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ROLES OF THE GLUTATHIONE- AND THIOREDOXIN-DEPENDENT REDUCTION SYSTEMS IN THE *ESCHERICHIA COLI* AND *SACCHAROMYCES CEREVISIAE* RESPONSES TO OXIDATIVE STRESS

Orna Carmel-Harel and Gisela Storz
National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; e-mail: ornah@box-o.nih.gov; storz@helix.nih.gov

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■ **Abstract** The glutathione- and thioredoxin-dependent reduction systems are responsible for maintaining the reduced environment of the *Escherichia coli* and *Saccharomyces cerevisiae* cytosol. Here we examine the roles of these two cellular reduction systems in the bacterial and yeast defenses against oxidative stress. The transcription of a subset of the genes encoding glutathione biosynthetic enzymes, glutathione reductases, glutaredoxins, thioredoxins, and thioredoxin reductases, as well as glutathione- and thioredoxin-dependent peroxidases is clearly induced by oxidative stress in both organisms. However, only some strains carrying mutations in single genes are hypersensitive to oxidants. This is due, in part, to the redundant effects of the gene products and the overlap between the two reduction systems. The construction of strains carrying mutations in multiple genes is helping to elucidate the different roles of glutathione and thioredoxin, and studies with such strains have recently revealed that these two reduction systems modulate the activities of the *E. coli* OxyR and SoxR and the *S. cerevisiae* Yap1p transcriptional regulators of the adaptive responses to oxidative stress.

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INTRODUCTION

The tripeptide glutathione (GSH) and the small protein thioredoxin are reductants in many cellular reactions. For example, GSH functions to reduce cellular disulfide bonds, often in conjunction with glutaredoxin proteins (Figure 1*A*). In eukaryotic cells, GSH is also an electron donor for the antioxidant enzyme glutathione peroxidase (Figure 1*B*). Similarly, thioredoxin proteins act as disulfide oxidoreductases and are the electron donors for thioredoxin peroxidases in both prokaryotic and eukaryotic cells (Figure 1). Glutathione disulfide (GSSG), which is formed upon oxidation, is reduced by glutathione reductase at the expense of

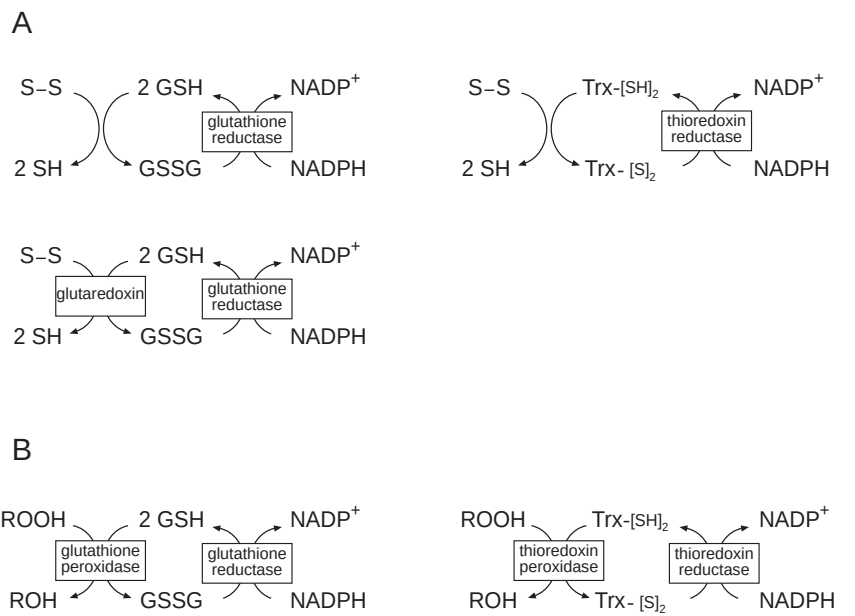

Figure 1 Roles of the glutathione (GSH)- and thioredoxin (Trx)-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* defenses against oxidative stress. (*A*) Disulfide bond reduction by GSH, by GSH together with glutaredoxin, and by Trx. (*B*) Peroxide elimination by GSH- or Trx-dependent peroxidases.

NADPH. Oxidized thioredoxin is reduced by thioredoxin reductase at the expense of NADPH. In both cases, NADPH is regenerated by glucose-6-phosphate dehydrogenase. Most glutaredoxins and all thioredoxins and thioredoxin reductases contain a conserved motif of two cysteines separated by two amino acids (CXXC) at their active sites. The redox potentials determine whether enzymes are more reducing or more oxidizing and differ significantly among the various enzymes. In general, however, thioredoxins are better disulfide bond reductants than glutaredoxins. The roles of GSH and thioredoxin in maintaining the cellular thiol/disulfide status in both prokaryotic and eukaryotic cells are the subject of several reviews [for example, see Holmgren (43) and Rietsch & Beckwith (82)]. Here we examine the roles of GSH and thioredoxin in protecting bacteria and yeast against oxidative stress.

Oxidative stress occurs when cells are exposed to elevated levels of reactive oxygen species such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and alkyl hydroperoxides (ROOH), which include cumene hydroperoxide and *t*-butyl hydroperoxide. Oxidative stress can lead to DNA damage and mutations, lipid peroxidation, the disassembly of iron-sulfur clusters, disulfide bond formation, and other types of protein oxidation [reviewed by Jamieson (50) and by Storz & Imlay (92)]. Given the GSH and thioredoxin requirements for disulfide bond reduction and for glutathione-and thioredoxin-dependent peroxidase activities, as well as the fact that GSH is the major cellular thiol, the GSH- and thioredoxin-based systems have been proposed to protect cells against oxidative stress (67, 88). The characterization of *E. coli* and *S. cerevisiae* strains lacking components of the GSH- and thioredoxin-based reduction systems, together with studies of the expression of the corresponding genes in response to oxidative stress (summarized in Table 1), is allowing the antioxidant roles of these systems to be evaluated in detail.

This review focuses on the GSH- and thioredoxin-based reduction systems in *E. coli* and *S. cerevisiae*; however, all the components of the two systems are well conserved in higher eukaryotes. For example, the *E. coli* and *S. cerevisiae* glutaredoxin reductase proteins show 46% and 50% identity to human glutathione reductase, respectively. Thus, much of what is being learned about the roles of the GSH and thioredoxin systems in the bacterial and yeast responses to oxidative stress is likely to hold true for other organisms.

ESCHERICHIA COLI

E. coli cells have adaptive responses to H_2O_2 and to $O_2^{\bullet -}$-generating compounds [reviewed by Storz & Imlay {92)]. Cells treated with low concentrations of H_2O_2, paraquat, or menadione become resistant to otherwise lethal doses of these oxidants. In large part, the adaptive response to H_2O_2 is regulated by the OxyR transcription factor, and the adaptive response to $O_2^{\bullet -}$-generating compounds is regulated by the SoxR and SoxS transcription factors.

TABLE 1 The glutathione- and thioredoxin-dependent reduction systems in *Escherichia coli* and *Saccharomyces cerevisiae*[a]

Gene	Locus[b]	Activity	Oxidant sensitivity[c]	Regulation
Escherichia coli				
gshA	AAC75735	γ-Glutamyl-cysteine synthase	$H_2O_2^{wt}$, CHP^{wt}, $diamide^{s}$	
gshB	AAC75984	Glutathione synthase	$Diamide^{s}$	
gor	AAC76525	Glutathione reductase	$H_2O_2^{wt}$, $CHP^{(s)}$, $tBHP^{wt}$, $PQ^{(s)}$, $diamide^{s}$	H_2O_2-inducible by OxyR
grxA	AAC73936	Glutaredoxin 1		H_2O_2-inducible by OxyR
grxB	AAC74148	Glutaredoxin 2		
grxC	AAC76634	Glutaredoxin 3		
nrdH	AAC75720	Nucleotide reductase		
trxA	AAC76786	Thioredoxin 1	$H_2O_2^{s}$	
trxC	AAC75635	Thioredoxin 2	$H_2O_2^{wt}$	H_2O_2-inducible by OxyR
trxB	AAC73974	Thioredoxin reductase	$H_2O_2^{r}$	
ahpC	AAC73706	Alkyl hydroperoxide reductase	$H_2O_2^{(s)}$, CHP^{S} ($\Delta ahpCF$)	H_2O_2-inducible by OxyR
ahpF	AAC73707	Alkyl hydroperoxide reductase	$H_2O_2^{(s)}$, CHP^{S} ($\Delta ahpCF$)	H_2O_2-inducible by OxyR
tpx	AAC74406	Thioredoxin peroxidase		
bcp	AAC75533	Thioredoxin peroxidase		
Saccharomyces cerevisiae				
GSH1	YJL101C	γ-Glutamyl-cysteine synthetase	$H_2O_2^{s}$, CHP^{wt}, $tBHP^{s}$	H_2O_2-inducible by Yap1p
GSH2	YOL049W	Glutathione synthetase	$H_2O_2^{wt}$, $tBHP^{wt}$	
GLR1	YPL091W	Glutathione reductase	$H_2O_2^{S}$, $CHP^{(s)}$, $tBHP^{(s)}$, $PQ^{(s)}$, $diamide^{S}$	H_2O_2-inducible by Yap1p

GRX1	YCL035C	Glutaredoxin 1	$H_2O_2^{wt}$, MEN^{s}	
GRX2/TTR1	YDR513W	Glutaredoxin 2	$H_2O_2^{s}$, MEN^{wt}	H_2O_2-inducible by Yap1p
GRX3	YDR098C	Glutaredoxin 3		
GRX4	YER174C	Glutaredoxin 4		
GRX5	YPL059W	Glutaredoxin 5	$H_2O_2^{s}$, MEN^{s}	
GPX1	YKL026C	Glutathione peroxidase	$H_2O_2^{wt}$, $tBHP^{wt}$	
GPX2	YBR244W	Glutathione peroxidase	$H_2O_2^{wt}$, $tBHP^{wt}$	H_2O_2-inducible by Yap1p
GPX3	YIR037W	Glutathione peroxidase	$H_2O_2^{s}$, $tBHP^{s}$	
TRX1	YLR043C	Thioredoxin 1		
TRX2	YGR209C	Thioredoxin 2	$H_2O_2^{S}$, $diamide^{r}$	H_2O_2-inducible by Yap1p and Skn7p
TRR1	YDR353W	Thioredoxin reductase	$H_2O_2^{s}$, $tBHP^{s}$	H_2O_2-inducible by Yap1p and Skn7p
TRX3	YCR083W	Thioredoxin 3	$H_2O_2^{wt}$	
TRR2	YHR106W	Thioredoxin reductase	$H_2O_2^{s}$	
TSA1	YML028W	Thioredoxin peroxidase	$H_2O_2^{s}$, $tBHP^{s}$	H_2O_2-inducible by Yap1p and Skn7p
AHP1	YLR109W	Thioredoxin peroxidase	$tBHP^{s}$	H_2O_2-inducible by Yap1p and Skn7p
	YDR453C	Thioredoxin peroxidase	$H_2O_2^{wt}$, $tBHP^{wt}$, $diamide^{wt}$	
	YBL064C	Thioredoxin peroxidase	$H_2O_2^{wt}$, $tBHP^{wt}$, $diamide^{wt}$	
	YIL010W	Thioredoxin peroxidase	$H_2O_2^{wt}$, $tBHP^{wt}$, $diamide^{wt}$	

[a]References are given in the text. The table should be interpreted with the caution that the data was compiled from several different studies.

[b]Locus information for *E. coli* and *S. cerevisiae* is taken from http://www.ncbi.nlm.nih.gov and http://genome-www.stanford.edu/cgi-bin/SGD/search, respectively.

[c]Oxidant sensitivities reported for exponentially growing cells. CHP, cumene hydroperoxide; *t*BHP, *t*-butyl hydroperoxide; MEN, menadione; PQ, paraquat; wt, wild type; (s), somewhat sensitive; s, sensitive; S, very sensitive; r, resistant.

Roles of Glutathione, Glutathione Reductase, and Glutaredoxins

GSH, L-γ-glutamyl-L-cysteinylglycine, is synthesized in two steps by γ-glutamylcysteine synthetase and glutathione synthetase. Mutations in the gene encoding γ-glutamylcysteine synthetase (*gshA*) in *E. coli* K strains were isolated in a screen for reduced growth in the presence of diamide, which oxidizes GSH, and in a screen for resistance to the DNA-damaging agent nitrosoguanidine, which requires activation by GSH [3, 34; reviewed by Fuchs (23)]. *E. coli* K strains carrying mutations in the gene encoding glutathione synthetase (*gshB*) were isolated by selecting for mutants that were sensitive to diamide and for mutants that were defective in deoxyribose and cysteine synthesis (3, 24). The *gshA* and *gshB* mutants both are devoid of GSH but have nearly wild-type growth rates in rich and minimal media (3, 24, 34). These results indicate that GSH is dispensable for normal *E. coli* growth. Exponentially growing *gshA* mutant cells also show wild-type resistance to H_2O_2 and cumene hydroperoxide (34). Stationary-phase *gshA* mutant cells are twofold more sensitive to H_2O_2 than wild-type (13), and GSH may also protect cells from the damaging effects of radiation in the presence of oxygen (37). In general, though, GSH does not appear to be a critical antioxidant defense in *E. coli*. The expression of *gshA* and *gshB* has not been studied in detail.

E. coli strains carrying mutations in the gene encoding glutathione reductase (*gor*) were isolated in a screen for colonies with reduced glutathione reductase activity and in a selection for diamide sensitivity (18, 101). *gor* mutants show wild-type growth rates, and it is interesting that the ratio of GSH to total glutathione (GSH + GSSG) is not altered significantly from that of the wild-type. This finding suggests that GSSG disulfide can be reduced independently of glutathione reductase (101). Increased GSH synthesis in *gor* mutants may also help to explain how high GSH levels are maintained (1). *gor* single mutants are somewhat more sensitive to the $O_2^{\bullet -}$-generating compound paraquat and to cumene hydroperoxide but not to *t*-butyl hydroperoxide (1, 61). Increased sensitivity to H_2O_2 owing to the lack of glutathione reductase could be uncovered only in a catalase mutant background (6); however, the transcription of *gor* is clearly induced by H_2O_2 in an OxyR-dependent manner (14, 68).

E. coli glutaredoxin 1 (encoded by *grxA*) was discovered as an activity that could provide electrons to ribonucleotide reductase in strains lacking thioredoxin 1 (42). The glutaredoxin 2 (encoded by *grxB*) and glutaredoxin 3 (encoded by *grxC*) activities were found when mutant strains lacking glutaredoxin 1 and thioredoxin 1 still showed GSH oxidoreductase activity (4). One additional glutaredoxin-like protein (encoded by *nrdH*), which is reduced by thioredoxin reductase rather than by GSH, was detected by sequence homology (53). *grxA* and *grxC* single mutants have wild-type growth rates (81, 85). *grxB* and *nrdH* single mutants have not been described, and the oxidant sensitivities of the *grxA* and *grxC* strains have not been reported. The expression of *grxB*, *grxC*, and *nrdH* in response to oxidative stress

also has not been studied, but *grxA* expression is clearly induced by H_2O_2 in an OxyR-dependent manner (96, 109).

Roles of Thioredoxins and Thioredoxin Reductase

E. coli thioredoxin 1 (encoded by *trxA*) was discovered as an electron donor for ribonucleotide reductase, and thioredoxin reductase (encoded by *trxB*) was discovered as an activity required to reduce oxidized thioredoxin 1 [reviewed by Holmgren (43)]. Thioredoxin 2 (encoded by *trxC*) was subsequently identified on the basis of sequence similarity (69, 91). Strains carrying individual mutations in the *trxA*, *trxB*, and *trxC* genes are viable. *trxC* mutants do not show altered sensitivity to H_2O_2 (83), *trxA* mutants are sensitive to H_2O_2 in both the exponential and stationary phases, and *trxB* mutants are sensitive in stationary phase (83, 95). It is interesting that *trxB* mutants show increased resistance to H_2O_2 in exponential phase. Because the *trxB* mutants also have increased expression of a *katG*::*lacZ* fusion, the increased resistance may be caused by activation of the OxyR transcription factor, as discussed below. The expression of the thioredoxin genes does not parallel the sensitivities of the mutant phenotypes; *trxA* expression is not increased by H_2O_2 (68), whereas *trxC* expression is clearly induced in an OxyR-dependent manner (83). The expression of *trxB* in response to oxidative stress has not been examined.

Strains carrying various combinations of mutations affecting both the GSH- and thioredoxin-based reduction systems recently have been constructed in *E. coli* (81). The thiol/disulfide status of these mutants was monitored by assays of cytosolic alkaline phosphatase activity. Periplasmic alkaline phosphatase requires two intrachain disulfide bonds for activity. The enzyme is inactive if it is aberrantly expressed in the reducing cytosol of wild-type cells. Thus, increased cytosolic alkaline phosphatase activity is indicative of increased cytosolic disulfide bond formation. *trxA gor* and *trxA gshA* double mutants had significantly higher levels of alkaline phosphatase activity than the *trxA*, *gor*, and *gshA* single mutants, indicating that both the GSH- and thioredoxin-based reduction systems contribute to the reduction of disulfide bonds in the cytosol and that the systems can partially substitute for each other. Systematic measurements of the oxidant sensitivities of these mutant strains have not been carried out.

Roles of an Alkyl Hydroperoxide Reductase and Thioredoxin Peroxidases

An alkyl hydroperoxide reductase (encoded by *ahpCF*) activity that converts lipid hydroperoxides and other ROOH to the corresponding alcohols, using either NADH or NADPH as the reducing agent, was discovered in constitutively active *oxyR* mutant strains that were more resistant to cumene hydroperoxide than were wild-type cells (49). The enzyme was found to be composed of two components, a 22-kDa AhpC subunit that acts on the substrate and a 52-kDa AhpF flavoprotein that can use either NADH or NADPH to reduce oxidized AhpC. The

C-terminal domain of AhpF is homologous to thioredoxin reductase and contains one redox-active disulfide center (99). Because the N-terminal domain of AhpF also contains a redox-active disulfide center, AhpF can be regarded as a fusion between a thioredoxin-like protein and a thioredoxin reductase-like protein (80). The alkyl hydroperoxide reductase was proposed to be a prokaryotic equivalent of the eukaryotic glutathione peroxidase, but AhpC homologs have been discovered in numerous eukaryotes (11). *E. coli* strains in which the *ahpCF* operon has been deleted are slightly sensitive to H_2O_2 and extremely sensitive to cumene hydroperoxide (93), indicating a clear antioxidant role for the alkyl hydroperoxide reductase. The expression of the *ahpCF* messenger RNA is regulated by OxyR (98).

Two additional peroxidase activities, which require thioredoxin and thioredoxin reductase rather than AhpF for reduction, have been described in *E. coli* (alternately denoted thioredoxin peroxidases or thiol peroxidases). A 20-kDa peroxidase (encoded by *tpx*) was discovered as a periplasmic activity that could protect against DNA damage and glutamine synthetase inactivation caused by metal-catalyzed oxidation (7). The 18-kDa bacterioferritin-comigratory protein (encoded by *bcp*) was suspected to be a peroxidase on the basis of its homology to AhpC, and indeed, it was found to have an antioxidant activity similar to that of the *tpx*-encoded protein (52). *tpx* and *bcp* mutants grow slightly more slowly than wild-type strains under aerobic conditions (8, 52). *tpx* mutants also are hypersensitive to the $O_2^{\bullet -}$-generating compound paraquat and slightly sensitive to H_2O_2 and *t*-butyl hydroperoxide (8). *bcp* mutants are hypersensitive to H_2O_2 and *t*-butyl hydroperoxide (52). The levels of the bacterioferritin-comigratory protein appear to increase during exponential growth, but the expression of *tpx* and *bcp* has not been studied in detail.

Regulation of OxyR Activity

Two types of mutations affecting the gene encoding the OxyR transcription factor were isolated. The locus was first identified via dominant point mutations that conferred increased resistance to H_2O_2 and cumene hydroperoxide and led to elevated expression of H_2O_2-inducible proteins (14). Subsequently, recessive deletion mutations that conferred sensitivity to H_2O_2 and cumene hydroperoxide and prevented induction of a subset of the H_2O_2-inducible proteins were constructed. The sequence of OxyR revealed that the 34-kDa transcription factor shares homology with the LysR family of bacterial regulators (15).

In vitro transcription and DNA footprinting experiments showed that the tetrameric OxyR protein exists in two forms, reduced and oxidized, and that only the oxidized form activates transcription (94; Figure 2*A*, see color insert). Mass-spectrometric analysis and thiol/disulfide titrations of purified OxyR later revealed that two conserved cysteine residues (C199 and C208) are in a free-thiol form in reduced OxyR and in a disulfide-bonded form in oxidized OxyR (109). Mutation of either C199 or C208 abolishes the ability of the transcription factor to sense H_2O_2 in vivo and in vitro. Thus, direct oxidation of OxyR by H_2O_2, which leads

to disulfide bond formation, is the mechanism by which *E. coli* cells sense H_2O_2 and induce the expression of antioxidant defense activities. The crystal structures of the reduced and oxidized forms of the C-terminal domain of OxyR indicate that the formation of the C199-C208 bond leads to a substantial conformational change (H-J Choi, S-J Kim, S Cho, J-R Woo, G Storz, S-E Ryu, unpublished data).

Alkylation assays showed that, in logarithmically growing wild-type cells treated with H_2O_2, the OxyR protein is completely oxidized within 30 s and remains oxidized for ~5 min, paralleling the increase and decrease of messenger RNAs transcribed from OxyR- regulated genes (5, 97). These experiments also showed that the minimum H_2O_2 concentration required to completely oxidize OxyR in vivo is 5 μM. Given the millimolar levels of GSH inside the cell compared with the submicromolar levels of OxyR, the reaction between OxyR and H_2O_2 must be highly specific.

The finding that OxyR was activated/oxidized for only a defined time period raised the question of how the protein was reduced (109). In vitro transcription assays with purified components showed that glutaredoxin 1 together with GSH and thioredoxin 1 together with thioredoxin reductase can catalyze the reduction of OxyR. In vivo, the OxyR-regulated response is prolonged in *gor* and *grxA* single mutants but not in *trxA* or *trxB* single mutants. These results indicate that, in the cell, oxidized OxyR is reduced by glutaredoxin 1. Because the *gor* and *grxA* genes are themselves activated by oxidized OxyR, the OxyR response is autoregulated. Consistent with overlapping functions of the GSH and thioredoxin systems in preventing disulfide bond formation in the *E. coli* cytoplasm, OxyR is partially active in the absence of oxidative stress in *trxA gor* and *trxA gshA* double-mutant strains. These mutant cells have a decreased GSH/GSSG ratio but do not have elevated levels of H_2O_2 (5). Thus OxyR may be activated by either of two different mechanisms in the cell: increases in the H_2O_2 concentration or decreases in the thiol/disulfide ratio. The observation that OxyR is constitutively active in some mutants that are defective in the disulfide bond reduction systems may help to explain why *trxB* mutants show increased resistance to H_2O_2 and why *trxA gshA* mutants show elevated expression of the OxyR-regulated *grxA* gene (26, 95).

Regulation of SoxR Activity

The divergently transcribed *soxRS* genes also were identified by two types of mutations: dominant point mutations that led to resistance to $O_2^{\bullet -}$-generating compounds and to constitutive expression of $O_2^{\bullet -}$-inducible proteins and reporter gene fusions, and recessive deletion and insertion mutations that led to hypersensitivity to $O_2^{\bullet -}$-generating compounds and prevented $O_2^{\bullet -}$-dependent induction (35, 100). The 17-kDa SoxR protein has homology to the mercury-dependent MerR regulator of *E. coli*, and the 13-kDa SoxS protein has homology to the AraC class of prokaryotic transcriptional activators (2, 106). The regulation of the *soxRS* regulon occurs in two steps (75, 107). Under conditions of oxidative stress, the

constitutively expressed SoxR protein is converted to an active form that activates *soxS* transcription. Increased levels of SoxS in turn lead to increased expression of antioxidant activities such as manganese superoxide dismutase.

Chemical analyses and visible and electron paramagnetic resonance spectroscopy showed that SoxR is a homodimer with two [2Fe-2S] centers per dimer (39, 40, 105). The oxidation of the reduced $[2Fe\text{-}2S]^{1+}$ form of SoxR to a $[2Fe\text{-}2S]^{2+}$ form appears to be the mechanism of SoxR activation (20, 22, 27, 28, 39, 41; Figure 2*B*, see color insert). Electron paramagnetic resonance spectroscopy of whole cells indicated that overproduced wild-type SoxR protein is oxidized within 2 min after cells are treated with $O_2^{\bullet -}$-generating compounds and that constitutively active mutant SoxR proteins are predominantly in the oxidized form even in the absence of stress (20, 27, 41). The nature of the oxidant that reacts with the [2Fe-2S] cluster in SoxR is still under debate. The *soxRS* regulon is induced by redox cycling reagents that generate $O_2^{\bullet -}$. Thus, the SoxR protein might be oxidized directly by $O_2^{\bullet -}$. However, because the generation of $O_2^{\bullet -}$ is accompanied by the consumption of cellular reductants, it is also possible that SoxR activity is modulated by alterations in NADPH, reduced flavodoxin, or reduced ferredoxin levels (64).

The in vivo electron paramagnetic resonance studies showed that oxidized SoxR is rapidly reduced once the oxidative stress is removed (20), but the mechanism of SoxR reduction is not yet understood. Kobayashi & Tagawa (57) have reported the purification of an NADPH-dependent SoxR reductase; however the corresponding gene has not been identified. SoxR activity also appears to be modulated by the assembly/disassembly of the [2Fe-2S] clusters. The SoxR apoprotein binds to the *soxS* promoter but does not activate *soxS* expression. Aerobic exposure to monothiols such as GSH leads to disruption of the SoxR [2Fe-2S] clusters in vitro (19). In contrast, the presence of dithiols such as dithiothreitol and the dithiol enzyme thioredoxin in vitro promotes cluster assembly into apo-SoxR (21). *gshA*, *trxA*, and *trxB* single-mutant strains showed wild-type induction of a *soxS-lacZ* fusion in response to paraquat, but *soxS-lacZ* induction was reduced in a *gor trxA* double mutant (19, 21). These results indicate that both the GSH and thioredoxin reduction systems are required to maintain the SoxR [2Fe-2S] clusters, but how the thiols affect iron-sulfur cluster assembly remains to be elucidated.

SACCHAROMYCES CEREVISIAE

S. cerevisiae cells also have adaptive responses to both H_2O_2- and $O_2^{\bullet -}$-generating compounds [reviewed by Jamieson (50)]. The mechanisms by which these responses are regulated are less well elucidated than those of *E. coli*; however, several studies have clearly implicated the Yap1p and Skn7p transcription factors in regulating the expression of genes induced by H_2O_2.

Roles of Glutathione, Glutathione Reductase, and Glutaredoxins

S. cerevisiae strains with reduced levels of GSH caused by mutations in the gene encoding γ-glutamylcysteine synthetase (*GSH1*) were first isolated in a screen for resistance to nitrosoguanidine [55, 56; reviewed by Grant & Dawes (30)]. GSH-deficient strains carrying mutations in either *GSH1* or the gene encoding glutathione synthetase (*GSH2*) were also identified in a screen for sensitivity to methylglyoxal, which is normally degraded by the GSH-dependent glyoxalase system (76). The *GSH1* gene was cloned by complementation of a *gsh1* mutation (77), and the *GSH2* gene was detected in the *S. cerevisiae* genome by its homology to mammalian, frog, plant, *Schizosaccharomyces pombe*, and *E. coli* glutathione synthetase sequences (33, 46). Unlike *E. coli gshA* mutants, *S. cerevisiae gsh1* mutants have decreased growth rates compared with wild-type strains and are unable to grow in minimal medium in the absence of exogenous GSH (31, 76, 104). This auxotrophy is most likely caused by a requirement for a reductant in normal cellular processes because growth can be restored by the thiol reductants dithiothreitol, β-mercaptoethanol, and cysteine (31). It is interesting that *gsh1* mutants are sensitive to H_2O_2 and *t*-butyl hydroperoxide but not to cumene hydroperoxide (31, 47). In contrast to *gsh1* mutants, *gsh2* mutants show some growth in the absence of exogenous GSH and are not sensitive to H_2O_2 or *t*-butyl hydroperoxide (33). γ-Glutamylcysteine, the intermediate that accumulates in the *gsh2* strains, appears to have some of the antioxidant activities of GSH. The transcription of *GSH1* is induced by H_2O_2 in a *YAP1*-dependent manner, and bacterially expressed Yap1p was shown to bind to the *GSH1* promoter (90, 104). *GSH2* expression has not been studied in detail, but glutathione synthetase activity is elevated upon H_2O_2 treatment (46).

The gene encoding the *S. cerevisiae* glutathione reductase (*GLR1*) was cloned on the basis of the sequence of cyanogen bromide-generated peptide fragments of commercially available yeast glutathione reductase and the sequence of a C-terminal domain which is highly conserved among glutathione reductases (17). *glr1* mutants show wild-type growth rates but accumulate increased levels of GSSG (29, 74). The mutants are very sensitive to H_2O_2 and diamide and are partially sensitive to cumene hydroperoxide, *t*-butyl hydroperoxide, and paraquat (29). It is interesting that *glr1* mutants that also lack the genes for thioredoxin 1 and thioredoxin 2 are nonviable (74). Thus, yeast cells require the presence of either the GSH- or the thioredoxin-dependent reducing systems for growth. *GLR1* expression is induced by H_2O_2 and diamide in a Yap1p-dependent manner in exponential phase (29). Yap1p also mediates stationary-phase induction of the gene (32).

Two genes encoding glutaredoxin proteins (also denoted thioltransferases) with two cysteines (CXXC) at the active site (*GRX1* and *GRX2/TTR1*) and three genes encoding glutaredoxins with one cysteine (CXXS) at the active site (*GRX3, GRX4*, and *GRX5*) were detected in the completed sequence of the *S. cerevisiae* genome

on the basis of homology to glutaredoxins in other organisms. The functions of the five glutaredoxins were investigated by studies of mutant phenotypes and gene expression (65, 84). *grx1, grx2, grx3,* and *grx4* single mutants as well as a *grx1 grx2* double mutant all showed wild- type growth rates in both rich and minimal media. In contrast, the growth rate of a *grx5* single mutant was decreased in both types of media, and *grx2 grx5* as well as *grx3 grx4 grx5* mutants were nonviable. The *grx1* mutant is sensitive to the $O_2^{\bullet -}$-generating compound menadione but not to H_2O_2, whereas the *grx2* mutant is sensitive to H_2O_2 but not to menadione (65). The *grx5* mutant is sensitive to both of these oxidants (84). Together these results suggest an interesting functional relationship among the glutaredoxins which remains to be elucidated. The presence of three single-cysteine glutaredoxins, Grx3p, Grx4p, and Grxp5, which can act only on GSH-modified proteins, suggests that significant levels of these mixed disulfides are formed and therefore must be reduced in yeast. The expression of both *GRX1* and *GRX2* is induced by various stress conditions, including exposure to H_2O_2 and menadione, as well as osmotic shock and heat stress, whereas transcription of *GRX3, GRX4*, and *GRX5* is not induced by any of these stresses.

Roles of Glutathione Peroxidases

Glutathione peroxidases are considered to be one of the major defenses against peroxides in mammalian cells. This activity is not present in bacteria but has been detected in *S. cerevisiae* (25). Recently, genes (*GPX1, GPX2,* and *GPX3*) encoding three glutathione peroxidase homologs were identified in the completed sequence of the *S. cerevisiae* genome. The functions of the three genes were investigated by phenotypic assays of null-mutant strains and by studies of *GPX1, GPX2,* and *GPX3* expression (45). These experiments showed that the *gpx3* null mutant is hypersensitive to H_2O_2 and *t*-butyl hydroperoxide but that *gpx1* and *gpx2* single mutants and *gpx1 gpx2* double mutants do not exhibit obvious mutant phenotypes. The expression of *GPX3* was not induced by any stresses tested. *GPX1* was induced by glucose starvation, and GPX2 was induced by H_2O_2 and *t*-butyl hydroperoxide in a Yap1p-dependent manner.

Roles of Thioredoxins and Thioredoxin Reductases

The genes (*TRX1* and *TRX2*) encoding two thioredoxin activities found in *S. cerevisiae* were cloned subsequently on the basis of the partial amino acid sequences available for the proteins (72). *trx1* and *trx2* single mutants have wild-type growth rates and cell morphology (72); however, the *trx2* single mutant is extremely sensitive to H_2O_2 (58). It is surprising that *trx2* mutants are very resistant to diamide (74). This phenotype is not understood. *trx1 trx2* double mutants are auxotrophic for methionine and have decreased rates of DNA replication and corresponding increases in cell size and generation time (72). The methionine auxotrophy may be caused by reduced 3′-phosphoadenosine 5′-phosphosulfate reductase activity, but the replication phenotype is not understood, especially given

that ribonucleotide reductase activity does not appear to be limited (73). *TRX1* expression has not been studied extensively, but *TRX2* is strongly induced by H_2O_2 and diamide in a Yap1p- and Skn7p-dependent manner (58, 70). The H_2O_2 sensitivity of *trx2* mutants and strong *TRX2* induction by oxidants indicate that the thioredoxin 2 protein plays a key role in the *S. cerevisiae* defenses against oxidative stress.

The yeast thioredoxin reductase 1 protein was purified in an assay for activities that could reduce the *TSA1*-encoded thioredoxin peroxidase described below (9). Antibodies raised against the purified protein were then used to clone the corresponding *TRR1* gene. Subsequent studies showed that *trr1* mutant strains are sensitive to H_2O_2 and *t*-butyl hydroperoxide (63, 66) and that *TRR1* transcription is induced by H_2O_2 in a Yap1p- and Skn7p-dependent manner (12, 62).

The presence of one additional thioredoxin gene (*TRX3*) and one additional thioredoxin reductase gene (*TRR2*) was detected by searches of the *S. cerevisiae* genome data bank (79). The corresponding two proteins have N-terminal domain extensions with characteristics of mitochondrial import signals, and expression data showed that the proteins are indeed present in mitochondrial fractions. A *trx3* mutant exhibited the same growth rate and H_2O_2 sensitivity as wild-type strains. In contrast, the *trr2* mutant was hypersensitive to H_2O_2. The expression of *TRX3* and *TRR2* has not been studied extensively, but both promoters contain sequences that match Yap1p binding sites and the stress response elements bound by the Msn2p and Msn4p transcription factors.

Roles of Thioredoxin Peroxidases

The products of five yeast open reading frames on the yeast genome show homology to the AhpC subunit of the *E. coli* alkyl hydroperoxide reductase. The first of these proteins (encoded by *TSA1*) was purified on the basis of providing protection against oxidative damage to glutamine synthetase (54). Because the 25-kDa protein could protect against an oxidation system containing thiols but not against an oxidation system lacking thiols, the protein was named a thiol-specific antioxidant protein (TSA). Upon further characterization, Tsa1p was shown to be a peroxidase that reduces H_2O_2 and ROOH by the use of hydrogens provided by thioredoxin, thioredoxin reductase, and NADPH (9). The activity was thus renamed thioredoxin peroxidase (TPx). The *TSA1* gene was isolated by using antibodies raised against the purified TSA/TPx protein (10)

Another member of the AhpC-TSA gene family (*AHP1*) was discovered independently by three different groups. Lee et al (63) isolated *AHP1* in a search for genes whose overexpression would rescue the *t*-butyl hydroperoxide hypersensitivity of a *skn7* deletion strain. Ahp1p was also identified as being present in a complex with an *Arabidopsis thaliana* thioredoxin carrying a cysteine mutation that blocks release of the substrate when this thioredoxin derivative is expressed in yeast (102). In addition, Ahp1p was identified in an assay for other thioredoxin peroxidase activities (51). As was found for the *TSA1*-encoded protein, the

AHP1-encoded protein requires thioredoxin and thioredoxin reductase for its activity in vivo (63) and in vitro (51). In vitro assays of the *TSA1*- and *AHP1*-encoded activities indicated that Tsa1p preferentially reduces H_2O_2 rather than ROOH such as cumene hydroperoxide and *t*-butyl hydroperoxide, whereas Ahp1p preferentially reduces ROOH rather than H_2O_2 (51, 78). The *tsa1* null mutant is hypersensitive to killing by H_2O_2 and *t*-butyl hydroperoxide (10, 78). In contrast, the *ahp1* null mutant is hypersensitive only to *t*-butyl hydroperoxide (63, 78). Expression of both *TSA1* and *AHP1* is induced by H_2O_2 in a Yap1p- and Skn7p-dependent manner (63).

The products of three other open reading frames (YDR453C, YBL064C, and YIL010W) show homology to Tsa1p and Ahp1p. These proteins were purified recently and were also shown to have thioredoxin-dependent peroxidase activity (78). Fusions between each of the peroxidases and the green fluorescent protein indicate that Tsa1p, Ahp1p, and YDR453C are located in the cytosol, YBL064C is located in mitochondria, and YIL010W is located in the nucleus (78). However, others have suggested that Ahp1p is in peroxisomes, given Ahp1p's homology to other peroxisomal proteins (63). Strains lacking YDR453C show a reduced growth rate, but the YDR453C, YBL064C, and YIL010W single-mutant strains do not show significant sensitivity to H_2O_2, *t*-butyl hydroperoxide, or diamide (78). The YDR453C, YBL064C, and YIL010W transcripts are expressed at significantly lower levels than the *TSA1* and *AHP1* mRNAs, but the expression of YDR453C and YBL064C also is induced by H_2O_2 and diamide (78). Further studies of the relative activities of the five thioredoxin peroxidases and their in vivo substrates need to be carried out in order to elucidate the individual functions of the peroxidases.

Regulation of Yap1p Activity

The Yap1p transcription factor was discovered on the basis of its binding to a site recognized by the mammalian AP-1 transcription factor (TGACTCA) (36), and the corresponding *YAP1* gene was cloned by using antibodies directed against purified Yap1p (71). The *YAP1* gene also was isolated independently in multicopy screens for resistance to sulfometuron methyl and cycloheximide [*PDR4* (44)], resistance to 4-nitroquinoline-*N*-oxide, trenimon, and nitrosoguanidine [*SNQ3* (38)], and resistance to the iron chelators 1,10-phenanthroline and 1-nitroso-2-naphthol [*PAR1* (86)]. Subsequently, *yap1* mutant strains were found to be hypersensitive to H_2O_2 (87), and Yap1p was shown to be required for the *S. cerevisiae* adaptive response to H_2O_2 and for the H_2O_2-, *t*-butyl hydroperoxide-, and diamide-dependent activation of *TRX2* (58, 90).

Mutational studies showed that a C-terminal domain, often referred to as a cysteine-rich domain (CRD) owing to the presence of three cysteine-serine-glutamate (CSE) repeats, is required for Yap1p-dependent gene activation and resistance to H_2O_2 and diamide (59, 103). A second domain, which also contains several cysteines and is located at the N terminus, is required for H_2O_2 but not diamide resistance (16). Confocal-microscopy studies of Yap1p fused to the green

fluorescent protein revealed that Yap1p is present throughout the cell during normal growth but is concentrated in the nucleus when cells are treated with diamide (59). Yap1p derivatives with deletions of the C-terminal cysteine-rich domain are constitutively located in the nucleus, and strains expressing the truncated Yap1p are resistant to diamide. Subsequent studies demonstrated that Yap1p contains a nuclear export signal within the C-terminal cysteine-rich domain and that, in the absence of stress, Yap1p is actively exported from the nucleus via the export receptor Crm1p (also denoted Xpo1p) (60, 108). Two-hybrid studies showed an interaction between Yap1p and Crm1p in normally growing cells but not in cells exposed to diamide. These findings have led to the model depicted in Figure 2*C*. In the absence of stress, Yap1 is actively exported from the nucleus. Under conditions of oxidative stress, Yap1p can no longer interact with Crm1p and hence is no longer exported to the cytoplasm. The increased nuclear concentration of Yap1p then leads to increased expression of the Yap1p target genes. It is tempting to speculate that Yap1p might be a direct sensor of oxidative stress, analogous to OxyR and that oxidation of cysteines in the C-terminal cysteine-rich domain prevents Yap1p interaction with Crm1p, but biochemical data on the oxidation state of Yap1p still are lacking.

Another clue to the mechanism of Yap1p activation has come from the recent finding that Yap1p is concentrated in the nucleus and is constitutively active in *trx1 trx2* mutants (48). There are several explanations for these effects of the *trx1 trx2* mutations. The lack of thioredoxins might change the redox status of the whole intracellular environment and thus indirectly cause Yap1p activation. In support of this possibility, the effects of the *trx1 trx2* mutations on Yap1p activation are observed only under aerobic conditions, and *trx1 trx2* mutants show increased intracellular oxidation as determined by measuring the fluorescence of an oxidant-sensitive probe. Alternatively, the thioredoxins might be required to directly reduce oxidized Yap1p, analogous to how glutaredoxin 1 reduces oxidized OxyR. A combination of the two possibilities also needs to be considered. The isolation of a *trr1* mutant in a screen for elevated *TRX2* expression in the absence of oxidative stress is consistent with a role for the thioredoxin-based reducing system in modulating Yap1p activity (O Carmel-Harel, R Stearman, G Storz, unpublished results). Interestingly however, the GSH-based reducing system does not appear to have a role in regulating Yap1p because *gsh1* mutants still have an adaptive response to H_2O_2, and *grx1 grx2* mutants do not show constitutive Yap1p activation (48, 89).

Several genes whose expression is regulated by Yap1p are also regulated by Skn7p. In fact, *yap1* single mutants, *skn7* single mutants, and *yap1 skn7* double mutants are equally sensitive to H_2O_2, and the oxidative-stress-dependent induction of some targets is abolished in both of the single-mutant strains (62, 70). These findings indicate that the Yap1p and Skn7p factors act in concert at some promoters, although the transcription factors also have separate functions. Skn7p shows similarity to the DNA binding domain of eukaryotic heat shock factors and to the receiver domain found in prokaryotic two-component regulators. However, it is

not understood how Skn7p activity is regulated in response to oxidative stress and whether the GSH- and thioredoxin-dependent systems impact on Skn7p.

CONCLUDING REMARKS

Many components of the GSH- and thioredoxin-dependent reduction systems have now been identified in *E. coli* and *S. cerevisiae*, in part as a result of the completed genomic sequences of the two organisms. A combination of biochemical and genetic studies has shown that there is extensive redundancy for many of the enzymes and significant overlap between the two reduction systems. As a consequence, the interpretation of some results is complicated. Nevertheless, several general conclusions can be drawn with respect to the roles of the GSH- and thioredoxin-based systems in protection against oxidative stress. First, the expression of the genes encoding a subset of the components, such as *E. coli trxC* and *S. cerevisiae TRX2*, is strongly induced by oxidative stress. Second, the lack of individual components of the reducing pathways can result in wild-type sensitivity, increased sensitivity, or decreased sensitivity to oxidants. *E. coli trxC* mutants show wild-type resistance to H_2O_2, whereas *S. cerevisiae trx2* mutants are extremely sensitive and *E. coli trxB* mutants show increased resistance. Third, GSH and thioredoxin modulate the activities of the *E. coli* OxyR and SoxR and the *S. cerevisiae* Yap1p transcription factors. Thus, both reduction systems are integral parts of the bacterial and yeast responses to oxidative stress.

We have described the mutant phenotypes reported in several different studies (summarized in Table 1), and a few cautionary comments are warranted. The genetic backgrounds of many of the mutant strains differ. This precludes the direct comparison of results from different laboratories and may explain why differing phenotypes occasionally have been reported. A systematic study of the oxidant sensitivities of isogenic strains containing single mutations in the genes encoding all of the components of the two reduction systems would be interesting and would allow direct assessment of the antioxidant contributions of the different enzymes. As described above, the interpretation of some phenotypes also is complicated by the fact that the lack of some components leads to the activation of the OxyR and Yap1p transcription factors, resulting in increased expression of other antioxidant activities. In addition, it should be noted that under certain conditions, reductants can act as oxidants (91). Finally, the GSH and thioredoxin pathways impact on many aspects of cell metabolism. Glutaredoxins and thioredoxins are required for deoxyribonucleoside triphosphate synthesis, thioredoxins affect the yeast cell cycle, and GSH conjugation is an effective means of eliminating many toxic compounds, including oxidants. Thus, further genetic experiments are important but their outcomes should be interpreted with care.

Many critical questions remain to be answered. More must be learned about the cellular roles of the different isozymes. Why do *E. coli* cells possess three glutaredoxins and one glutaredoxin-like protein, and why does *S. cerevisiae* possess

five glutaredoxins? Differences in substrate affinity, redox potential, expression, and subcellular localization are likely to be part of the answer. How do the roles of the GSH-based system and the thioredoxin-based system differ? Why is the activity of OxyR modulated by glutaredoxin and GSH while the activity of Yap1p is regulated, directly or indirectly, by thioredoxin? What are the in vivo substrates for the different glutaredoxin and thioredoxin proteins? What are the redox statuses of the different cellular compartments? How is the cellular redox status altered under conditions of oxidative stress?

The characterization of additional mutants and further application of the biochemical assays described above should help to provide answers to some of these questions. However, it is likely that new genetic screens and new assays will have to be developed to identify additional components, to monitor the redox states of intracellular proteins, and to isolate the substrates of the glutaredoxin and thioredoxin proteins. For example, a fluorescent indicator of the redox statuses of different cellular compartments is a much needed tool. Nevertheless, further studies of *E. coli* and *S. cerevisiae* clearly will provide important new insights into the cellular redox status and oxidative-stress responses that will be relevant to other organisms for which the roles of GSH and thioredoxin cannot be evaluated so conclusively.

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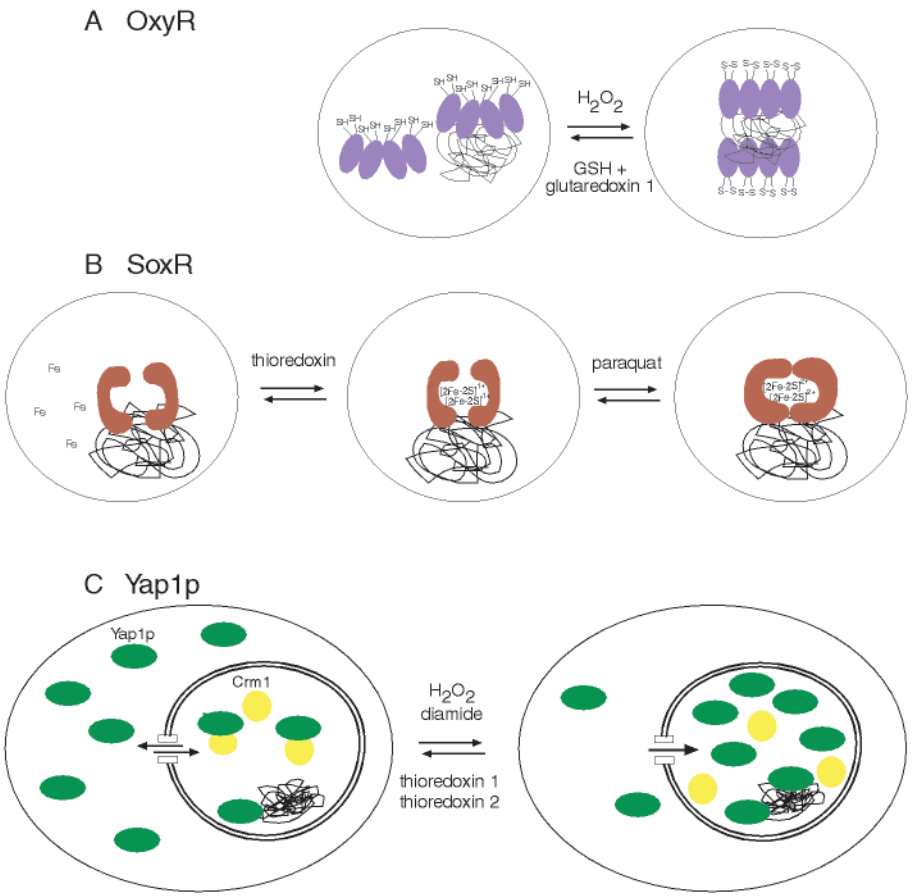

Figure 2 Regulation of OxyR, SoxR, and Yap1p by oxidative stress and the GSH- and thioredoxin-dependent reduction systems. (*A*) Direct oxidation of OxyR by H_2O_2 results in a conformational change which leads to enhanced expression of antioxidant activities. The reduced dithiol form of OxyR can bind some, but not all, OxyR targets while the oxidized-disulfide form binds to all promoters. Oxidized OxyR is reduced by glutaredoxin 1 together with GSH. (*B*) The $[2Fe\text{-}2S]^{1+}$ clusters in reduced SoxR are oxidized to $[2Fe\text{-}2S]^{2+}$ when cells are exposed to O_2-generating compounds. Oxidized SoxR then activates *soxS* transcription; increased SoxS levels lead to elevated expression of antioxidant genes. The reduced, oxidized and apoprotein forms of SoxR can all bind to the *soxS* promoter, but only oxidized SoxR can activate transcription. Thioredoxin stimulates the assembly of the SoxR [2Fe-2S] cluster. (*C*) Yap1p localization is regulated by oxidative stress. During normal growth, Yap1p associates with Crm1p and is actively exported to the cytoplasm. In diamide- or H_2O_2-treated cells, Yap1p can no longer bind Crm1p and accumulates in the nucleus where it activates the expression of antioxidant defenses. The lack of thioredoxin 1 and thioredoxin 2 leads to Yap1p accumulation in the nucleus in the absence of oxidative stress.

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