target genes is correlated with the arrest of HP-treated cells first in S, and later in G2/M. We further showed that inactivation of this complex by forkhead gene disruption is sufficient to change the cell cycle arrest in MD cells from G1- to the S-phase arrest observed normally in HP-treated cells. Together, this evidence suggest that Mcm1p–Fkh2p–Ndd1p complex inactivation may occur during treatment with HP, and it could play a causative role in the cell cycle arrest, possibly together with other factors.

It was previously reported that the DNA damage checkpoint protein Rad9p contributes to a G2/M arrest in response to HP treatment (Flattery-O'Brien and Dawes, 1998). However, this does not explain the S-phase delay we observe after HP treatment. Another mechanism that was
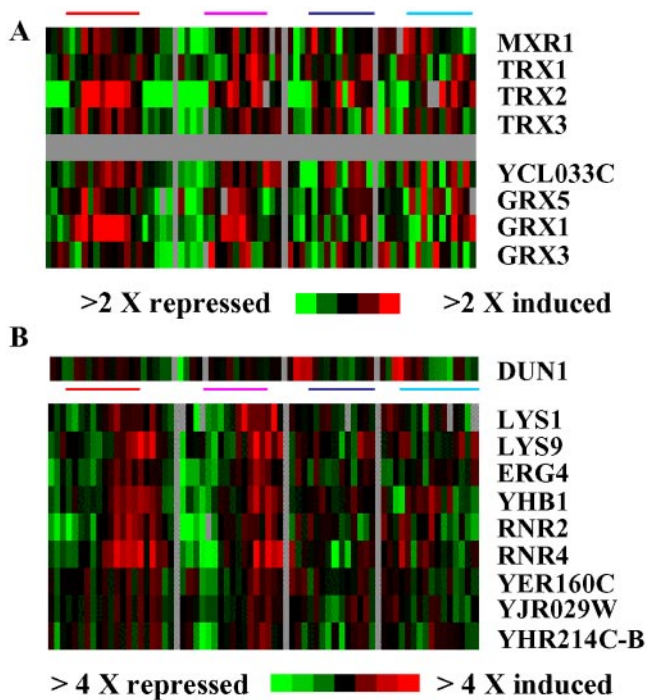
Figure 7. Single gene differences between HP and MD responses. (A) HP-specific induction of thiol protein reduction systems. Expression profiles of genes involved in thiol-disulfide exchange reactions. Horizontal lines represent times of exposures to HP (red and magenta lines) and MD (blue and light blue lines). (B) Differential expression of DUN1 and its targets. Expression profiles of DUN1 (top) and its targets (bottom) during HP and MD time courses. HP1 and HP2 are designated with red and magenta lines, respectively; MD1 and MD2 are designated with blue and light blue lines, respectively. The color scale used to represent variations in transcript abundance is shown in the key at the bottom of the figure. Gray represents missing values.

found by others to be activated by HP might be more relevant to the cell cycle arrest phenotype we observe. Transcriptional activation of the *RNR* genes, which are responsible for the rate-limiting step of dNTP and DNA synthesis (Huang and Elledge, 1997), and induction of the *MAG1* gene, which encodes a DNA-dealkylating enzyme, are markers of the activation of the DNA replication checkpoint (Zhu and Xiao, 2001). This pathway senses blocks in DNA replication and transmits the signal through Mec1p, Rad53p, and Dun1p, delaying mitotic entry (Weinert *et al.*, 1994; Navas *et al.*, 1995; Paulovich and Hartwell, 1995; Desany *et al.*, 1998). The occurrence of *RNR* and *MAG1* gene activation that we observe after exposure to HP, and the dependence of the cell cycle arrest in treatment initiation before S suggests that activation of the replication checkpoint is among the first events occurring during HP-induced oxidative stress and may be responsible for inactivating forkhead-associated transcription. Two lines of evidence support this hypothesis: first, Rad53p phosphorylation was shown to be induced after exposure to HP during S, but not after, and to lead to an S-phase delay (Leroy *et al.*, 2001); second, ionizing radiation was shown to cause a G2/M arrest accompanied by repression of forkhead-associated transcription in wild-type cells, but not in *MEC1* mutants (Gasch *et al.*, 2001).

How might HP lead to different cell cycle effects than MD? One possibility, discussed above, relates HP exposure to interference with DNA replication. Other possibilities, per-

haps related, are suggested by the remarkably few individual genes that are differentially expressed after treatment with the two oxidative stress agents and represent functional categories that are distinct between MD and HP. Several of these are known as important for protecting cells from oxidation of thiol groups and generation of aberrant disulfide bonds (e.g., *GRX1*, *GRX4*, *GRX5*, *TRX1*, *TRX2*, *TRX3*, *MXR1*, and *YCL033C*). There is ample precedent in the literature pointing to protein modification by thiol group oxidation as a specific outcome of HP exposure (reviewed in Rhee *et al.*, 2000). Both HP and superoxide ions can potently modify thiol groups in various molecules in vitro. However, HP is able to directly react with glutathione, unlike superoxide ions, thus potentially perturbing this important antioxidant system and affecting its protective capacities (Winterbourn and Metodiewa, 1999). Some of the genes shown in our study to be induced by HP were identified in previous studies as well. Such was the glutaredoxin-encoding *GRX1* gene that was reported to respond primarily to HP (Grant *et al.*, 2000). In addition, *YCL033C*, which encodes a methionine (R)-sulfoxide reductase, and is coexpressed with GRX1, was previously reported to protect cells from oxidative protein damage, specifically that caused by HP (Kryukov *et al.*, 2002). Together with *GRX3* and *GRX5*, these genes define a small HP-specific regulon.

It therefore is tempting to speculate that HP might cause a distinct cell cycle arrest due to its ability to modify protein cysteines or methionines. There is evidence in the literature for such regulatory modifications caused by HP. In *Escherichia coli*, the mechanism by which HP exposure causes induction of the HP regulon is through the formation of an intramolecular disulfide bond within the product of the *oxyR* gene, which converts it to a transcriptional activator (Zheng *et al.*, 1998). In yeast, the transcriptional regulator Yap1p is modified by the glutathione peroxidase-like enzyme Gpx3p in response to HP exposure (Delaunay *et al.*, 2002).

Functional Conservation of Forkhead Protein Roles in Oxidative Stress Responses in Yeast and Mammals

In mammals, members of the FOXO protein subfamily, are the orthologs of *C. elegans Daf-16* longevity transcription factor (Lin *et al.*, 1997; Ogg *et al.*, 1997). Both Daf-16 and the FOXO proteins are known to protect cells from oxidative stress (Kops *et al.*, 2002; Nemoto and Finkel, 2002; Murphy *et al.*, 2003). Their mode of response to oxidative stress, however, is still unclear. Reports exist showing both that HP decreases nuclear localization of FOXO proteins (Nemoto and Finkel, 2002), or increases it (Brunet *et al.*, 2004). Similarly, the effects of FOXO proteins on cell cycle progression are conflicting. Whereas most reports suggest that active FOXO proteins cause a cell cycle arrest (either a G1 arrest [Medema *et al.*, 2000] or a G2/M arrest [Furukawa-Hibi *et al.*, 2002; Tran *et al.*, 2002]), one study showed that an active FOXO protein actually facilitates the M/G1 transition (Alvarez *et al.*, 2001). For another forkhead protein, FOXM1b, such a cell cycle promoting affect is the accepted mode of action (Wang *et al.*, 2002).

The apparent involvement of yeast Fkh2p in cell cycle regulation and in responses to oxidative stress presents a striking similarity to combined properties of mammalian forkhead proteins and suggests functional conservation. This presents opportunities for further study of this involvement, which might shed light on the conserved functions of forkhead proteins in higher organisms and vice versa. Regulatory mechanisms, or even structural features of the proteins relevant to oxidative stress might be conserved as well.

The results presented in this article allow for a better understanding of the varied effects of oxidative stress. We have provided evidence suggesting that the Mcm1p–Fkh2p–Ndd1p G2/M transcriptional complex is involved in mediating the effects of oxidative stress on the yeast cell cycle. Unraveling this mechanism expands the known conservation in the cellular responses toward oxidative stress, and the roles of forkhead proteins in them, from *C. elegans* to *S. cerevisiae*.

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