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Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*

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Summary

The *Saccharomyces cerevisiae* Yap1p transcription factor is required for the H_2O_2-dependent activation of many antioxidant genes including the *TRX2* gene encoding thioredoxin 2. To identify factors that regulate Yap1p activity, we carried out a genetic screen for mutants that show elevated expression of a *TRX2–HIS3* fusion in the absence of H_2O_2. Two independent mutants isolated in this screen carried mutations in the *TRR1* gene encoding thioredoxin reductase. Northern blot and whole-genome expression analysis revealed that the basal expression of most Yap1p targets and many other H_2O_2-inducible genes is elevated in Δ*trr1* mutants in the absence of external stress. In Δ*trr1* mutants treated with H_2O_2, the Yap1p targets, as well as genes comprising a general environmental stress response and genes encoding protein-folding chaperones, are hyperinduced. However, despite the elevated expression of genes encoding antioxidant enzymes, Δ*trr1* mutants are extremely sensitive to H_2O_2. The results suggest that cells lacking thioredoxin reductase have diminished capacity to detoxify oxidants and/or to repair oxidative stress-induced damage and that the thioredoxin system is involved in the redox regulation of Yap1p transcriptional activity.

Introduction

Aerobically growing cells are continuously challenged by the formation of reactive oxygen species (ROS) that arise from an incomplete reduction of molecular oxygen during respiration. If not eliminated, ROS are dangerous to cells as they can damage all cellular components (reviewed by Halliwell and Gutteridge, 1999). Most organisms possess protective antioxidant molecules and enzymes, and many cells are able to adapt to oxidative stress by increasing the levels of antioxidant enzymes. For example, *Saccharomyces cerevisiae* cells treated with low doses of H_2O_2 adapt and become resistant to otherwise lethal doses of H_2O_2 by increasing the transcription of many antioxidant enzymes (reviewed by Jamieson, 1998).

Yap1p (yeast AP-1) is critical in regulating the *S. cerevisiae* adaptive response. The transcription factor was identified as a functional homologue of mammalian AP-1 on the basis of its ability to bind to an AP-1 recognition element (Harshman *et al.*, 1988). Later studies showed that *yap1* null mutants are hypersensitive to oxidative stress (Schnell *et al.*, 1992). Expression analysis of individual genes showed that Yap1p regulates the expression of several genes whose products play major roles in the oxidative stress tolerance. These targets include *GSH1* encoding γ-glutamylcysteine synthetase (Wu and Moye-Rowley, 1994; Stephen *et al.*, 1995), *GLR1* encoding glutathione reductase (Grant *et al.*, 1996), *GPX2* encoding glutathione peroxidase (Inoue *et al.*, 1999), *TRX2* encoding thioredoxin 2 (Kuge and Jones, 1994; Morgan *et al.*, 1997), *TRR1* encoding thioredoxin reductase (Charizanis *et al.*, 1999; Lee *et al.*, 1999a), and the *TSA1*- and *AHP1*-encoded thioredoxin peroxidases (Lee *et al.*, 1999b). Whole-genome expression analysis by two-dimensional protein gels (Lee *et al.*, 1999a) and DNA microarrays (Gasch *et al.*, 2000) showed that Yap1p regulates the expression of many more genes in response to treatment with H_2O_2 as well as the superoxide-generating compound menadione and the thiol-oxidant diamide. A Yap1p recognition element was defined by studies of Yap1p binding to the *TRX2* (5′-TTAG/CTAA) (Kuge and Jones, 1994) and *GSH1* (5′-TTAGTCA) (Wu and Moye-Rowley, 1994) promoters and is present at many of the promoters of genes whose expression is induced in a Yap1p-dependent fashion.

The amino-terminus of Yap1p contains a bZip DNA-binding domain, which is conserved among the AP-1 family of proteins, including the mammalian cJun and cFos and *S. cerevisiae* Gcn4p (Moye-Rowley *et al.*,

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1989). A carboxy-terminal cysteine-rich domain (c-CRD), containing three Cys–Ser–Glu repeats, was found to be critical for Yap1p-dependent gene activation and resistance to H_2O_2 and diamide (Kuge *et al*., 1997; Wemmie *et al*., 1997). A second, more amino-terminal, cysteine-rich domain (n-CRD), also containing three cysteine residues, is only required for resistance to H_2O_2 (Coleman *et al*., 1999). There is no increase in Yap1p protein levels and only a modest increase in Yap1p DNA-binding activity in response to oxidative stress (Kuge *et al*., 1997). Instead, the localization of the transcription factor changes dramatically (Kuge *et al*., 1997). In the absence of stress, Yap1p is present in both the cytoplasm and the nucleus. Upon treatment with H_2O_2 and diamide, the protein is concentrated in the nucleus. Recent studies have shown that Yap1p localization is controlled by Crm1p-mediated nuclear export and that there are nuclear export signals within both the n-CRD and the c-CRD (Kuge *et al*., 1998; Yan *et al*., 1998; Coleman *et al*., 1999). Site-directed mutagenesis has indicated that cysteines within both the n-CRD and the c-CRD are critical for resistance and for the appropriate subcellular localization of Yap1p after oxidative stress (Kuge *et al*., 1998; Yan *et al*., 1998; Coleman *et al*., 1999). These results raise the intriguing possibility that the cysteines serve as redox sensors that regulate the accessibility of the nuclear export signals.

To gain insight into the regulation of Yap1p transcriptional activity, we carried out a genetic screen designed to identify new genes involved in the Yap1p regulatory pathway. This genetic screen allowed us to isolate genes conferring induced expression to the Yap1p-dependent thioredoxin 2 (*TRX2*) gene in the absence of oxidative stress. Here, we report the isolation of mutations in the *TRR1* gene encoding thioredoxin reductase 1 and the effects of the *trr1* mutations on the expression of Yap1p targets.

Results

Isolation of mutants with induced TRX2 *expression in the absence of* H_2O_2

The *TRX2* gene, which encodes thioredoxin 2, is strongly induced by H_2O_2 in a Yap1p-dependent manner (Kuge and Jones, 1994). To isolate mutants with altered Yap1p-dependent expression of *TRX2*, we constructed a fusion between the *TRX2* promoter and the *HIS3* gene. The promoterless *HIS3* gene and the *TRX2–HIS3* fusion were then integrated into haploid YPH499 cells that carry a chromosomal *HIS3* deletion, generating YRS92 and YRS94 respectively. Residual expression from the promoterless *HIS3* gene was sufficient to allow both strains to grow on SC minimal medium lacking histidine (SC–His). However, YRS92 did not grow on SC–His containing either 10 mM or 50 mM concentrations of the His3p inhibitor 3-amino-1,2,4,-triazole (3-AT), and YRS94 only showed some residual growth in the presence of 50 mM 3-AT inhibitor (Fig. 1). Consistent with H_2O_2-dependent induction of *TRX2* expression, YRS94 cells showed strong growth on SC–His plates containing 0.4 mM H_2O_2 in addition to 50 mM 3-AT (data not shown).

To isolate mutants that constitutively express the *TRX2–HIS3* fusion, YRS94 was mutagenized with methanesulphonic acid ethyl ester (EMS) and plated on SC–His containing 75 mM 3-AT. Two mutants, YRS94#4 and YRS94#11, which showed strong growth in the presence of 50 mM and 75 mM 3-AT in the absence of H_2O_2 (Fig. 1), were chosen for further study.

To determine whether the ability of YRS94#4 and YRS94#11 to grow on the SC–His medium in the presence of 75 mM 3-AT resulted from a *cis*-acting mutation at the *TRX2* promoter fused to the *HIS3* gene or from a *trans*-acting mutation, we examined the expression of the endogenous *TRX2* gene by Northern blots (Fig. 2A). In the absence of H_2O_2, the *TRX2* mRNA levels were elevated in YRS94#4 and YRS94#11 mutant cells compared with wild-type cells grown in YPD (Fig. 2A, lanes 3 and 5 compared with lane 1). Upon a 30 min exposure to 0.1 mM H_2O_2, *TRX2* expression was further induced in the wild-type cells (Fig. 2A, lane 2) and in the YRS94#4 (Fig. 2A, lane 4) and YRS94#11 (Fig. 2A, lane 6) mutant cells. On average, compared with YRS94, the basal levels of the *TRX2* mRNA were threefold higher in YRS94#4 and fourfold higher in YRS94#11. *TRX2* expression was induced threefold for YRS94 and YRS94#4 and twofold for YRS94#11 upon treatment with H_2O_2.

Similar results were obtained by monitoring β-galactosidase activity in cells transformed with a plasmid containing the *lac*Z reporter gene under the control of the *TRX2* promoter (Fig. 2B). In the absence of H_2O_2, the basal β-galactosidase activity of the YRS94#4 and YRS94#11 mutants was significantly higher than the basal activity of the wild-type strain (Fig. 2B). Upon a 30 min exposure to 0.1 mM H_2O_2, the wild-type and mutant strains showed increases in β-galactosidase activity. A m*TRX2–lacZ* plasmid carrying mutations in the Yap1p binding sites of the *TRX2* promoter was not expressed in either wild-type or mutant cells, indicating that the enhanced *TRX2* basal expression is Yap1p dependent (Fig. 2B). Taken together, these results suggest that the mutation leading to elevated expression of the *TRX2–HIS3* and *TRX2–lacZ* fusions and the endogenous *TRX2* gene is located in a *trans*-acting regulatory element.

The basal *TRX2* mRNA levels in the mutants are approximately equal to the induced *TRX2* mRNA levels found in the wild-type strain. In contrast, the basal

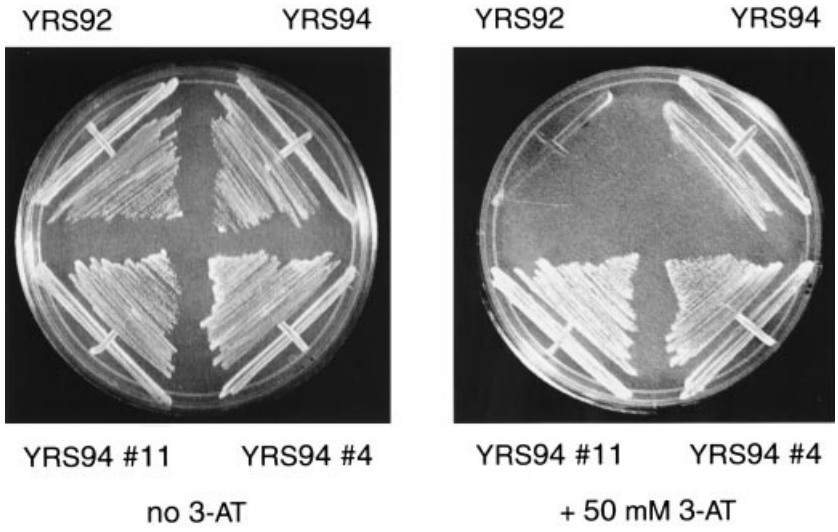

Fig. 1. Growth of parent strain and mutants selected to express constitutively a *TRX2–HIS3* gene fusion. YSR92 carrying a promoterless *HIS3* gene, YSR94 carrying a *TRX2–HIS3* fusion and the YRS94#4 and YRS94#11 mutant strains were streaked on SC–His or on SC–His containing 50 mM 3-AT. Plates were incubated for 2 days at 30°C.

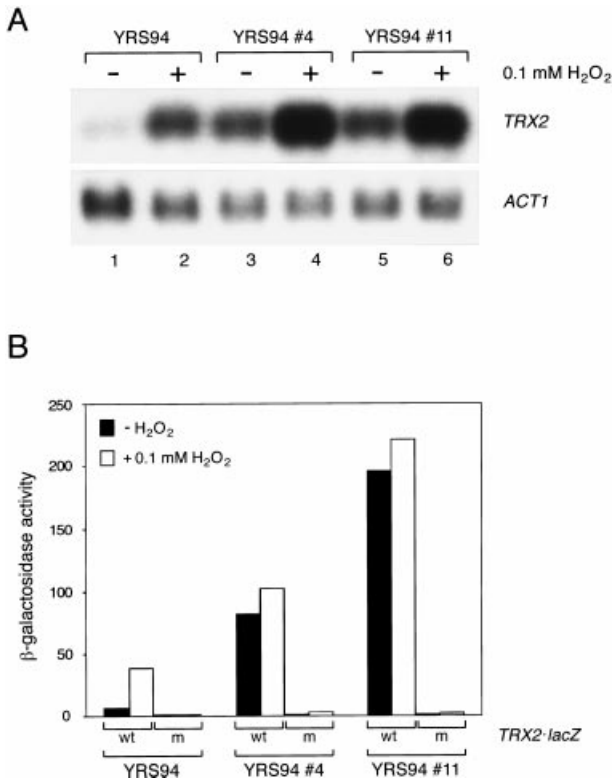

Fig. 2. *TRX2* expression in mutants YRS94#4 and YRS94#11. Cultures in early log phase (OD_{600} = 0.4) were split and grown in the absence or the presence of 0.1 mM H_2O_2 for 30 min.
A. Northern blot analysis of total cellular RNA isolated from untreated and treated cells. The experiment was repeated three times, and a representative blot is shown.
B. β-Galactosidase activity in extracts prepared from untreated and treated cells carrying a wild-type (wt) or mutant (m) *TRX2–lacZ* fusion. The results shown are from a representative experiment.

β-galactosidase levels in the mutants are higher than the induced β-galactosidase levels in the wild-type strain. We also observed significantly more induction of the *TRX2* mRNA levels than of the β-galactosidase activity by H_2O_2 in YRS94#4 and YRS94#11. These discrepancies between the Northern blots and the β-galactosidase assays may result from the greater stability of the β-galactosidase protein compared with the *TRX2* mRNA.

To characterize the YRS94#4 and YRS94#11 mutant strains further, we also tested their sensitivity to H_2O_2 using a zone inhibition assay. Surprisingly, despite the elevated basal *TRX2* mRNA levels, both mutants were found to be more sensitive to 2.5 M H_2O_2 than the wild-type strain (Table 1).

The MAT**a** mutant strains also were mated with the MAT**α** wild-type strain BY4709 to determine whether the mutation(s) in YRS94#4 and YRS94#11 are dominant or recessive. The heterozygous diploids had both normal basal and induced *TRX2* expression (data not shown) as well as wild-type sensitivity to H_2O_2, indicating that the YRS94#4 and YRS94#11 mutation(s) are recessive and should be complemented by the intact genes from a wild-type genomic library.

Table 1. H_2O_2 sensitivity of YRS94 parent and mutant strains.

	Zones of inhibition (mm) with 2.5 M H_2O_2[a]	
Strain	No plasmid	pYOH8
YRS94	23	22
YRS94#4	57	24
YRS94#11	58	25
YRS94 Δ*trr1*	55	26

a. Total diameter of the growth inhibition zone caused by the addition of H_2O_2. The values are from a representative assay.

Identification of the gene mutated in YRS94#4 and YRS94#11

To identify the mutation(s) involved in the constitutive expression of *TRX2*, an *S. cerevisiae* genomic library was transformed into mutant YRS94#4. Transformants were screened by replica plating onto plates containing 1, 2 and 4 mM H_2O_2. Plasmids isolated from colonies that grew on 2 and/or 4 mM H_2O_2 were retransformed into YRS94#4 and YRS94#11 and tested for complementation of the parental H_2O_2 sensitivity. Fourteen plasmids allowed YRS94#4 to grow in the presence of 2 mM H_2O_2 and conferred wild-type growth in a zone of inhibition assay (data not shown). These same plasmids also complemented YRS94#11 (data not shown), suggesting that the two strains carry mutations in the same gene(s).

The 14 complementing plasmids corresponded to one of two types of plasmids carrying an overlapping region of chromosome IV. Twelve had a 5.6 kb insert in one orientation, whereas two had a 7.9 kb insert in the opposite direction. The overlap of these two genomic fragments narrowed the region required for complementation down to position 1, 179, 342–1, 184, 927 on chromosome IV. Examination of the genes residing in this overlap region suggested that *TRR1* might be the gene mutated in the two strains. Using polymerase chain reaction (PCR) with primers flanking the *TRR1* gene, DNA fragments were generated from YRS94, YRS94#4 and YRS94#11 genomic DNA. The PCR product from each strain was sequenced directly revealing single point mutations in *TRR1* in both YRS94#4 and YRS94#11 (Fig. 3). YRS94#4 carries a mutation of a G to an A at

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S.cerevisiae  -----MVHNKVTIIGSGPAAHTAAIYLARAEIKPILYEGMMANGIAAGGQLTTTTEIENF  55
S.pombe       -----MTHNKVVIIGSGPAGHTAAIYLARGELKPVMYEGMLANGIAAGGQLTTTTDVENF  55
A.thaliana    MNGLETHNTRLCIVGSGPAAHTAAIYAARAELKPLLFEGWMANDIAPGGQLTTTTDVENF  60
E.coli        --MGTTKHSKLLILGSGPAGYTAAVYAARANLQPVLITGMEK-----GGQLTTTTEVENW  53
                    * *****  *** * **    *    *        ********  **

S.cerevisiae  PGFPDGLTGSELMDRMREQSTKFGTEIITETVSKVDLSSKPFKLWTEFNEDAEPVTTDAI  115
S.pombe       PGFPDGINGTTLTENFRAQSLRFGTEIITETVSKLDLSSRPFKYWLEGAEEEEPHTADSV  115
A.thaliana    PGFPEGILGVELTDKFRKQSERFGTTIFTETVTKVDFSSKPFKLFTDS----KAILADAV  116
E.coli        PGDPNDLTGPLLMERMHEHATKFETEIIFDHINKVDLQNRPFRLNGDN----GEYTCDAL  109
              ** *    *  *          * * *      * *    **              *

                                      D (#4)
                                      ↓
S.cerevisiae  ILATGASAKRMHLPGEE----TYWQKGISACAVCDGAVPIFRNKPLAVIGGGDSACEEAQ  171
S.pombe       ILATGASARRLHITGED----TYWQAGISACAVCDGAVPIYRNKPLAVVGGGDSAAEEAQ  171
A.thaliana    ILAIGAVAKRLSFVGSGEVLGGFWNRGISACAVCDGAAPIFRNKPLAVIGGGDSAMEEAN  176
E.coli        IIATGASARYLGLPSEE----AFKGRGVSACATCDG--FFYRNQKVAVIGGGNTAVEEAL  163
              * * ** *                    * ***** ***     **    ** ***  * ***

S.cerevisiae  FLTKYGSKVFMLVRKDHLRASTIMQKRAE---KNEKIEILYNTVALEAKGDGK--LLNAL  226
S.pombe       FLTKYGSKVYVLVRRDKLRASPIMAKRLL---ANPKVEVLWNTVAEEAQGDGK--LLNNL  226
A.thaliana    FLTKYGSKVYIIHRRDAFRASKIMQQRAL---SNPKIDVIWNSSVVEAYGDGERDVLGGL  233
E.coli        YLSNIASEVHLIHRRDGFRAEKILIKRLMDKVENGNIILHTNRTLEEVTGDQMG-VTGVR  222
               *    * *    * *  **  *   *       *        *    *  **

                          D (#11)
                          ↓
S.cerevisiae  RIKNTKKNEETDLPVSGLFYAIGHTPATKIVAGQVDTDEAGYIKTVPGSS----LTSVPG  282
S.pombe       RIKNTNTNEVSDLQVNGLFYAIGHIPATKLVAEQIELDEAGYIKTINGTP----RTSIPG  282
A.thaliana    KVKNVVTGDVSDLKVSGLFFAIGHEPATKFLDGGVELDSDGYVVTKPGTT----QTSVPG  289
E.coli        LRDTQNSDNIESLDVAGLFVAIGHSPNTAIFEGQLELEN-GYIKVQSGIHGNATQTSIPG  281
                    * * *** **** * *            **      *       ** **

S.cerevisiae  FFAAGDVQDSKYRQAITSAGSGCMAALDAEKYLTSLE-------                   319
S.pombe       FFAAGDVQDKVFRQAITSAGSGCQAALLAMHYLEELEDTD----                  322
A.thaliana    VFAAGDVQDKKYRQAITAAGTGCMAALDAEHYLQEIGSQEGKSD                  333
E.coli        VFAAGDVMDHIYRQAITSAGTGCMAALDAERYLDGLADAK----                  321
               ****** *   ***** ** ** *** *  **
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Fig. 3. Position of mutations in YRS94#4 and YRS94#11. The thioredoxin reductase protein sequences of *S. cerevisiae* (AAB64789), *S. pombe* (CAA17692), *A. thaliana* (Q39243) and *E. coli* (BAA35620) and were aligned using CLUSTAL W (1.8) software. Identical amino acid residues are indicated by the asterisks, the redox-active centre is indicated by the box, and the arrowheads point to the amino acid changes in YRS94#4 (G138D) and YRS94#11 (G243D).

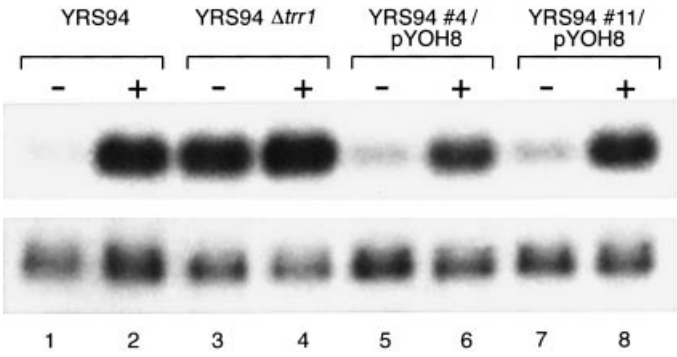


Fig. 4. Effect of *TRR1* deficiency on *TRX2* expression. Cultures in early log phase ($OD_{600} = 0.4$) were split and grown in the absence or the presence of 0.1 mM H_2O_2 for 30 min. Northern blot analysis of total cellular RNA isolated from untreated or treated cells. The experiment was repeated twice, and a representative blot is shown.

position 413, resulting in the conversion of a glycine to an aspartic acid at position 138. YRS94#11 carries a mutation of a G to an A at position 728, resulting in the conversion of a glycine to an aspartic acid at position 243.

The *TRR1* gene encodes the 34 kDa thioredoxin reductase protein that catalyses the reduction of oxidized thioredoxin at the expense of NADPH. The deduced amino acid sequence of Trr1p is 49% identical to the *Escherichia coli* and 61% identical to the *Arabidopsis thaliana* thioredoxin reductases whose structures have been solved (Dai *et al*., 1996; Lennon *et al*., 1999). The amino acids comprising the thioredoxin reductase active site as well as the NADPH and FAD binding sites are conserved with the *E. coli*, *A. thaliana* and *Schizosaccharomyces pombe* homologues. The two conserved glycines that are mutated in YRS94#4 and YRS94#11 are both near the CAVC active site in the three-dimensional structures of the bacterial and plant homologues.

Effect of Δtrr1 *mutation on* TRX2 *expression*

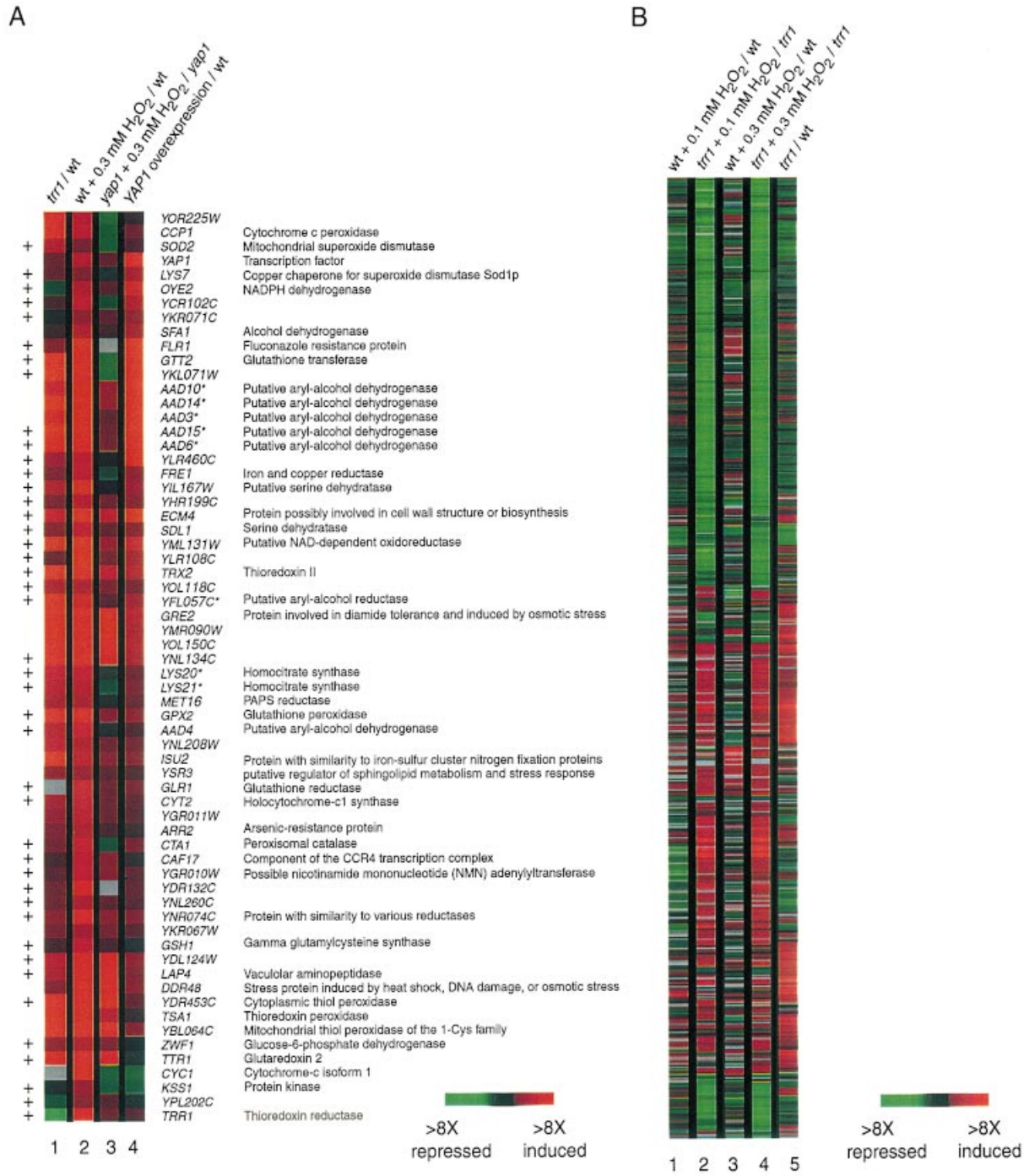
To examine the role of *TRR1* in the Yap1p-dependent regulation of gene expression, we constructed a Δ*trr1* strain by replacing the entire *TRR1* open reading frame (ORF) in YRS94 with the kanMX4 gene cassette. Consistent with a previous report (Machado *et al*., 1997), the YRS94 Δ*trr1* mutant had reduced growth rates compared with the wild-type strain and was hypersensitive to H_2O_2 (Table 1). We also observed that the mutant was unable to grow on glycerol as a carbon source (data not shown). The YRS94 Δ*trr1* strain was tested for *TRX2* expression by Northern blot analysis (Fig. 4). Similar to the YRS94#11 mutant, YRS94 Δ*trr1* exhibited an ≈ fourfold higher basal level of *TRX2* mRNA compared with YRS94 (Fig. 4, lane 3 compared with lane 1). The expression was further induced ≈ twofold upon treatment with H_2O_2 (Fig. 4, lane 4). The basal and induced levels of β-galactosidase activity for the YRS94 Δ*trr1* strain carrying the *TRX2–lacZ* fusion plasmid were almost identical to the levels observed for YRS94#11 (data not shown). Although it is formally possible that the YRS94#4 and YRS94#11 strains contain recessive mutations in genes other than *TRR1*, the similarities in the YRS94#4, YRS94#11 and YRS94 Δ*trr1* phenotypes indicate that the defects reported for YRS94#4 and YRS94#11 result from the inactivation of thioredoxin reductase.

The wild-type *TRR1* gene was also cloned into a yeast centromeric plasmid to generate pYOH8. YRS94#4 and YRS94#11 transformed with pYOH8 had basal and induced *TRX2* expression similar to those measured in the wild-type strain (Fig. 4) and showed wild-type resistance to H_2O_2 (Table 1). These results verify that the YRS94#4 and YRS94#11 mutant phenotypes are a result of at least partial loss of thioredoxin reductase function.

Effect of Δtrr1 *mutation on global gene expression*

Whole-genome expression analysis has proved to be a powerful tool in examining the global effects of specific mutations and specific environmental conditions (reviewed by Brown and Botstein, 1999). In yeast, this technique allows every ORF in the genome to be analysed for differences in mRNA transcript levels when a pair of strains or culture conditions is compared. Hierarchical clustering of gene expression data organizes genes according to similar expression profiles and indicates which genes are co-regulated (Eisen *et al*., 1998). In addition, the results of these assays can be compared with the large collection of array experiments carried out with isogenic strains.

To gain more insight into the effects of the Δ*trr1* deletion, we used DNA microarrays to compare the whole-genome expression patterns of wild-type (DBY7286) and Δ*trr1* mutant (DBY7286 Δ*trr1*) cells left untreated or treated with 0.1 and 0.3 mM H_2O_2. Figure 5A shows a cluster diagram of the expression of genes previously identified as potential Yap1p targets (Gasch *et al*., 2000). The data are shown for DBY7286 Δ*trr1* versus untreated DBY7286 (Fig. 5A, column 1), DBY7286 treated with 0.3 mM H_2O_2 versus untreated DBY7286 (Fig. 5A, column 2) (Gasch *et al*., 2000), DBY7286 Δ*yap1* treated with 0.3 mM H_2O_2 versus untreated DBY7286


A
trr1 / wt
wt + 0.3 mM H2O2 / wt
yap1 + 0.3 mM H2O2 / yap1
YAP1 overexpression / wt
YOR225W
CCP1 Cytochrome c peroxidase
+ SOD2 Mitochondrial superoxide dismutase
YAP1 Transcription factor
+ LYS7 Copper chaperone for superoxide dismutase Sod1p
+ OYE2 NADPH dehydrogenase
+ YCR102C
+ YKR071C
SFA1 Alcohol dehydrogenase
+ FLR1 Fluconazole resistance protein
+ GTT2 Glutathione transferase
+ YKL071W
AAD10* Putative aryl-alcohol dehydrogenase
AAD14* Putative aryl-alcohol dehydrogenase
AAD3* Putative aryl-alcohol dehydrogenase
+ AAD15* Putative aryl-alcohol dehydrogenase
+ AAD6* Putative aryl-alcohol dehydrogenase
+ YLR460C
+ FRE1 Iron and copper reductase
+ YIL167W Putative serine dehydratase
+ YHR199C
+ ECM4 Protein possibly involved in cell wall structure or biosynthesis
+ SDL1 Serine dehydratase
+ YML131W Putative NAD-dependent oxidoreductase
+ YLR108C
+ TRX2 Thioredoxin II
+ YOL118C
+ YFL057C* Putative aryl-alcohol reductase
GRE2 Protein involved in diamide tolerance and induced by osmotic stress
YMR090W
YOL150C
+ YNL134C
+ LYS20* Homocitrate synthase
+ LYS21* Homocitrate synthase
MET16 PAPS reductase
+ GPX2 Glutathione peroxidase
+ AAD4 Putative aryl-alcohol dehydrogenase
YNL208W
ISU2 Protein with similarity to iron-sulfur cluster nitrogen fixation proteins
YSR3 putative regulator of sphingolipid metabolism and stress response
+ GLR1 Glutathione reductase
+ CYT2 Holocytochrome-c1 synthase
YGR011W
ARR2 Arsenic-resistance protein
+ CTA1 Peroxisomal catalase
+ CAF17 Component of the CCR4 transcription complex
+ YGR010W Possible nicotinamide mononucleotide (NMN) adenylyltransferase
+ YDR132C
+ YNL260C
+ YNR074C Protein with similarity to various reductases
YKR067W
+ GSH1 Gamma glutamylcysteine synthase
+ YDL124W
+ LAP4 Vacuolar aminopeptidase
DDR48 Stress protein induced by heat shock, DNA damage, or osmotic stress
+ YDR453C Cytoplasmic thiol peroxidase
TSA1 Thioredoxin peroxidase
YBL064C Mitochondrial thiol peroxidase of the 1-Cys family
+ ZWF1 Glucose-6-phosphate dehydrogenase
+ TTR1 Glutaredoxin 2
CYC1 Cytochrome-c isoform 1
+ KSS1 Protein kinase
+ YPL202C
+ TRR1 Thioredoxin reductase
1 2 3 4
>8X repressed
>8X induced
B
wt + 0.1 mM H2O2 / wt
trr1 + 0.1 mM H2O2 / trr1
wt + 0.3 mM H2O2 / wt
trr1 + 0.3 mM H2O2 / trr1
trr1 / wt
1 2 3 4 5
>8X repressed
>8X induced

Δ*yap1* (Fig. 5A, column 3) (Gasch *et al*., 2000) and untreated DBY7286 with a *YAP1*-overexpressing vector versus untreated DBY7286 with a control vector (Fig. 5A, column 4) (DeRisi *et al*., 1997). Genes that are expressed at higher levels in the Δ*trr1* deletion in response to H_2O_2 treatment and after *YAP1* overexpression are indicated as red boxes according to the depicted scale. This comparison showed that, in the Δ*trr1* mutant strain, ≈ 70% of the putative Yap1p-induced genes are expressed at basal levels that are ≥ 1.5-fold higher than the basal levels in the wild-type strain in the absence of H_2O_2 treatment. Interestingly, other H_2O_2-inducible stress genes, many of which are targets of the transcription factors Msn2p and Msn4p but not Yap1p (such as *CTT1* and *HSP12*), are also expressed at higher basal levels in untreated DBY7286 Δ*trr1* (data not shown). Together, these results suggest that, under standard growth conditions, the Δ*trr1* mutant experiences stress similar to that induced in cells by the presence of exogenously added H_2O_2, possibly because of increased internal levels of ROS and/or alterations in the cellular redox potential.

We also compared the expression of genes that are induced or repressed > twofold in Δ*trr1* mutants treated with 0.1 and 0.3 mM H_2O_2 (Fig. 5B, columns 2 and 4) with the expression of the corresponding genes in the wild-type cells treated with 0.1 and 0.3 mM H_2O_2 (Fig. 5B, columns 1 and 3). The comparison showed that ≈ 50% of the putative Yap1p targets are induced ≥ 1.5-fold more strongly by 0.1 and 0.3 mM H_2O_2 in DBY7286 Δ*trr1* compared with DBY7286. In a previous study, we discovered a large set of ≈ 900 genes, termed the environmental stress response (ESR), that are similarly activated or repressed by a large variety of environmental insults including heat shock, oxidative stress, osmotic shock and starvation (Gasch *et al*., 2000). On average, ESR genes were induced or repressed twofold more strongly in the H_2O_2-treated Δ*trr1* cells than in the untreated Δ*trr1* and H_2O_2-treated wild-type cells. We suggest that the effects of exogenously added H_2O_2 are more severe in the Δ*trr1* mutant as a result of the diminished capacity of the mutant cells to detoxify H_2O_2 or other oxidants and/or to repair oxidative stress-induced damage. However, we did not observe a significant difference in viability between the wild type (95%) and the Δ*trr1* mutant (92%) 30 min after exposure to the low 0.3 mM concentration of H_2O_2.

We also found that genes encoding multiple classes of protein-folding chaperones are strongly induced in treated DBY7286 Δ*trr1* but not in the treated wild-type or the untreated Δ*trr1* mutant strains. The hyperinduced chaperone genes include members of the Hsp70 family (such as *SSE1* and *SSA2*), the Hsp90 family (such as *HSP82*, *STI1* and *CPR6*), the Hsp10/60 family and the ESR group of chaperones (such as *HSP42*, *HSP104*, *HSP78*, *SSA3* and *SSA4*). The hyperinduction of these genes, which are generally required under conditions that generate denatured proteins (Morano *et al*., 1998), suggests that Δ*trr1* mutants treated with H_2O_2 have increased levels of misfolded proteins compared with treated wild-type cells.

Discussion

Previous studies have shown that the Yap1p transcription factor is a major regulator of the *S. cerevisiae* response to H_2O_2. To identify factors that regulate Yap1p activity, we carried out a genetic screen for mutants that show elevated expression of a *TRX2–HIS3* fusion in the absence of H_2O_2. Two independent strains recovered in our screen carried mutations in the *TRR1* gene encoding thioredoxin reductase. Whole-genome expression analysis revealed that the basal expression of Yap1p targets is elevated in Δ*trr1* mutants and that the Yap1p targets are induced more strongly by H_2O_2 in Δ*trr1* mutants than in the wild-type strain. Our results, together with the finding that strains lacking both thioredoxin 1 and thioredoxin 2 (*trx1 trx2* mutants) have constitutive Yap1p activity in the absence of stress (Izawa *et al*., 1999), strongly implicate the thioredoxin system, consisting of thioredoxins and thioredoxin reductase, in modulating the activity of the Yap1p transcription factor.

Fig. 5. Effect of *TRR1* deficiency on global gene expression patterns. Cultures in early log phase (OD_{600} = 0.4) were split and grown in the absence or the presence of 0.1 mM or 0.3 mM H_2O_2 for 30 min.

A. Poly A^+ RNA was isolated from untreated DBY7286 and DBY7286 Δ*trr1*, labelled and hybridized to microarrays. The expression profile was then compared with the expression profiles of DBY7286 and DBY7286 Δ*yap1* treated with 0.3 mM H_2O_2 (Gasch *et al*., 2000) and DBY7286 overexpressing Yap1p (DeRisi *et al*., 1997). Column 1 corresponds to untreated DBY7286 Δ*trr1* versus untreated DBY7286; column 2 corresponds to DBY7286 treated with 0.3 mM H_2O_2 versus untreated DBY7286; column 3 corresponds to DBY7286 Δ*yap1* treated with 0.3 mM H_2O_2 versus untreated DBY7286 Δ*yap1*; and column 4 corresponds to untreated DBY7286 overexpressing *YAP1* versus untreated DBY7286. Grey boxes denote missing data. A plus sign (+) to the left of the columns indicates genes that contain ≥ one putative Yap1p binding site (5′-TTAG/CTAA or 5′-TTAGTCA) within 1000 bp of the initiating codon, and an asterisk in the gene annotation indicates genes likely to cross-hybridize. The gene names and functions to the right of the columns are taken from http://genome-http://www.stanford.edu/Saccharomyces.

B. Poly A^+ RNA was isolated from untreated and treated DBY7286 and DBY7286 Δ*trr1*, labelled and hybridized to microarrays. Column 1 corresponds to DBY7286 treated with 0.1 mM H_2O_2 versus untreated DBY7286; column 2 corresponds to DBY7286 Δ*trr1* treated with 0.1 mM H_2O_2 versus untreated DBY7286 Δ*trr1*; column 3 corresponds to DBY7286 treated with 0.3 mM H_2O_2 versus untreated DBY7286; column 4 corresponds to DBY7286 Δ*trr1* treated with 0.3 mM H_2O_2 versus untreated DBY7286 Δ*trr1*; and column 5 corresponds to untreated DBY7286 Δ*trr1* versus untreated DBY7286. The experiment was repeated twice, and the complete data set is available at http://www-genome.stanford.edu/trr1.

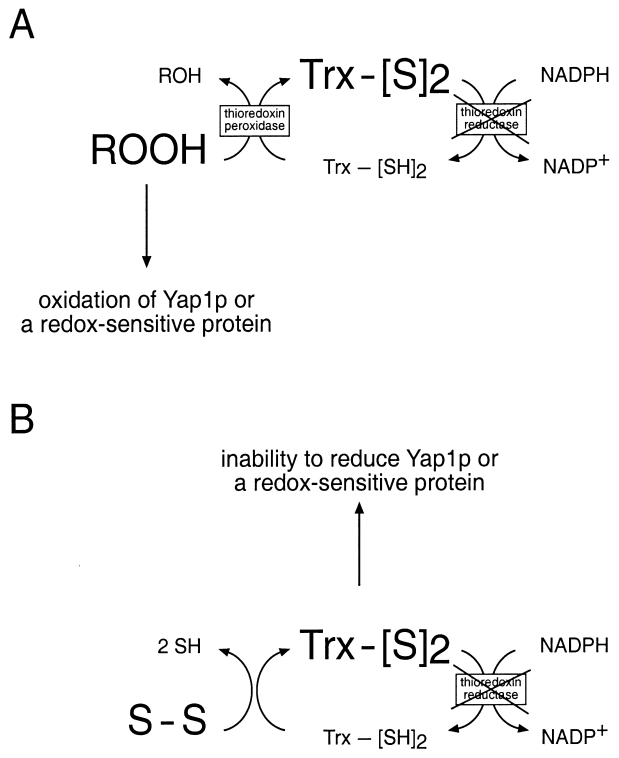


Fig. 6. Models for the role of thioredoxin reductase.
A. Peroxide elimination by thioredoxin-dependent peroxidases.
B. Disulphide bond reduction by thioredoxin.

There are several possible models for the effects of the *trr1* and *trx1 trx2* mutations. The lack of thioredoxin reductase or thioredoxin 1 and 2 might result in decreased ability of the mutant cells to detoxify H_2O_2 or other oxidants. For example, the absence of reduced thioredoxin will impair the ability of the thioredoxin-dependent peroxidases, encoded by *TSA1*, *AHP1*, *YDR453C*, *YBL064C* and *YIL010W*, to remove peroxides from the cell. In this model, increased peroxide levels would lead to the oxidation of Yap1p itself or another, as yet unidentified, redox-sensitive protein that modulates Yap1p activity (Fig. 6A). Alternatively, the thioredoxin system might be acting to reduce Yap1p or the hypothesized redox-sensitive protein. In this model, Yap1p or the putative Yap1p regulator would be oxidized by the peroxides present in the cell in the absence of external stress, but the oxidized protein would not be efficiently reduced, leading to the accumulation of activated Yap1p (Fig. 6B). A combination of the two models is also likely, and both models are consistent with the results of the microarray experiments.

The two models for the effects of the *trr1* mutation may also explain the conundrum that Δ*trr1* mutants are hypersensitive to H_2O_2 despite the elevated levels of many antioxidant activities. Although the expression of the thioredoxin-dependent peroxidases is elevated, the enzymes may not be able to remove peroxides without reduction by the thioredoxin system. The accumulation of proteins with abnormal disulphide bonds may also contribute to the increased H_2O_2 sensitivity. Regardless of the model, an important conclusion from our study is that strains with elevated expression of genes encoding antioxidant enzymes may still be hypersensitive to oxidative stress.

The reducing environment of the cell is maintained by both the thioredoxin system and the glutaredoxin system, consisting of glutaredoxins, glutathione and glutathione reductase. In *E. coli*, the activity of the redox-sensitive OxyR transcription factor is primarily modulated by the glutaredoxin system (Zheng *et al*., 1998). Our results and the findings that Yap1p is constitutively active in *trx1 trx2* mutants implicate the thioredoxin system in modulating Yap1p activity in *S. cerevisiae*. Izawa *et al*. (1999) found that a deficiency in *S. cerevisiae* glutaredoxin 1 and glutaredoxin 2 (*grx1 grx2*) does not lead to the constitutive activation of Yap1p and suggested that the glutaredoxin system does not modulate Yap1p activity. The lack of a *grx1 grx2*-dependent Yap1p phenotype might be explained by the presence of three additional glutaredoxin proteins present in yeast. However, we have found that a deletion of the single gene encoding glutathione reductase (*glr1*) also does not affect Yap1p activity (data not shown). It is not clear why OxyR activity is regulated by the glutaredoxin system whereas Yap1p activity is regulated by the thioredoxin system, but it will be interesting to see whether the activities of other transcription factors are modulated specifically by one or the other reducing system.

Our genetic screen has defined the *TRR1*-encoded thioredoxin reductase as an activity that is important for the redox regulation of Yap1p. It is likely that yet other proteins are involved. Our *TRX2–HIS3* screen was not saturated and may reveal additional activities that modulate Yap1p activity. It will also be interesting to carry out biochemical analysis to determine whether Yap1p is oxidized directly and, if so, whether thioredoxin 1 or thioredoxin 2 reduce Yap1p *in vivo*.

Experimental procedures

Yeast strains, growth conditions and techniques

The strains and plasmids used in this study are listed in Table 2. YPH499 was the wild-type strain for all genetic experiments, BY4709 was used in the mating experiments, and DBY7286 and its derivative DBY7286 Δ*trr1* were used in the microarray experiments. *S. cerevisiae* strains were grown in either YPD rich media (Sherman, 1991) for non-selective growth or drop-out SC minimal media (Bio101) for selective growth. Yeast cells were transformed using the lithium acetate–PEG method as described previously (Gietz and Schiestl, 1995).

Construction and mutagenesis of YRS94

The promoter region upstream of the *TRX2* (YGR209C) gene (from chromosome VII position 913, 508–913, 222) was PCR amplified [Trx2-Prm1A (5′-CGACTTCTAGATCAGCA TAAC TTGAGTGCCAGTG) and Trx2-Prm1B (5′-CGA TCTGGAT CCTATTGATGTGTTATTTAAAGATATCGTA GAC); *Xba*I and *Bam*HI restriction sites are underlined] from genomic *S. cerevisiae* DNA isolated from the wild-type strain F113. The promoter fragment included the complete intergenic region between the upstream ORF (YGR210C) and the −1 nucleotide from the Trx2p initiating methionine. PCRs using 5 units of *Pfu* polymerase (Stratagene), 0.35 μg of genomic DNA, 0.25 mM dNTPs and 0.5 mM primers per 100 μl reaction volumes were run in a preheated thermal cycler at 94°C for 3 min, followed by five cycles at 94°C for 30 min, 62°C for 30 min, 68°C for 60 min and 20 cycles at 94°C for 30 min, 56°C for 30 min, 68°C for 60 min. The reaction products were purified using Qia-Quick columns (Qiagen), digested with *Xba*I and *Bam*HI and cloned into the corresponding sites (dephosphorylated) of the yeast integrating plasmid pCM105, which contains a promoterless *HIS3* gene and *URA3* as a selectable marker. The sequence of the pPTRX2-HIS3 clone was verified on both strands before introduction into yeast. YPH499 was transformed with *Nco*I-digested pPTRX2-HIS3 with selection for uracil prototrophy. YRS94 (1×10^8 cells ml^{-1}) was mutagenized with EMS (20 μl; Sigma) in two separate mutagenesis reactions. A total of $\approx 2 \times 10^8$ cells were plated on SC–His supplemented with 75 mM 3-AT (Fluka). In addition to inhibiting His3p, 3-AT has been reported to inhibit yeast catalases (Kowaltowski *et al.*, 2000) and possibly contributed to oxidative stress on the selection plates. About 50 colonies that grew after 4 days at 30°C were rescreened. Two mutants that grew well on SC–His + 75 mM 3-AT plates and in YPD liquid culture and had altered sensitivity to H_2O_2 were chosen for further characterization.

Complementation of YRS94#4

An *S. cerevisiae* genomic library (ATCC 37323) in YEp13 was transformed into YRS94#4 with selection for leucine prototrophy. The ≈125 000 transformants (> 10-fold representation of the yeast genome) were screened by replica plating onto SC-Leu containing 1 mM H_2O_2. Approximately 650 large colonies were observed with a high background of smaller colonies. As a control, transformants were also replicated on SC–Leu–Trp, and roughly 650 colonies were obtained without a background of smaller colonies. The large colonies identified on the SC–Leu + 1 mM H_2O_2 plates were replicated onto plates with 2 or 4 mM H_2O_2. Plasmids isolated from 16 of the colonies that grew on 2 mM and/or 4 mM H_2O_2 were retransformed into YRS94#4 and tested for complementation of the parental H_2O_2 sensitivity. Fourteen plasmids restored wild-type resistance to H_2O_2. The remaining two plasmids did not restore wild-type growth and were therefore probably false positives.

Sequencing and cloning of TRR1

The *TRR1* gene (from −611 to 1557) was PCR amplified [TRR1-F1 (5′-GCAGTAAGCTTAGCGCAACTAGTGACAGA ACG) and TRR1-R1 (5′-CGACTCTGCAGATATAACCAGCA ACGCATCGTGTAGG); *Hin*dIII and *Pst*I restriction sites are underlined] from genomic *S. cerevisiae* DNA isolated from strains YRS94, YRS94#4 and YRS94#11. The pool of PCR products for each strain was sequenced directly. In addition, the purified PCR fragment from YRS94 was digested with *Hin*dIII and *Pst*I and cloned into the corresponding restriction sites of pRS415 to generate pYOH8.

TRR1 *gene disruptions*

Gene deletions were performed by the one-step PCR-based gene disruption technique (Wach, 1996). A 1.6 kb PCR

Table 2a. Yeast strains and plasmids used in this study.

Strain	Source	Genotype
YPH499	Sikorski and Hieter (1989)	*MAT***a** *ura3-52 lys2-801*^{amber} *ade2-101*^{ochre} *trp1-Δ63 his3-Δ200 leu2-Δ1*
YRS92	This study	YPH499 *URA3*::pCM105
YRS94	This study	YPH499 *URA3*::pPTRX2-HIS3
YRS94 Δ*trr*1	This study	YRS94 *trr1-Δ0*
DBY7286	Spellman *et al.* (1998)	*MAT***a** *GAL2 ura3*
DBY7286 Δ*trr*1	This study	DBY7286 *trr1-Δ0*
F113	Laboratory stock	*MAT***a**, *can1, ino1-13, ura3-52*
BY4709	Brachmann *et al.* (1998)	*MATα ura3-Δ0*

Table 2b.

Plasmid	Source	Description
pCM105	Laboratory stock	A derivative of pCM99 (Dancis *et al.*, 1994) containing a modified multicloning site at the 5′ side of the *HIS3* reporter gene
pPTRX2-HIS3	This study	*TRX2* promoter cloned into the *Xba*I and *Bam*HI sites of pCM105
pYOH8	This study	*TRR1* cloned into the *Hin*dIII and *Pst*I sites of pRS415 (Sikorski and Hieter, 1989)
pYOH10	This study	The 4.2 kb *Nco*I *TRX2–lacZ* fragment (filled in) from TRXLACZ (Kuge and Jones, 1994) cloned into the *Sma*I site of pRS415
pYOH11	This study	The 4.2 kb *Nco*I *TRX2–lacZ* fragment (filled in) from mTRXLACZ (Kuge and Jones, 1994) cloned into the *Sma*I site of pRS415

fragment containing the kanMX4 gene was PCR amplified [TRR1-A (5′-GCTATACAGCAAATAGCGAACAGTACGAAA GTAAACATCATATTATCAATACGTACGCTGCAGGTCGAC) and TRR1-B (5′-TTGGATAAGTATACAAAAATTTGAGTGTA TCTATTTTATAATGGAAAATATCGATGAATTCGAGCTCG), kanMX4 sequence is underlined] from pFA6a (Wach *et al.*, 1994) and then transformed into YRS94 and DBY7286 with selection for G418 (Life Technologies) resistance. Deletions were confirmed by PCR using the appropriate primers directed against the kanMX4 region as well as sequences flanking the *TRR1* gene.

Northern blot analysis

Yeast cells from overnight cultures were diluted to OD_{600} of 0.05 in the appropriate media and incubated with shaking at 30°C until they reached an OD_{600} of 0.4. The cells were then aliquoted and incubated in the absence or presence of H_2O_2 (0.1 mM) for 30 min. Total RNA was prepared by the hot phenol method (Schmitt *et al.*, 1990), followed by treatment with Trizol reagent according to the manufacturer's protocol (Life Technologies). The RNA pellet was resuspended in 10 mM Tris-HCl, pH 7.4. Aliquots (10 μg) of total RNA for each tested condition were separated by electrophoresis on a 1.4% agarose–formaldehyde gel, transferred to Nytran Super-Charge membrane (Schleicher and Schuell) and hybridized with ^{32}P-labelled DNA-specific probes. *TRX2* transcripts were probed using a ^{32}P end-labelled oligonucleotide (5′-TACCGG CAACTATACCGTTGGA). A DNA fragment generated by PCR from the ORF of *ACT1* [ACT1–1 (5′-TCGGCAA TACCTGGGAA CATGGTGG) and OCH-212 (5′-TGAA CACGGTATTGTCACCAACTGG)] was labelled using the Prime-It II random primer labelling kit (Stratagene). Hybridizations with the *ACT1* probe served as a control for RNA loading. The intensities of the *TRX2* and *ACT1* bands were quantified on a phosphorimager (Molecular Dynamics) to calculate the expression levels.

Microarrays

The growth conditions and procedure for total RNA extraction were as described above for the Northern analysis. Poly A^+ mRNA was isolated using oligotex beads (Qiagen) according to the manufacturer's instructions. Probe preparation, microarray production, hybridization and data analysis were performed as described previously (DeRisi *et al.*, 1997; Spellman *et al.*, 1998; Gasch *et al.*, 2000). The complete data set is available at http://www-genome.stanford.edu/trr1. Quantification of the induction of the Yap1p target genes was carried out by comparing individual data points. Quantification of the induction of the ESR response was carried out by averaging the values for the wild type and the Δ*trr1* mutant and then comparing the averages.

Sensitivity assay

Filter disks (3 mm; Whatman) soaked with 10 μl of 1.0, 2.5 or 5.0 M H_2O_2 were placed on lawns made by plating 100 μl of early log phase cells ($OD_{600} = 0.4$). The zones of inhibition around the filter disks were measured after 2 days of incubation at 30°C.

β-Galactosidase assay

Cells (1×10^7) were harvested in an Eppendorf tube, washed once with water, resuspended in 800 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM 2-mercaptoethanol, pH 7.0) and placed on ice. Cells were permeabilized by the addition of 10 μl of 0.1% SDS and 20 μl of chloroform. β-Galactosidase activity was determined by mixing the cell extract with a stock solution (4 mg ml^{-1}) of ONPG and incubating the mixture at 30°C for 20–30 min. The reaction was then quenched by adding 400 μl of 1 M sodium carbonate. Unbroken cells and cell debris were removed by centrifugation for 2 min, and colour intensity was measured. The activity was calculated as Miller units $= [OD_{420} \times 1000]/[OD_{600} \times \text{time (min)} \times \text{volume (ml)}]$.

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