The Response to Heat Shock and Oxidative Stress in *Saccharomyces cerevisiae*

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ABSTRACT A common need for microbial cells is the ability to respond to potentially toxic environmental insults. Here we review the progress in understanding the response of the yeast *Saccharomyces cerevisiae* to two important environmental stresses: heat shock and oxidative stress. Both of these stresses are fundamental challenges that microbes of all types will experience. The study of these environmental stress responses in *S. cerevisiae* has illuminated many of the features now viewed as central to our understanding of eukaryotic cell biology. Transcriptional activation plays an important role in driving the multifaceted reaction to elevated temperature and levels of reactive oxygen species. Advances provided by the development of whole genome analyses have led to an appreciation of the global reorganization of gene expression and its integration between different stress regimens. While the precise nature of the signal eliciting the heat shock response remains elusive, recent progress in the understanding of induction of the oxidative stress response is summarized here. Although these stress conditions represent ancient challenges to *S. cerevisiae* and other microbes, much remains to be learned about the mechanisms dedicated to dealing with these environmental parameters.

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ORGANISMS are constantly challenged by ever-changing variables in their environment, including fluctuating nutrient levels, osmotic imbalance, exposure to toxic molecules, and nonoptimal temperatures. While multicellular or motile organisms can usually alter these conditions by a change in location or physiology, single-celled organisms such as yeast are at the mercy of their situation, and must adapt or perish. Even in the absence of lethal stresses, subtle differences in the ability to tolerate environmental fluctuations can confer a competitive advantage in a mixed niche such as the surface of a ripening fruit. Here we will review the current state of knowledge of the response of *Saccharomyces cerevisiae* to two important environmental challenges: heat and oxidative stress. Studies on the response to heat and oxidative stress have provided important insight into nearly every aspect of eukaryotic cell biology, as we hope will be clear from our discussion.

Control and Regulation of the Heat Shock Response

The most fundamental stress experienced by a yeast cell is the ambient temperature. *Saccharomyces cerevisiae* exhibits optimal growth between 25° and 30° (77°–86°F), the anthropomorphic equivalent of a sunny day. However, at

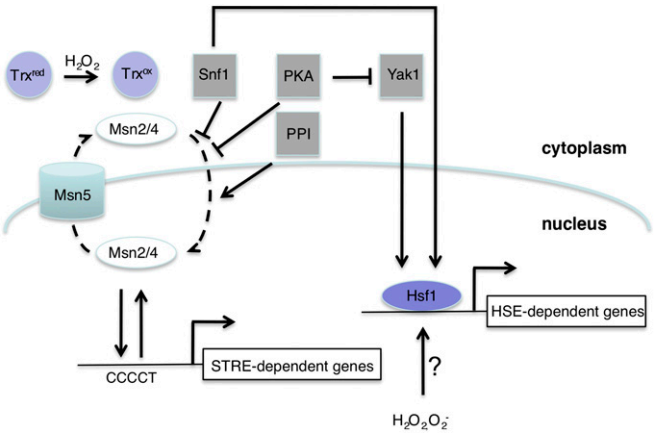

Figure 1 Regulation of Msn2/4 and Hsf1. Regulation of Msn2 by growth control proteins and oxidative stress is diagrammed. H_2O_2 triggers the oxidation of cytoplasmic thioredoxin proteins (Trx^{ox}) from their normally reduced status (Trx^{red}). This induces the recruitment of Msn2 into the nucleus where it can interact with its cognate binding site (stress response element, STRE) and activate expression of target gene transcription. Hsf1 is constitutively nuclear and prebound to many target genes containing heat shock elements (HSEs) in their promoters. Hsf1 is also regulated by the nutrient-sensing kinases, Snf1 and PKA, and by oxidative stress through unknown mechanisms.

temperatures >36–37° (nearing 100°F), yeast cells activate a protective transcriptional program termed the heat shock response (HSR) and alter other components of their physiology, including membrane composition and carbohydrate flux. *S. cerevisiae* and other mesophilic yeasts maintain growth at temperatures up to ~42° (107°F), but are unable to cope with chronic exposure to higher temperatures (indeed, yeast RNA polymerase II is inactive at temperatures >42°; Yamamoto *et al.* 2008). As yeast cells may experience this range of temperature over the course of a day/night cycle, investigation of the HSR induced by shift from 30° to 37° (the classic "heat shock") in a laboratory setting is physiologically relevant. Over the last 25 years, much has been revealed about how yeast cells respond to heat shock, including the transcription factors that govern changes in gene expression and the metabolic reprogramming that enables cells to withstand chronic exposure to sublethal temperatures. In the decade since the previous incarnation of this review series, the scope has been both narrowed, by detailed analysis of molecular interactions between players, and expanded, through genome-level surveys of gene expression and protein–protein interactions. These recent advances will be discussed in this chapter, with an emphasis on the integration and interplay between the HSR and other stress response pathways. Because the literature is extensive, the reader is directed to several prior reviews that discuss the relevance of past findings in greater depth (Piper 1997; Trott and Morano 2003).

The major players: heat shock transcription factor 1 and Msn2/4

In eukaryotes, the heat shock transcription factor (HSF) protein family is the primary modulator of the HSR. In *S. cerevisiae* (and likely other related yeasts), a second transcription factor represented by the *MSN2* and *MSN4* genes also contributes substantially to heat shock gene expression. Indeed, microarray analysis using conditional and knockout mutants, respectively, of the *HSF1*, *MSN2*, and *MSN4* genes suggests that these three factors are responsible for the bulk if not the entirety of the HSR (Figure 1). The Msn2/4 regulon is much broader than heat shock-induced transcripts and includes oxidative stress and metabolic and other cytoprotective genes, leading to its characterization as the "environmental stress response" (ESR), described in more detail below.

HSE type	architecture	example genes
perfect	nn**GAA**nn**TTC**nn**GAA**nn	***HSP26, HSP104, SSA1***
gap	nn**GAA**nn**TTC**nnnnnnn**GAA**nn	***HSP82, CPR6, CUP1***
step	nn**GAA**nnnnnnn**GAA**nnnnnnn**GAA**nn	***HSP12, SSA3, YDJ1***

Figure 2 Divergence in HSE architecture. Three different types of HSE have been described on the basis of spacing and positioning, as described in the text. Many genes within the Hsf1 regulon have annotated HSEs within the promoters; representative examples are shown (Yamamoto *et al.* 2005). n, any nucleotide.

HSF1: Four distinct HSF isoforms have been described in mammals. HSF1 is primarily dedicated to regulating the HSR, while HSF2 plays a role in developmental gene expression. The roles of HSF3 and HSF4 are less well understood but evidence suggests these factors may functionally interact with HSF1 to modulate gene expression (reviewed in Akerfelt *et al.* 2010). Invertebrates including the "lower" eukaryotes express a single, essential HSF equivalent to mammalian HSF1 (Akerfelt *et al.* 2010). The fundamental architectural elements of this transcription factor are conserved, consisting of an amino-terminal winged helix-turn-helix DNA binding domain (DBD) followed by a leucine zipper motif required for trimerization and activation, a loosely defined, serine-rich "regulatory" domain, and a carboxy-terminal transcriptional activation domain (Trott and Morano 2003). *S. cerevisiae HSF1* is unusual in that it includes an amino-terminal extension of ~150 amino acids that acts as a second potent transcriptional activation domain (Sorger 1990). Hsf1 recognizes a pentameric heat shock element (HSE) defined as repeating units of the sequence nGAAn (Sorger and Pelham 1987). Early investigations defined the HSE as a triple inverted repeat, consistent with the fact that Hsf1 binds DNA as a trimer (Sorger and Pelham 1987). The discovery that Hsf1 activates the yeast metallothionein gene *CUP1* in response to heat shock and oxidative stress challenged this paradigm, as the *CUP1* promoter contains an atypical HSE with two nGAAn repeats followed by a pentameric spacer and another repeat (Tamai *et al.* 1994; Liu and Thiele 1996) (Figure 2). Subsequent work revealed that variations of this "noncanonical" HSE were widespread in the yeast genome (Yamamoto *et al.* 2005), greatly expanding the range of possible Hsf1 targets (see Figure 2).

HSE architecture dramatically dictates Hsf1 behavior, as the noncanonical HSE with 5-bp "spacers" between the nGAAn units is cooperatively bound by HSF that need not be phosphorylated nor oligomerized (trimerized) (Hashikawa *et al.* 2006), two conditions previously thought to be absolute prerequisites for function. Moreover, a novel C-terminal regulatory domain (CTM) identified in Hsf1 is required for transcription from "perfect" promoters bound noncooperatively by a single trimer, but not for the noncanonical gene targets (Hashikawa *et al.* 2006). These findings demonstrate previously unappreciated complexity in Hsf1 function in *S. cerevisiae* and help explain the unexpectedly large number of Hsf1-responsive genes uncovered in genomic analyses (see *Global analysis of the HSR*, below).

Msn2/4: Msn2 and its close homolog Msn4 (referred to as Msn2/4) were identified in the mid-1990s as proteins required for expression of a wide array of genes in response to multiple types of stress, but not for viability under normal conditions (Martinez-Pastor *et al.* 1996; Schmitt and McEntee 1996). Analysis of the promoter of the cytosolic catalase gene (CTT1) indicated the presence of an element termed the stress responsive element (STRE) that consisted of a pentameric core of CCCCT (Wieser *et al.* 1991). The STRE was demonstrated to be the recognition site for Msn2/4 and to be required for induction of *CTT1* and many other genes to various stresses (Martinez-Pastor *et al.* 1996). Msn2 is the dominant factor of the two, although overexpression of *MSN4* can partially alleviate gene expression and growth phenotypes associated with *msn2*Δ cells (Schmitt and McEntee 1996). Msn2/4 targets are regulated by growth phase and nutrient status (Figure 1); *i.e.*, induced after the diauxic shift or in late stationary/quiescent phase cells (Trott and Morano 2003). Nutrient-responsive transcriptional control is mediated by the glucose-responsive cyclic AMP (cAMP)-protein kinase A signaling pathway that phosphorylates Msn2/4 under nonstress conditions to block its nuclear import, thereby inhibiting target gene activation (Gorner *et al.* 1998). While PKA is an important negative regulator of Msn2/4 function, other signal transduction pathways modulate the activity of these transcription factors. The AMP-dependent protein kinase Snf1 also phosphorylates and downregulates Msn2 (De Wever *et al.* 2005). Experiments from several labs have implicated the protein phosphatase PP1 in dephosphorylation of the PKA- and Snf1-triggered changes in phosphorylation (Mayordomo *et al.* 2002; De Wever *et al.* 2005; Lenssen *et al.* 2005). PP1 appears to directly dephosphorylate Msn2 but also to negatively influence Snf1 activity, thus providing two different routes of control to Msn2 activity. Utilization of these multiple distinct but related pathways to control Msn2/4 function is likely to provide the means through which Msn2 regulation is tied into general cellular stress.

Nucleocytoplasmic shuttling of Msn2/4 is rapid and oscillates even during stress conditions, suggesting that modulators such as cAMP/PKA influence the sensitivity and frequency of nuclear localization rather than absolutely restricting transport from one compartment to another (Jacquet *et al.* 2003). Msn2 is degraded in the nucleus upon constitutive or chronic activation, providing a potential adaptation mechanism for periods of prolonged nutrient limitation or environmental stress (Lallet *et al.* 2004). Remarkably, elimination of Msn2/4 allows cells to dispense with the "essential" cAMP/PKA pathway, suggesting that one or more Msn2/4 targets antagonize PKA function and are detrimental to optimal growth (Smith *et al.* 1998). Several lines of evidence point to the Yak1 kinase as the prime candidate for this role. First, Msn2/4 controls *YAK1* expression (Smith *et al.* 1998); second, Yak1 kinase activity is directly inhibited by phosphorylation by PKA (Lee *et al.* 2008); and third, deletion of *YAK1* also suppresses PKA hypomorphic mutations (Garrett and Broach 1989). In addition, Yak1 phosphorylates the PKA regulatory subunit, Bcy1, biasing its localization to the cytoplasm. In contrast to the negative regulation exerted on Msn2/4 by PKA, Yak1 phosphorylates and activates Msn2/4 and Hsf1 under low glucose conditions (Griffioen *et al.* 2001; Lee *et al.* 2008). This finding makes this kinase one of the few regulatory proteins known to influence both Hsf1 and Msn2/4 (Griffioen *et al.* 2001; Lallet *et al.* 2004; Lee *et al.* 2008).

Sensing thermal stress: How do yeast cells sense heat shock and activate the appropriate transcriptional response? In 1991 Craig and Gross postulated that heat shock proteins (HSPs) could act as the cellular thermometer, but this conjecture lacked substantial supporting evidence in eukaryotic systems (Craig and Gross 1991). Moreover, this idea is predicated on the fact that HSPs are sensing a physiological disturbance distinct from those caused by other stresses. Insight into this question is provided by the observation that forced misfolding of nascent proteins by incorporation of the proline analog azetidine 2-carboxylic acid (AZC) results in G1 arrest in yeast cells in a manner similar to heat shock (Rowley *et al.* 1993; Trotter *et al.* 2001). AZC treatment also results in transcriptional activation of a set of genes closely matching the Hsf1, but not the Msn2/4 regulon, and repression of ribosomal protein synthesis, an Hsf1-dependent process (Trotter *et al.* 2002). Treatment with sublethal concentrations of ethanol (6–8%) also induces Hsf1 but not Msn2/4 (Takemori *et al.* 2006). Lastly, inhibition of proteasomal degradation with the specific inhibitor MG132 is also an effective activator of Hsf1 (Lee and Goldberg 1998). Together these data suggest that accumulation of misfolded proteins triggers Hsf1 activation, but it is not clear whether it is the accumulation of misfolded forms of existing proteins, nascent chains, or both that is the proximal inducer. While cycloheximide pretreatment predictably blocks Hsf1 induction by AZC incorporation, the same experiment has not been done with ethanol (Trotter *et al.* 2002). Both treatments, as well as transient heat shock, fail to induce the unfolded protein response (UPR) in the ER, which is stimulated by misfolding of resident proteins (Cox and Walter

1996). Moreover, heat shock at 37° does not result in bulk protein aggregation (Nathan *et al.* 1997). These findings support a model whereby misfolding of newly synthesized polypeptides might be sensed as increased substrate load on ribosome-proximal HSPs/chaperones. How is this signal then transmitted to Hsf1? Although a small number of Hsf1 post-translational regulators have been uncovered (see below), substantial genetic data in yeast support a model wherein select HSPs, including Hsp70, Hsp90, and their cofactors, repress Hsf1 activation (Nelson *et al.* 1992; Duina *et al.* 1998; Liu *et al.* 1999; Harris *et al.* 2001). Moreover, pharmacological inhibition of Hsp90 with specific small molecules such as the ansamycins (geldanamycin and macbecin) or radicicol results in Hsf1 activation in yeast, as it does in higher eukaryotes (Zou *et al.* 1998; Harris *et al.* 2001). Given the high protein synthetic capacity of actively growing cells, it is conceivable that a transient heat shock could impact the folding of enough nascent chains or newly released polypeptides to effectively compete for components of the Hsp70/Hsp90 machinery with Hsf1. However, yeast Hsf1 is largely trimerized, nuclear, and DNA bound in unstressed cells (McDaniel *et al.* 1989; Hahn *et al.* 2004); therefore, the chaperone repression model has to be revised: protein misfolding (in the nucleus) must be sensed by nuclear chaperones or cytoplasmic misfolding events must titrate chaperones that transit between the two compartments, depleting the nuclear pool. In support of this latter argument, both Hsp70 (Ssa4) and Hsp90 (Hsp82 and Hsc82) can localize to the nucleus in response to environmental stress (Chughtai *et al.* 2001; Quan *et al.* 2004; Tapia and Morano 2010).

In addition to the Hsf1 pathway, thermal stress also induces the Msn2/4 regulon and activates the cell wall integrity pathway. Although the mechanism of Msn2/4 activation by low glucose conditions through cAMP/PKA is well established, induction of this pathway by heat shock and other environmental stress is poorly understood (Thevelein *et al.* 2000). Like Hsf1, Msn2/4 is hyperphosphorylated in response to heat shock, but this modification is inhibited by cAMP, suggesting that it is not mediated by PKA (Garreau *et al.* 2000). Heat shock, oxidative and ethanol stress moderately reduce cAMP levels via destabilization of the Ras activator, Cdc25, leading to speculation that cAMP/PKA may yet be the nexus of ESR signaling and that additional phosphorylation events may act as subordinate modulators of the response (Wang *et al.* 2004). Activation of the cell wall integrity pathway by heat shock has been reviewed elsewhere, but importantly is insulated from Hsf1 or Msn2/4 activation and is not affected by cAMP/PKA (Levin 2005).

Modulators of the HSR

Chromatin modulation of heat shock gene expression: Because most of the HSR is by definition a transcriptional program, it is not surprising that chromatin status plays an important role in governing the magnitude and kinetics of the response. It is generally accepted that transcriptionally inert portions of eukaryotic genomes are maintained in densely packed, nucleosome-rich states. Moreover, association of the DNA with nucleosomal proteins is enhanced by deacetylation of lysine residues in the histone tails, as well as other epigenetic modifications, including methylation. The *HSP82* locus, one of two genes encoding the chaperone Hsp90, has been an informative model for how heat shock rapidly and reversibly activates gene expression. Early DNase I footprinting studies established that the *HSP82* promoter contains multiple HSEs, only one of which (HSE1) was constitutively bound by Hsf1 in unstressed cells (Gross *et al.* 1990). Mutation of HSE1 resulted in the appearance of two stable nucleosomes, suggesting that Hsf1 binding "precleared" the promoter (including the TATA box), priming it for transcription initiation (Gross *et al.* 1993). Upon heat shock, Hsf1 occupies additional low-affinity HSEs within the *HSP82* promoter in a cooperative manner, which can compensate for loss of HSE1 with regard to both nucleosome clearance and gene expression (Erkine *et al.* 1999). This mode of Hsf1 activation may be the norm, as ChIP-chip studies demonstrated that Hsf1 binds to many target genes (see below) constitutively, with additional binding occurring in response to heat shock (Hahn *et al.* 2004). Strikingly, nucleosome remodeling kinetics are exceedingly rapid—abundance of histone 4 within the *HSP82* locus is drastically reduced within 1 min of heat shock (Zhao *et al.* 2005a). Hsf1 target genes attenuate expression within 20–40 min after induction, and correspondingly, nucleosomes are found to reposition within the promoter, ORF, and 3′-UTR of *HSP82* in the same timeframe (Zhao *et al.* 2005a). The correlation between heat shock gene induction and nucleosome remodeling may not be absolute; the remodeling complex SWI/SNF is recruited to Hsf1 target genes and required for maximal gene expression, yet histone eviction occurs in *snf2*Δ mutants that abrogate complex function, albeit with delayed kinetics (Shivaswamy and Iyer 2008).

A genome-wide search for genes required for sustained growth during heat shock (39°) resulted in the identification of multiple components of the Rpd3L deacetylase complex (*RPD3*, *SIN3*, *UME1*, *SAP30*, *SDS3*, *DEP1*, and *PHO23*) (Ruiz-Roig *et al.* 2010). Rpd3L promotes transcription initiation at target promoters, where it functions in both activating and repressing contexts. Intriguingly, the complex is required for activation of Msn2/4-dependent gene expression in response to heat shock, where it contributes to the magnitude of the response; *i.e.*, genes are induced by heat shock but to a much lower level than in wild-type strains (Alejandro-Osorio *et al.* 2009). In contrast, Hsf1-dependent genes such as *HSP82* are activated independently of Rpd3L, which instead contributes to basal repression (Kremer and Gross 2009; Ruiz-Roig *et al.* 2010). Rpd3L is recruited to promoters upon stress, and it likely contributes to subsequent recruitment of Msn2 (Ruiz-Roig *et al.* 2010). Chromatin modification therefore plays a critical role in modulating

the kinetics and amplitude of stress gene expression, suggesting close collaboration between stress-specific transcription factors and more general gene control machinery.

Effects of heat shock on RNA metabolism: In addition to modulation of gene transcription, heat shock causes immediate post-transcriptional effects with significant physiological impact. Remarkably, upon 42° heat shock bulk poly(A)$^+$ RNA stably accumulates in the nucleus (Saavedra *et al.* 1996). This finding is at odds with the obvious robust translation of HSPs under the same conditions. In fact, heat shock transcripts, best exemplified by studies with the *SSA4* gene, are selectively exported through nuclear pores utilizing signals in the 5′- and 3′-untranslated regions (UTRs) of the message (Saavedra *et al.* 1996). A specific nuclear pore protein Rip1 is required for export of heat shock transcripts during thermal stress but not under normal growth conditions, defining a dedicated transport pathway for these important mRNAs (Saavedra *et al.* 1997). Recent work has defined novel ribonucleoprotein assemblies termed processing bodies (P-bodies) and stress granules (SGs) that appear to concentrate nontranslating mRNAs in exchangeable but sequestered pools in response to a variety of stress conditions (Parker and Sheth 2007). Heat shock induces the formation of SGs that contain translation initiation factors and nonheat shock mRNAs (for example, *PGK1*) capable of redistributing into the cytoplasm, and presumably reengaging in translation, upon recovery (Grousl *et al.* 2009). Heat shock-induced SGs also contain a subset of P-body components involved in RNA degradation including Dcp2 and Dhh1, yet are spatially distinct from other P-body markers (Grousl *et al.* 2009). These data demonstrate that cells restrict the translation of nonheat shock transcripts via at least two mechanisms: blocking mRNA transport from the nucleus and redirecting cytoplasmic mRNAs away from ribosomes and into subcellular complexes. Interestingly treatment of yeast cells with high ethanol concentrations (>10%) also leads to block of mRNA export and formation of SGs (Saavedra *et al.* 1996; Kato *et al.* 2011). It is tempting to speculate that this result may be yet another manifestation of the close relationship heat and ethanol share as protein denaturants that lead to activation of the HSR, but a plausible mechanism to account for this has not been put forward. In addition, the possibility that both treatments similarly affect other aspects of yeast physiology such as membrane fluidity cannot be excluded.

Modulation by trans-acting factors: Great progress has been made in understanding regulation of HSF1 in human and murine systems by post-translational modifications, including phosphorylation by multiple kinases and most recently reversible acetylation mediated by sirtuin deacetylase enzymes (Westerheide *et al.* 2009; Akerfelt *et al.* 2010). In contrast, relatively little is known in this regard in yeast. As described earlier, Msn2/4 is under tight control by the cAMP/PKA pathway (mediated in part by Yak1), and target serine residues phosphorylated by PKA have been identified in Msn2 whose mutation abrogates nutrient control of function (Gorner *et al.* 2002). Similarly, Hsf1 activates a subset of genes, including the copper metallothionein *CUP1* in response to low glucose conditions, and induction is lost in cells lacking the AMP kinase homolog Snf1 (Tamai *et al.* 1994). Snf1 was found to directly phosphorylate Hsf1 in response to glucose starvation, but not in response to heat shock, demonstrating that distinct pathways sense environmental changes and communicate this information to Hsf1 (Hahn and Thiele 2004). Hsf1 phosphorylation patterns are different in response to low sugar, heat shock, or oxidative stress, but kinases mediating phosphorylation in the latter two cases are yet to be discovered (Tamai *et al.* 1994; Liu and Thiele 1996; Hahn and Thiele 2004). It is not clear at this time whether such kinases would themselves be activated by the specific stress, as is Snf1, or if constitutively active kinases gain access to Hsf1 under stress conditions.

Global analysis of the HSR

As is the case for analysis of many transcriptional networks in yeast, the advent of DNA microarray technology offers unprecedented insight into the breadth of the heat shock response on a genome-wide scale. Two comprehensive studies utilized microarray approaches to reveal changes in gene expression in response to stressful conditions ranging from heat shock to osmotic stress to nutrient limitation. Gene expression of ~10% of the genome is remodeled during one or more stresses, highlighting the depth to which stress-based transcription penetrates the global transcriptome (Gasch *et al.* 2000; Causton *et al.* 2001). Abundance (probably reflecting transcriptional changes but could also include differences in mRNA stability (Castells-Roca *et al.* 2011) of ~600 genes was found to decrease during stress, while about half that number (300) are induced (Gasch *et al.* 2000; Causton *et al.* 2001). This comprehensive response has been termed the environmental stress response (ESR). Comparative arrays using an *msn2*Δ *msn4*Δ mutant demonstrated that a significant proportion of the induced genes rely on these transcription factors for activation (Gasch *et al.* 2000). Notably, many of these same targets are activated in response to multiple stresses, consistent with previous single-gene analyses. The HSR is therefore a subset of the ESR and is composed of genes requiring Hsf1, Msn2/4, both, or in a few isolated cases, neither for their expression during heat shock. Subsequent studies to identify direct Hsf1 targets cataloged ~72 genes whose heat shock induction at 39° was abrogated by a severe loss-of-function mutant, *hsf1-R206S/F256S* (Yamamoto *et al.* 2008). ChIP analysis to identify promoters directly bound by Hsf1 revealed a larger number (165) of potential Hsf1-responsive genes (Hahn *et al.* 2004). This result suggests that Hsf1 may contribute to the expression of nonheat shock genes and is consistent with the fact that it has been previously documented to bind many promoters constitutively. An alternative explanation may be that the “normal” conditions established

in the laboratory (rich medium, high glucose, aeration, 30°) may actually be perceived as a mild stress by yeast cells. While this interpretation would make the behavior of yeast Hsf1 more like its mammalian counterpart, a closer analogy may be *Drosophila* HSF1, which is also constitutively nuclear and prebound to many heat shock promoters (Yao *et al.* 2006).

A number of additional insights can be gleaned from these genome-wide studies. It is clear that the magnitude of the HSR is proportionate to the intensity of the stress: temperature shift from 25° to 37° results in a longer lasting HSR with greater amplitude of change in gene expression when compared to a 29°-to-33° shift (Gasch *et al.* 2000). There is a limit to this effect, as no significant differences in binding of Hsf1 to chromosomal loci are detected between 39° and 42° (Hahn *et al.* 2004). These data suggest that yeast cells are capable of sensing gradations in temperature stress up to a threshold point, after which the system, at least Hsf1, is maximally activated. A handful of Hsf1 target genes (*CUP1* and *HSP82*) are induced more strongly at 39° *vs.* 37°, demonstrating that the threshold is likely in the 39°–40° range (Santoro *et al.* 1998).

Another important insight is the breadth of target genes induced by the HSR. While protein chaperones and their cofactors are obvious and for the most part long-recognized targets, the HSR touches on many diverse aspects of cell physiology, including oxidant defense, cell wall remodeling, metabolism, and transport (Hahn *et al.* 2004). However, representatives from these various functional annotations are few, making it unlikely that heat shock dramatically impacts these processes. Rather, it may be that some of the proteins involved are thermolabile or perhaps rate limiting during heat shock, necessitating a transient increase in gene expression to support continued function under thermal duress. Transcriptional profiling revealed that a number of duplicated isozymes are differentially regulated by stress (Gasch *et al.* 2000; Causton *et al.* 2001). For example, of the three thioredoxins, *TRX1*, *TRX2*, and *TRX3*, only *TRX2* is significantly induced by stress. Likewise, one hexokinase gene, *HXK1*, is highly induced by stress, while the expression of *HXK2* remains largely unchanged. This theme is recapitulated with the major HSPs: *HSC82* (Hsp90) is constitutively expressed and abundant, while *HSP82* is significantly expressed only during heat shock. Similar expression patterns exist for Hsp70, Hsp110, and some Hsp90 co-chaperones.

In contrast, genes encoding other HSPs such as Hsp104 are abundant under normal growth conditions and substantially induced by heat shock. Finally, the significant overlap in expression programs observed among the different stressors that have been examined explains the phenomenon of "cross-protection," whereby exposure to one stress enhances tolerance of a subsequent stress of a different nature; *i.e.*, heat shock induces tolerance to oxidants or osmotic shock. This is especially apparent for Msn2/4-dependent genes, most of which exhibit a common gene expression pattern in response to diverse stressors, suggestive of a monolithic, rather than stress-specific response.

Cellular functions of the heat shock response and heat shock proteins

The preceding sections outlined advances in understanding control and regulation of the HSR, with an emphasis on transcription. While unstressed cells exhibit moderate resistance to a range of environmental stressors, mildly stressed cells significantly increase their ability to withstand future insult (Berry and Gasch 2008; Yamamoto *et al.* 2008). One of the products of the HSR that confers cytoprotection is the disaccharide trehalose. Work in the late 1990s identified trehalose as a powerful stabilizer of proteins and membranes in multiple biological systems, including yeast. The role of trehalose in heat shock survival has been extensively reviewed elsewhere, and the reader is directed to these sources for additional information (Singer and Lindquist 1998; Trott and Morano 2003; Crowe 2007). However it is the heat shock proteins that have received the most attention to date, as they promote cell survival under both stress and nonstress conditions. The mechanistic details of protein chaperone functions at the molecular level have been the subject of intense scrutiny, and we have learned much regarding their operations *in vitro*. In contrast, a comprehensive understanding of the cellular roles played by chaperones is only just emerging.

The yeast "chaperome": With the advent of whole genome sequencing, the chaperone complement of organisms can now be determined and defined as the chaperome (Morimoto 2008). This descriptor includes chaperones and other HSPs present under normal growth conditions as well as those whose abundance increases, or are solely produced during the HSR. Major HSPs were defined years ago on the basis of their abundance during heat shock, when they are easily detected by radioactive pulse–chase analysis because their synthesis is increased while that of the remainder of the proteome is repressed (Subjeck *et al.* 1982). These HSPs were named according to their apparent molecular masses and gave rise to the by-now familiar collection of Hsp100, Hsp90, Hsp70, Hsp60, and the small HSPs ubiquitous in eukaryotic cells. Work over the last decade has uncovered few new chaperones in yeast; instead a panoply of chaperone partner proteins, or "co-chaperones," have been elucidated that contribute to HSP functions in numerous ways. Nowhere is this more apparent than for the Hsp70 and Hsp90 chaperones, where work in yeast has led the way in identifying important new co-chaperones that play roles in protein folding, chaperone targeting, and substrate selection.

The Hsp70 chaperone system: The Hsp70 chaperone is the "workhorse" chaperone of eukaryotic cells [bacterial cells rely to a greater extent on the Hsp60/chaperonin family (GroEL/ES)], that interacts with proteins at all stages in

Table 1 Hsp70 chaperones in *Saccharomyces cerevisiae*

Gene name	Localization	Function	Reference
Ssa1	Cytosol	General folding	Werner-Washburne *et al.* (1987)
Ssa2	Cytosol	General folding	Werner-Washburne *et al.* (1987)
Ssa3	Cytosol	General folding	Werner-Washburne *et al.* (1987)
Ssa4	Cytosol	General folding	Werner-Washburne *et al.* (1987)
Ssb1	Ribosome	Nascent chain folding	Nelson *et al.* (1992)
Ssb2	Ribosome	Nascent chain folding	Nelson *et al.* (1992)
Ssz1	Ribosome	Nascent chain folding	Gautschi *et al.* (2002); Hundley *et al.* (2002)
Sse1	Cytosol	Substrate binding; NEF	Dragovic *et al.* (2006); Raviol *et al.* (2006); Shaner *et al.* (2006)
Sse2	Cytosol	Substrate binding; NEF	Dragovic *et al.* (2006); Raviol *et al.* (2006); Shaner *et al.* (2006)
Kar2	ER	General folding	Vogel *et al.* (1990)
Lhs1	ER	Substrate binding; NEF	Baxter *et al.* (1996); Craven *et al.* (1996); Hamilton and Flynn (1996); Steel *et al.* (2004)
Ssc1	Mitochondrion	Postimport folding	Craig *et al.* (1989)
Ssq1	Mitochondrion	Assembly Fe/S proteins	Dutkiewicz *et al.* (2003)
Ecm10	Mitochondrion	Postimport folding	Baumann *et al.* (2000)

their lifetimes (Frydman 2001). Hsp70s protect nascent polypeptides as they emerge from the ribosome, assist in targeting and translocation, and play important roles in either refolding damaged proteins or shepherding their ubiquitylation and degradation. Interactions with substrates occur through the C-terminal substrate binding domain, whose affinity for same is governed by allosteric movements in the N-terminal nucleotide binding domain (Vogel *et al.* 2006a,b). Hsp70•ATP loosely binds an extended, mostly hydrophobic, polypeptide segment until nucleotide hydrolysis, after which substrate diffusion is restricted by conformational shifts in the substrate binding domain (Jiang *et al.* 2005). Protein folding is promoted by iterative cycles of substrate binding and release until all hydrophobic regions are suitably buried. Two classes of Hsp70 cofactors regulate the speed of this cycle: diverse relatives of the bacterial DnaJ co-chaperone termed J proteins, which enhance ATP hydrolysis, and distinct classes of structurally unrelated proteins that act as nucleotide exchange factors.

As shown in Table 1, Hsp70s are found in the ER, mitochondria, and cytoplasm. In the latter two compartments, a "specialized" Hsp70 operates alongside a "general" counterpart. In the mitochondrial matrix, Ssc1 is involved in protein refolding after import, while Ssq1 is specifically required for maturation of iron-sulfur cluster-containing proteins. Two specialized Hsp70s exist in the cytoplasm and both are localized to the ribosome; Ssb1/2 participate in folding of nascent chains, whereas the divergent Ssz1 appears to have lost both nucleotide hydrolysis and substrate binding capabilities and instead promotes enhancement of Ssb1/2 function by the J protein Zuo1. Constitutive (Ssa1/2) and stress-inducible (Ssa3/4) Hsp70s carry out the bulk of Hsp70 functions in the cytoplasm, while the Kar2 protein plays the same role in the ER lumen. Two even more divergent Hsp70s exist in the ER (Lhs1) and cytoplasm (Sse1/2) and will be discussed below. Because general Hsp70s such as the Ssa family exhibit promiscuous polypeptide binding and refolding activity, other components must be responsible for conferring specificity and regulation.

On the basis of the highly conserved J domain signature, ~22 J proteins have been identified in *S. cerevisiae* (Kampinga and Craig 2010). These proteins are in general highly divergent outside the J domain and this diversity likely plays an important role in determining Hsp70 involvement in distinct cellular processes. For example, Zuo1, involved in translation, contains a ribosome-association domain that facilitates interaction with Ssz1 and Ssb (Gautschi *et al.* 2001), and Swa2, required for endocytosis, contains a clathrin-binding domain (Gall *et al.* 2000). Some J proteins, such as the major cytoplasmic J protein Ydj1, bind clients as well as accelerate Hsp70 ATP hydrolysis, while others bind specific clients only or not at all. J proteins also localize to different cellular locations, including the mitochondrion, ER lumen, and ER membrane, where they presumably increase the local concentration of Hsp70 activity to promote specific cellular processes. A good example of this is the Sec63 protein, which operates with the Sec61 translocon channel in protein import and insertion into the ER. Sec63 contains a J domain localized within the ER lumen that promotes recruitment and function of Kar2 to catalyze protein translocation (Feldheim *et al.* 1992). A similar case can be made for Swa2 (Aux1), which recruits Ssa to clathrin-coated vesicles where it participates in disassembly and uncoating (Gall *et al.* 2000). The J domain can therefore be considered an Hsp70 recruitment module that serves to integrate this powerful protein remodeler into cellular activities.

Four classes of nucleotide exchange factor (NEF) have been uncovered in yeast with orthologous human counterparts, and unlike the J proteins, they bear no sequence or structural homology with each other. One class is found only in the mitochondrial matrix, encoded by *MGE1* and resembling the GrpE NEF from *Escherichia coli* (Laloraya *et al.* 1994; Voos *et al.* 1994). The yeast homolog of the Bag family of human NEFs, termed Snl1, is tethered to the ER

Table 2 Hsp90 co-chaperones in *Saccharomyces cerevisiae*

Gene name	Function	Reference
STI1	TPR; Hsp70/Hsp90 bridging	Chang *et al.* (1997)
CNS1	TPR; unknown	Dolinski *et al.* (1998)
PPT1	TPR; Hsp90 regulation	Wandinger *et al.* (2006)
TOM70	TPR; mitochondrial import	Young *et al.* (2003)
CPR6	TPR; Hsp90 regulation	Duina *et al.* (1996)
CPR7	TPR; Hsp90 regulation	Duina *et al.* (1996)
AHA1	Hsp90 ATPase regulation	Panaretou *et al.* (2002)
SGT1	Targeting Hsp90 to kinetochore, SCF complex	Kitagawa *et al.* (1999)
SBA1	Hsp90 ATPase regulation	Bohen 1998; Fang *et al.* (1998)
CDC37	Kinase targeting chaperone	Dey *et al.* (1996)
TAH1	Targeting Hsp90 to Rvb1/2 complex	Zhao *et al.* (2005a)

membrane with its Bag domain facing the cytoplasm (Ho *et al.* 1998; Sondermann *et al.* 2002). Two yeast proteins are related to the human HSPBP1 NEF: Sls1 localized to the ER lumen (Kabani *et al.* 2000) and Fes1, which resides in the cytoplasm (Kabani *et al.* 2002). The most unusual group consists of the Lhs1 protein in the ER lumen (Craven *et al.* 1996; Saris *et al.* 1997) and the Sse1/2 proteins in the cytoplasm (Mukai *et al.* 1993), both of which share the general domain architecture of the typical Hsp70s with insertions and C-terminal extensions that increase their molecular mass leading to their categorization as members of the "Hsp110" family (based on the size of the human orthologs) (Easton *et al.* 2000; Shaner and Morano 2007). On the basis of *in vitro* studies, these proteins are incapable of folding substrates on their own and instead have evolved a novel Hsp70 binding interface, which they use to form highly stable Hsp70•Hsp110 heterodimers to enhance nucleotide exchange (Shaner *et al.* 2005; Yam *et al.* 2005; Dragovic *et al.* 2006; Raviol *et al.* 2006; Polier *et al.* 2008; Schuermann *et al.* 2008). However, the peptide binding domains of the Hsp110 family, while altered with respect to Hsp70, retain the ability to bind and protect unfolded polypeptides as exemplified by the yeast Ssa1 protein (Brodsky *et al.* 1999). This raises the possibility that this group may act in a manner similar to the client-binding J proteins to enhance substrate delivery and transfer to Hsp70 for subsequent folding. Unlike the J proteins that have been implicated in specific processes via genetic and biochemical interactions, no such targeted or exclusive activities have been described for the NEFs. However the fact that NEF activity has arisen multiple times in evolution strongly hints that the co-chaperones are likely to play nonredundant roles.

The Hsp90 chaperone system: In contrast to the Hsp70 chaperone system, which interacts with nearly any partially unfolded protein it encounters, the Hsp90 chaperone is much more selective and interacts with a small but growing list of protein "clients." These clients rely on Hsp90 for the final steps of maturation after initial interactions with Hsp70, effectively linking these two chaperone machines in an "assembly line" of protein maturation. Hsp90 is highly abundant, accounting for 1–2% of total protein during stress conditions (Borkovich *et al.* 1989). As with Hsp70, Hsp90 is an ATP binding protein whose chaperone cycle is governed by nucleotide cycling (see Taipale *et al.* 2010). Also analogous to Hsp70, a sizable number of co-chaperones regulate steps in the nucleotide cycle in yeast and other eukaryotes. Although a comprehensive treatment is beyond the scope of this chapter, these co-chaperones can be grouped into three classes: those that bind the ATPase domain of Hsp90, the Aha1 protein that binds the middle domain, and a handful of proteins containing tetratricopeptide repeats (TPRs) that bind the highly conserved MEEVD sequence at the extreme C terminus of Hsp90 (see Table 2). A canonical client maturation cycle has been elucidated *in vitro* for animal steroid hormone receptors with the "minimal" complement of Hsp70/Hsp40, the linker protein HOP (homologous to the TPR-containing proteins Sti1 and Cns1), Hsp90, and the protein p23 (yeast Sba1) that stabilizes the ATP-bound state (Dittmar *et al.* 1997). However, variations on this theme for other clients suggest that the exceptions may outnumber the rule. For example, many if not all protein kinases interact with Hsp90 at some point in their maturation but do so in concert with the protein Cdc37, which can partner with Hsp90 or promote kinase maturation independently (Lee *et al.* 2002; Mandal *et al.* 2007). In contrast, most other Hsp90 clients do not require Cdc37 for their activity. The immunophilin homologs Cpr6 and Cpr7 likewise play little to no role in kinase maturation yet are required for other Hsp90 activities such as steroid receptor activity and Hsf1 repression (Duina *et al.* 1996, 1998). Co-chaperones can also link Hsp90 to specific pathways, as the p23-like protein Sgt1 associates with both Hsp90 and the Skp1–Cul1–F-box-protein (SCF) ubiquitin ligase complex through the multifunctional kinetochore protein Skp1 (Kitagawa *et al.* 1999). Hsp90 activity is also modulated by phosphorylation in yeast, mediated by the TPR-containing protein phosphatase Ppt1 and the tyrosine kinase Swe1 (Wandinger *et al.* 2006; Mollapour *et al.* 2010).

Although only a small number of yeast Hsp90 clients have been experimentally verified, genomic and proteomic approaches are being used to identify novel clients with the

goal of trying to establish the complete substrate complement in a single organism (Zhao *et al.* 2005b). Two major criteria must be satisfied to warrant inclusion on the list: clients must associate with Hsp90 at some point in their lifetime and pharmacological (with potent and specific Hsp90 ATPase inhibitors) or genetic depletion must affect the protein's stability and or cellular function. The Picard laboratory in Geneva, Switzerland maintains a list of clients that is continuously updated and contains a number of endogenous yeast proteins (http://www.picard.ch/downloads).

Oligomeric HSPs: Hsp104 and the small HSPs: In contrast to the previously described chaperones, the yeast protein Hsp104 and a series of small HSPs function *in vivo* and *in vitro* as oligomeric complexes. Hsp104 is a member of the AAA^+ ATPase family, which includes the well-described bacterial Clp chaperones/proteases as well as nonchaperone proteins Sec18 (vesicle fusion) and Cdc48 (ERAD), which form hexameric rings with a large (~15 Å) central channel (Doyle and Wickner 2009). Unlike most other chaperones, Hsp104 is capable of extracting misfolded proteins from aggregates, followed by translocation through the central channel. Hsp104 cannot refold proteins alone, however, and relies on Hsp70 to fully rescue substrates and return them to the appropriate native conformation. In addition, Hsp70 likely participates in early steps of recognition and perhaps priming of polypeptide extraction, making the Hsp104/Hsp70 partnership nearly obligatory (Parsell *et al.* 1994). Hsp104 is also one of the few yeast protein chaperones absolutely required for thermotolerance: *hsp104*Δ cells are exquisitely sensitive to lethal heat shock (Sanchez and Lindquist 1990). This protection comes at a price, as Hsp104 contains two independent but linked ATPase domains that bind and hydrolyze up to 12 ATP molecules per cycle, with potentially hundreds of cycles required per extracted protein (Doyle and Wickner 2009). Interestingly, humans lack Hsp104 or analogous disaggregase activity, although yeast Hsp104 expressed in human cells confers thermoprotection (Chernoff *et al.* 1995; Mosser *et al.* 2004). Hsp104 is also a primary modulator of yeast prion/amyloid stability and inheritance, where it is involved in processing fibers, which ultimately generates additional prion "seeds" that enhance distribution and substrate conversion (Chernoff *et al.* 1995).

Small HSPs (sHSPs) represent a diverse family of proteins with passive chaperone activity that are classed together due to sequence similarity with the eye lens protein α-crystallin. All known sHSPs exist either transiently or stably in high molecular weight oligomeric structures, where they interact with unfolded substrates usually at a 1:1 monomer-to-substrate ratio (Wotton *et al.* 1996). Instead of preventing the aggregation of damaged proteins, sHSPs appear to "co-aggregate" with their substrates in a mixed oligomeric agglomeration that can be resolubilized with the help of additional chaperones (Haslbeck *et al.* 2005). In yeast, two major sHSPs have been characterized, Hsp26 and Hsp42. Hsp42 is expressed in unstressed cells and forms a large, stable, barrel-like oligomer, whereas Hsp26 undergoes a dynamic transition as part of its chaperone activity (Haslbeck *et al.* 1999; Stromer *et al.* 2004). Under normal temperatures, Hsp26 exists as a 24-mer but rapidly dissociates upon heat shock via a novel thermosensing domain into dimers that are capable of interacting with unfolded substrates (Stromer *et al.* 2004). Hsp26:substrate oligomers then reassemble into a novel heterooligomer. Refolding of sHSP-associated substrates requires the action of the ATP-dependent Hsp70 and Hsp104 chaperones, which extract polypeptides from their protected state in the aggregate (Haslbeck *et al.* 2005). Both Hsp26 and Hsp42 are required to maintain the yeast proteome in a soluble state during heat shock, although Hsp26 appears to play a limited role under normal growth conditions (Haslbeck *et al.* 2004). In spite of the dramatic transfer of a significant fraction of cellular proteins from a soluble and presumably functional state to an insoluble, aggregated state in *hsp26*Δ and *hsp42*Δ mutants, no obvious growth phenotypes have been reported. However, examination of these mutants via scanning electron microscopy reveals profound morphological cellular surface alterations reminiscent of aged or dehydrated cells (Haslbeck *et al.* 2004). This "wrinkled" phenotype is also shared by cells lacking another poorly understood low-molecular weight HSP unrelated to the sHSP family, Hsp12. Hsp12 is a highly abundant protein that exists in a mostly unfolded state in its soluble form, although a subset of the total Hsp12 pool associates with cellular membranes, where it acquires a helical structure and increases membrane stability (Welker *et al.* 2010). Not surprisingly, *hsp12*Δ cells are hypersensitive to severe heat shock and osmotic stress, demonstrating that the chaperone plays an important role in membrane protection (Welker *et al.* 2010).

Chaperone networks: The yeast genome contains at least 60 ≥known chaperones: 7 sHSPs, 14 belonging to the CCT/TRiC and prefoldin complexes, 2 Hsp90s, 14 Hsp70s, 1 Hsp60, 3 AAA^+ ATPases (Hsp104), and 22 Hsp40s (Gong *et al.* 2009). How is the action of all these protein remodelers orchestrated at the cellular level? A combination of transcriptome profiling, proteomics, and large-scale phenotypic analysis suggests that the majority of protein chaperone function in the yeast cell can be divided into two classes: those that participate in protein translation and nascent chain folding and those required for repair and recovery after severe stress (Albanese *et al.* 2006). The former group, termed chaperones linked to protein synthesis (CLIPS), physically associate with ribosomes, are transcriptionally downregulated during stress along with RPs, and when disrupted confer profound sensitivity to translation inhibitors such as hygromycin and cycloheximide but not to heat shock. In contrast, the HSPs are induced by heat shock and are required for survival after severe thermal stress. These categories are obviously not completely mutually exclusive, but the degree to which phenotypes are shared

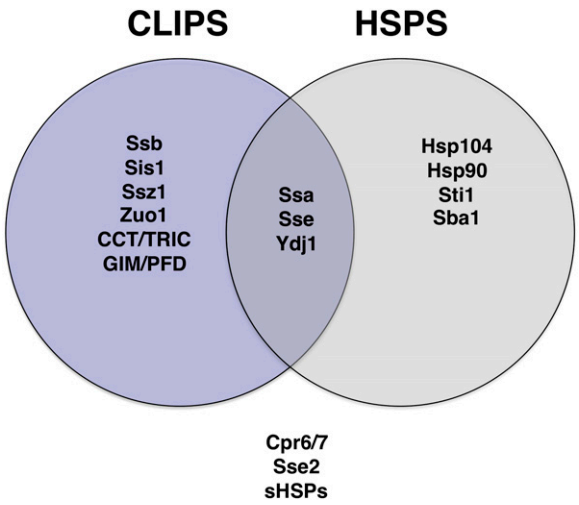

Figure 3 CLIPs and HSPS. Venn diagram depicting the intersection between chaperone networks, based on the work of Frydman and coworkers, is shown (Albanese *et al.* 2006). See text for details.

within the two classes is remarkable. The overlap between the CLIPS and HSPs is shown schematically in Figure 3.

The intersection between the HSR and other stress responses

HSR and oxidative stress response: The HSR, or more broadly defined the ESR, includes the reprogramming of a significant percentage of the total transcriptome. This suggests that heat shock impacts more than just protein stability. Indeed, the heat shock-induced ESR includes genes involved in metabolism, oxidant defense, and growth control. Tolerance of severe heat shock is in fact tightly linked to aerobic metabolism and oxidative stress. Yeast cells cultured anaerobically are 10^2- to 10^3-fold more resistant to heat shock than those grown in the presence of oxygen (Davidson *et al.* 1996). Cells lacking antioxidant enzymes such as catalase and superoxide dismutase (SOD) are conversely hypersensitive to heat shock (Davidson *et al.* 1996). Levels of reduced glutathione decline during aerobic, but not anaerobic, heat shock (Sugiyama *et al.* 2000b). These results suggest that the primary stress of heat shock induces a subsequent oxidative stress as a function of oxygen availability. In an aerobically growing cell, the main flux of oxygen is through the electron transport system localized to the mitochondrial network. A test of the hypothesis that heat induces oxidative stress by disruption of electron flow through the respiratory chain through deletion of the *COQ7* gene (encoding coenzyme Q) revealed increased heat sensitivity and nuclear mutation frequency (Davidson and Schiestl 2001). These effects are blocked by deletion of the NADH dehydrogenases *NDE1* and *NDE2*, required for electron transfer from NADH to the respiratory chain (Davidson and Schiestl 2001). Heat-induced oxidative stress is countered by induction of antioxidant genes, including the glutathione biosynthesis genes *GSH1* and *GSH2*, in a Yap1-dependent manner only under aerobic conditions (Sugiyama *et al.* 2000a). This conditional regulation all but guarantees that it is not the thermal stress but instead the resulting oxidative stress that is the proximal inducer of antioxidant gene transcription. The mitochondrial Mg^{2+}-SOD encoded by *SOD2* (also an ESR gene) is also required for tolerance to heat-induced oxidative stress (Sugiyama *et al.* 2000b). A recent study demonstrated that both Hsf1 and Yap1 are activated by the natural product celastrol, as is the case with the heavy metal cadmium, suggesting that adequate cellular defense against some noxious agents requires coordination of these two pathways (Trott *et al.* 2008). Although the mechanism behind this synchrony is unclear, it is interesting to note that another major mediator of the oxidative stress response (OSR) is the Skn7 transcription factor whose DNA binding domain is highly similar to that of Hsf1 (Morgan *et al.* 1997; Lee *et al.* 1999a). The relevance of the interaction between Skn7 and Hsf1 as it pertains to transcriptional induction in response to heat and oxidants is discussed later (*Transcription control of the OSR*). Superoxide anion selectively activates Hsf1 expression of the copper metallothionein *CUP1* and was shown to induce Hsf1 binding to DNA in cell-free extracts, similar to mammalian HSF1, which responds to treatment with hydrogen peroxide (Liu and Thiele 1996; Lee *et al.* 2000; Ahn and Thiele 2003). These findings demonstrate that HSFs can sense oxidative stressors. However, mammalian HSF1 contains two cysteine residues located within the DNA binding domain that mediate activation through the reversible formation of a disulfide bond, whereas yeast Hsf1 lacks any cysteine residues (Ahn and Thiele 2003). How then is yeast Hsf1 regulated by oxidants? It is likely that one or more cellular components acts as a sensor for thiol-reactive molecules, supported by the observation that glutathione treatment blocks superoxide-activated DNA binding by Hsf1 in cellular extracts, but not of purified Hsf1 (Lee *et al.* 2000). Identification of these factors will be important for resolving the molecular mechanisms behind this disparity and understanding the evolution of HSF as an ancillary component of the OSR.

HSR and cell wall integrity: The cell wall integrity (CWI) pathway responds to perturbations of the cell wall via activation of a MAP kinase pathway by plasma membrane-localized sensor proteins (reviewed thoroughly in Levin 2005). This pathway is also induced by transient heat shock or growth at 37°, suggesting that such temperatures destabilize the membrane or cell wall. Several Hsf1 and chaperone mutants retain viability at 30° but are temperature sensitive for growth at 37° (Zarzov *et al.* 1997; Morano *et al.* 1999; Shaner *et al.* 2008). Surprisingly, these phenotypes are apparently linked as both can be reversed by supplementation of the growth medium with 1 M sorbitol, an osmostabilizer that also suppresses mutants in the CWI pathway. This effect can be traced to defects in the terminal MAP kinase of the CWI pathway, Slt2 (Mpk1), a client of

Hsp90 that exhibits reduced activity in strains pharmacologically or genetically manipulated to be deficient in Hsp90 activity (Millson *et al.* 2005; Truman *et al.* 2007). Overexpression of Slt2, Hsp90, or the Slt2-dependent transcription factor Rlm1 also suppresses the temperature-sensitive phenotype of Hsf1 mutants, confirming that activation of the CWI pathway is necessary and sufficient to confer 37° growth (Truman *et al.* 2007). These findings challenge the conventional wisdom that strains defective in the HSR and protein chaperone function are thermosensitive due to proteome-wide folding defects. Instead, most if not all of the 37° growth phenotypes (but importantly not those due to severe heat shock at temperatures above 45°) can be attributed to dysfunction of the CWI pathway due to the dependence of Slt2 activity on the Hsp90 system.

Yeast as a model system for understanding the HSR

Exploiting yeast to understand the human HSR: *S. cerevisiae* has served as an ideal model system to dissect the intricacies of the eukaryotic HSR, which while thematically similar to the bacterial response to heat shock differs in terms of players and regulation. The ease of genetic manipulation and more recently advances in genomic transcriptional profiling and proteomic analyses has allowed a pace of discovery and investigation not possible in human cells. However, certain features of the human HSR are not represented in the yeast model. For example, as described earlier, yeast Hsf1 is constitutively trimerized and bound to many HS promoters in contrast to the situation in mammalian cells where HSF1 is maintained as an inactive monomer in the cytoplasm. In addition, while mammals express multiple HSF isoforms with distinct functions, fungi possess only a single Hsf1. Post-translational regulation of Hsf1 likely differs substantially between yeast and human cells. Lastly, higher eukaryotes do not possess a broad ESR mediated by non-Hsf1 factors. These disparities have been exploited to utilize yeast as a test system to understand intricacies of human HSF1 (hHSF1) function. Human HSF2, but not hHSF1, functionally complements a yeast *hsf1*Δ null strain, demonstrating that the hHSF2 isoform, which regulates developmentally controlled genes, retains its ancestral ability to control HSP genes (Liu *et al.* 1997). hHSF1 can be made functionally competent in yeast through disruption of a coiled-coil intramolecular interaction domain or substitutions within the DBD, suggesting that human cells normally modulate these interactions to allow stress-induced activation (Liu and Thiele 1999; Takemori *et al.* 2009). Analysis of hHSF1 in the yeast system has also revealed that a loop region within the highly conserved DNA binding domain influences promoter recognition and trimerization (Ahn *et al.* 2001). In a recent exciting development, the complementation system has been used as a drug discovery platform to identify a new class of HSR-activating compounds (Neef *et al.* 2010). These advances are of significant relevance to human health as modulation of the HSR shows promise for the treatment of diseases linked to protein folding such as neurodegenerative disorders (*e.g.*, Alzheimer's disease, Parkinson's disease, Lou Gehrig's disease) (Westerheide and Morimoto 2005). In addition, the collapse of protein homeostasis as organisms age is increasingly being recognized as a contributing factor to the pathologies of aging (Morimoto 2008). Restoration of protein biogenesis and repair through pharmacologic activation of the HSR is an intriguing possibility that will rely on mechanistic information obtained from yeast and other model eukaryotic systems.

The HSR in other yeasts: Analysis of the HSR in pathogenic fungi has followed closely on the heels of its elucidation in *S. cerevisiae*. Significant effort in understanding the HSR in the pathogenic, dimorphic yeast *Candida albicans* has revealed that the organism lacks a general stress response akin to the baker's yeast ESR (Enjalbert *et al.* 2003). This is consistent with work showing that the closest homologs of Msn2/4 in *C. albicans* play no role in stress resistance, nor do they contribute to the stress transcriptome (Nicholls *et al.* 2004). The question of whether an obligate animal pathogen requires a robust HSR is a relevant one, as *C. albicans* enjoys a controlled environment of 37°, with only occasional, modest increases in temperature (the febrile state). In fact, *C. albicans* exhibits an abridged HSR centered on the major HSPs and a handful of OSR genes, most of which are very modestly induced (two- to sixfold) relative to their baker's yeast counterparts (Enjalbert *et al.* 2003). Notably absent are the major changes in carbohydrate metabolism that promote sugar storage. *C. albicans* Hsf1 displays most of the characteristics of the *S. cerevisiae* protein and appears to play a major role in basal expression of key HSPs, including Hsp70 and Hsp90. Hsf1 is required for virulence, at least in part through its ability to induce HSP expression during stress, but potentially due to the requirement for basal levels of Hsp70/Hsp90 production (Nicholls *et al.* 2009, 2010). This distinction is difficult since Hsp90 (and by extension, Hsp70 due to extensive collaboration between the two chaperone machines) is likely required for signaling during infection. Moreover, Hsp90 has been identified as a key mediator of fungal drug resistance in *C. albicans* and *Aspergillus fumigatus* (see Cowen 2009 for a summary). This has prompted work to exploit the potential of therapeutic synergy between antifungals such as the azoles and the newer echinocandins and Hsp90 inhibitors such as geldanamycin and its derivatives (Cowen *et al.* 2009; Singh *et al.* 2009).

The Oxidative Stress Response

While elevated temperature represents the primary insult during heat shock (as described above), one of the major secondary consequences involves production of reactive oxygen species (ROS). All organisms are exposed to ROS during the course of normal aerobic metabolism or following exposure to radical-generating compounds (Halliwell 2006).

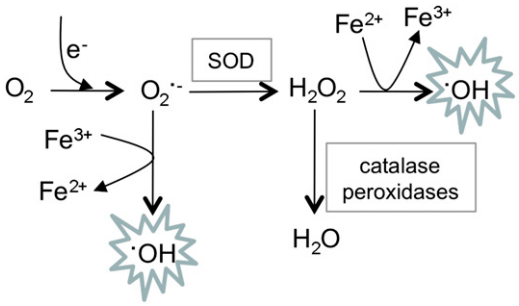

Figure 4 Generation of ROS. The superoxide anion (O_2^-) can be formed via electron leakage to oxygen from electron transport chains. Hydrogen peroxide (H_2O_2) is generated by the breakdown of superoxide catalyzed by superoxide dismutases (SODs). Hydrogen peroxide can be reduced by iron (Fe^{2+}) in the Fenton reaction to produce the highly reactive hydroxyl radical. In the Haber-Weiss reaction, superoxide can donate an electron to iron (Fe^{3+}), generating the hydroxyl radical and Fe^{2+}, which can further reduce hydrogen peroxide. Various antioxidant enzymes, including catalases and peroxidases, detoxify hydrogen peroxide to prevent such ROS generation.

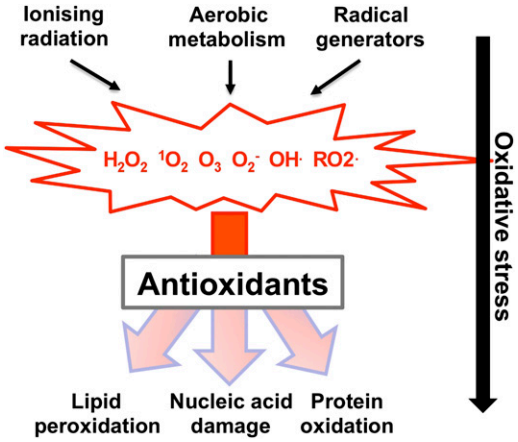

Figure 5 Oxidative stress. All organisms can be exposed to ROS during the course of aerobic metabolism or following exposure to ionizing radiation and radical-generating compounds. Antioxidant defense systems protect against ROS by detoxifying ROS as they are generated and by maintaining the intracellular redox environment in a reduced state. An oxidative stress occurs when the antioxidant and cellular survival mechanisms are unable to cope with the ROS or the damage caused by them. Oxidative stress can damage a wide variety of cellular components resulting in lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA.

Molecular oxygen is relatively unreactive and harmless in its ground state, but can undergo partial reduction to form a number of ROS, including the superoxide anion and hydrogen peroxide (H_2O_2), which can further react to produce the highly reactive hydroxyl radical (Figure 4). ROS are toxic agents that can damage a wide variety of cellular components resulting in lipid peroxidation, protein oxidation, and genetic damage through the modification of DNA. An oxidative stress is said to occur when the antioxidant and cellular survival mechanisms are unable to cope with the ROS or the damage caused by them (Figure 5). Various disease processes, including cancer, cardiovascular disease, arthritis, and aging have been shown to involve oxidative damage. Oxidative damage is also of particular concern to industry, since the oxidation of fats and oils is the process underlying food rancidity, and yeast cells used in the baking and brewing industries are exposed to oxidative stresses during freezing and drying. *S. cerevisiae* responds to an oxidative stress using a number of cellular responses that ensure the survival of the cell following exposure to oxidants. These include defense systems that detoxify ROS, reduce their rate of production, and repair the damage caused by them. Many responses are ROS specific, but there are also general stress responses that are typically invoked in response to diverse stress conditions.

Sources of ROS generation in yeast cells

ROS generation naturally arises from environmental insults and from side reactions of normal aerobic metabolism. Mitochondrial respiration is thought to provide the main source of ROS in eukaryotic cells via the process of oxidative phosphorylation (Murphy 2009). To generate ATP, electrons are transported along protein complexes that constitute the electron transport chain to the ultimate acceptor, molecular oxygen, with the formation of water. Leakage of these electrons from the respiratory chain can result in the reduction of oxygen, generating ROS in yeast cells (Figure 5). Similarly, the use of oxygen as a terminal electron acceptor during oxidative protein folding means that the ER is also a significant source of ROS (Tu and Weissman 2004). Other metabolic processes that can potentially generate endogenous ROS in yeast, depending on the growth conditions, include peroxisomal fatty acid degradation in the β-oxidation pathway (Hiltunen *et al.* 2003) and oxidative deamination of amino acids by D-amino acid oxidases during growth on D-amino acids as carbon sources (Pollegioni *et al.* 2007). Yeast cells can also become exposed to ROS produced by neutrophils and macrophages during immunological defense mechanisms and following exposure to numerous exogenous agents including xenobiotics, carcinogens, and UV and ionizing radiation (Halliwell 2006).

Commonly used model ROS compounds

Many oxidative stress studies have made use of single compounds such as hydrogen peroxide (H_2O_2) as a model oxidant. However, given that cells can respond to ROS via oxidant-specific responses, it has been argued that no single oxidant can truly be said to be representative of "oxidative stress" (Temple *et al.* 2005). Genome-wide screens of the yeast deletion collection have examined different ROS and further shown that there are both core functions that are required for a broad range of oxidative-stress conditions as well as ROS-specific functions that are only required during particular oxidant conditions (Thorpe *et al.* 2004). A brief discussion of the more frequently used model ROS compounds is provided here.

Hydroperoxides: Due to its ease of use including water solubility and relative stability, H_2O_2 is most widely used as

a model oxidative stress condition. H_2O_2 is a ubiquitous molecule formed as a byproduct of aerobic respiration and following exposure to diverse biological and environmental factors. It can damage cells by promoting oxidative stress but also plays important roles as a signaling molecule in the regulation of many biological processes (Veal *et al.* 2007). H_2O_2 must be removed from cells to avoid Fenton and Haber-Weiss reactions leading to the formation of highly reactive hydroxyl radicals (OH) (Figure 4). Organic hydroperoxides are often used to invoke lipid oxidation. For example, cumene hydroperoxide ($C_9H_{12}O_2$) is an aromatic lipid soluble hydroperoxide that is widely used as an intracellular source of ROS (Thorpe *et al.* 2004). It can generate highly reactive free radicals such as the alkoxy radical, resulting in high mutagenicity and toxicity (Simic *et al.* 1989). *tert*-butyl hydroperoxide ($(CH_3)_3COOH$) is less hydrophilic than hydrogen peroxide and is frequently used as a model alkyl hydroperoxide. Linoleic acid hydroperoxide (LoaOOH) has been used as a model lipid hydroperoxide in yeast and is toxic to yeast cells at very low concentrations compared to H_2O_2 and other organic peroxides (Evans *et al.* 1997). The importance of examining diverse hydroperoxides is emphasized by recent findings that indicate that hydrogen peroxide and *tert*-butyl hydroperoxide induce different cellular signaling responses (Iwai *et al.* 2010).

The superoxide anion: The superoxide anion (O_2^-) is generated by one electron reduction of oxygen (Figure 4). It is the major ROS product resulting from electron leakage from the mitochondrial electron transport chain (Halliwell 2006). The superoxide anion is not highly reactive itself but can act as a precursor for other ROS via dismutation to hydrogen peroxide and can generate the highly reactive hydroxyl radical via metal-catalyzed reactions. The superoxide anion can readily be generated in yeast cells using redox-cycling drugs such as menadione and paraquat, which transfer electrons to molecular oxygen.

Thiol-reactive compounds: Given the importance of redox homeostasis during oxidative stress conditions, thiol-reactive compounds are frequently used to induce oxidative stress. These include compounds that indirectly cause an oxidative stress by binding to and depleting thiol groups, as well as compounds which directly oxidize thiol groups. For example, 1-chloro-2,4-dintrobenzene (CDNB) is a substrate for glutathione transferases (Sheehan *et al.* 2001). It does not oxidize thiols in yeast but causes oxidative stress by depleting cellular glutathione (GSH), presumably leading to an accumulation of endogenous ROS (Wheeler *et al.* 2002). Diamide is a membrane-permeable, thiol-specific oxidant, which promotes the formation of disulfides (Kosower and Kosower 1995). It has frequently been used to induce oxidative stress in yeast where it causes a rapid oxidation of glutathione that shifts the redox state of the glutathione redox couple to a more oxidized form (Muller 1996).

Heavy metal stress: The availability of free redox active metals such as iron and copper can have a profound influence on the generation of cellular ROS (Liochev and Fridovich 1999). For example, reduced Fe^{2+} can generate the highly reactive hydroxyl radical via the Fenton reaction (Figure 4). Oxidized Fe^{3+} can be reduced by the superoxide anion further exacerbating the production of hydroxyl radicals in the Haber-Weiss reaction. Hence it is particularly important that free metal levels are tightly controlled, but this is confounded by oxidant attack of [4Fe-4S] clusters in proteins that can themselves provide a source of free iron (De Freitas *et al.* 2003). Cadmium is a highly toxic metal and a well-established human carcinogen, which is often interpreted as causing an oxidative stress (Brennan and Schiestl 1996). It is capable of entering cells via the same transport systems used by the essential heavy metals. Once inside the cell, the main mechanism for toxicity is through the depletion of GSH and binding to sulfhydryl groups (Wysocki and Tamas 2010). It can also displace iron and copper from various cytoplasmic and membrane proteins, increasing the levels of unbound free copper and iron ions, contributing to oxidative stress via Fenton reactions.

Transcriptional control of the OSR

Mounting the defensive response to elevated levels of ROS is a crucial step in preventing cell death from loss of physiologically appropriate redox balance. A key feature in this response is the transcriptional reprogramming of gene expression to provide the requisite changes in proteins to return the redox status of the cell back to an acceptable range. Predictably then, transcriptional regulators that lead to induction of antioxidant proteins have been identified and the focus of much study.

Yap1: A primary determinant in the antioxidant response is the transcription factor Yap1 (Harshman *et al.* 1988). This basic region-leucine zipper-containing positive transcriptional regulator was the second member of the bZip transcription factors discovered in *S. cerevisiae*, after its relative Gcn4 (Harshman *et al.* 1988; Jones *et al.* 1988). Early work established that Yap1 was likely to be a positive regulator of gene expression (Harshman *et al.* 1988; Jones *et al.* 1988) and was capable of conferring a multiple or pleiotropic drug resistance phenotype when overproduced (Leppert *et al.* 1990; Hussain and Lenard 1991). Several groups found that Yap1 was critical for tolerance to oxidants such as H_2O_2 and diamide, as well as heavy metals like cadmium (Schnell and Entian 1991; Kuge and Jones 1994; Wu and Moye-Rowley 1994). These data provided the first association of Yap1 and its central role as a determinant of oxidative stress tolerance.

Nuclear localization of Yap1 responds to oxidative stress: Genetic evidence provided a clear linkage between the presence of Yap1 and normal resistance to oxidants. Elegant experiments using the then recently developed green fluorescent protein (GFP) determined that in the absence of

oxidants, Yap1 was found primarily in the cytoplasm (Kuge *et al.* 1997). Challenge of the cells with diamide led to the rapid accumulation of Yap1 in the nucleus, with concomitant induction of target gene expression. The carboxy terminus of Yap1 was found to be both necessary and sufficient for diamide-induced Yap1 nuclear localization. Three of the six cysteines contained in Yap1 were present in this C-terminal domain, which was designated the cysteine-rich domain (CRD) in recognition of the relative enrichment of this redox active amino acid.

While these data provided a consistent and simple explanation for control of Yap1 activity by diamide, regulation of this factor during H_2O_2 stress was more complex. Loss of the carboxy terminus of Yap1 led to a hypersensitive phenotype when these strains were challenged with H_2O_2 (Wemmie *et al.* 1997). Additionally, deletion mutant derivatives lacking the second CRD located in the amino terminus of the factor were diamide hyperresistant but H_2O_2 hypersensitive. Together, these data indicate that different segments of Yap1 are required for this transcription factor to carry out its normal function in the presence of these different oxidants.

Work on the *Schizosaccharomyces pombe* Yap1 homolog called pap1$^+$ provided the first demonstration of the presence of a *trans*-regulator of these bZip transcription factors (Toda *et al.* 1991, 1992). Crm1 was first found as a factor required for normal chromosome structure (Adachi and Yanagida 1989). This same hypomorphic allele of *CRM1* exhibited a staurosporine hyperresistant phenotype, which was ultimately demonstrated to be due to localization of pap1$^+$ to the nucleus (Toone *et al.* 1998). Experiments in *S. cerevisiae* showed that Crm1 in this yeast was also required for nuclear export of Yap1 under nonstressed conditions (Kuge *et al.* 1998; Yan *et al.* 1998). Biochemical experiments localized the region of Yap1 required to associate with Crm1 to the CRD segment of the transcription factor. This association required the presence of reduced cysteine residues in the CRD. Oxidation or replacement of cysteine residues with other amino acids blocked binding of Yap1 and Crm1, correlating with constitutive nuclear localization of Yap1. This work led to the key conclusion that retention of Yap1 in nucleus in response to oxidant exposure was the result of control of nuclear export of Yap1, rather than the direct regulation of Yap1 import. Later work mapping the bipartite nuclear localization signal of Yap1 to its extreme amino terminus confirmed this view of the central importance of nuclear export in control of Yap1 activity (Isoyama *et al.* 2001).

Most of the studies analyzing regulated nuclear trafficking of Yap1 employed diamide as the oxidant to trigger the response of this protein. While mutagenesis experiments demonstrated that mutant derivatives of Yap1 exhibited dramatically different phenotypes in the presence of diamide *vs.* H_2O_2, the basis for this oxidant-specific response of Yap1 was unknown. This murky view began to change with the consideration of the thioredoxin-encoding gene *TRX2* as a key determinant in the Yap1-dependent induction of H_2O_2 resistance.

TRX2 had already been found to be required for H_2O_2 resistance and to be responsive to Yap1 (Kuge and Jones 1994). Analysis of a range of mutant forms of Yap1 indicated that alterations in the CRD caused constitutive activation of an artificial Yap1-responsive reporter gene but prevented the ability of these same mutants to support H_2O_2-induced *TRX2* transcription (Coleman *et al.* 1999). These observations indicated that CRD mutants were able to elevate the expression of genes involved in diamide resistance but other genes (such as *TRX2*) placed additional requirements on Yap1 for transcriptional activation. Further experiments in this study provided direct evidence that the amino-terminal cluster of cysteine residues were also required for response to H_2O_2. The two cysteine-rich domains were then designated the n-CRD and c-CRD to denote their relative amino- or carboxy-terminal location.

While the presence of both CRD regions was necessary for normal H_2O_2 regulation of *TRX2* transcription, the molecular mechanism underlying this bipartite requirement remained unknown. Direct analysis of oxidative folding of Yap1 provided crucial insight into the roles of the n- and c-CRD regions (Delaunay *et al.* 2000). Use of nonreducing SDS–PAGE demonstrated that a disulfide bond could form between the two CRDs consisting of cysteine 303 (located in the n-CRD) and cysteine 598 (found in the c-CRD). Importantly, this disulfide was not observed to form when cells were stressed with diamide. This group went on to show that H_2O_2-induced but not diamide-triggered oxidation required the participation of another protein called Gpx3 to properly fold Yap1 (Delaunay *et al.* 2002). Gpx3 (aka Orp1) is one of several glutathione peroxidase homologs found in *S. cerevisiae* (Inoue *et al.* 1999a). Unlike its homologs for which evidence exists that these proteins act as *bona fide* glutathione peroxidases (Avery and Avery 2001), Gpx3 has a primary role as a sensor protein and is covalently linked via its cysteine 36 to C598 of Yap1 upon H_2O_2 stress (Delaunay *et al.* 2002). This linkage is believed to stimulate the inter-CRD disulfide bond formation between C303 and C598, a key modification required for the Yap1 response to H_2O_2.

While formation of the Gpx3 C36-C598 Yap1 disulfide is required for normal regulation of Yap1 by H_2O_2, it is not sufficient. Mutants lacking additional cysteines present in either the n-CRD (C310) or the c-CRD (C629) exhibited major defects in H_2O_2 resistance (Coleman *et al.* 1999; Delaunay *et al.* 2000). An explanation for these data were provided by solution of the structure of the H_2O_2-induced disulfide-bonded form of Yap1 by NMR (Wood *et al.* 2004). This structure demonstrated the presence of a pair of disulfide bonds linking C303-C598 and C310-C629. This structure predicted that this dually disulfide-bonded form of Yap1 would be incapable of interacting with Crm1 as the binding site for this export factor is occluded by the H_2O_2-induced structure of Yap1.

Along with Gpx3, a second protein required for H_2O_2-induced folding of Yap1 was found. This protein was designated Ybp1 (Yap1-binding protein) and is required for *in vivo* folding of Yap1 in the presence of H_2O_2 but not diamide (Veal *et al.* 2003; Gulshan *et al.* 2004). A homolog of Ybp1, called Ybp2, is also present in *S. cerevisiae* but its function is presently unclear. Overproduction of Ybp2 (aka Ybh1) can bypass a *gpx3*Δ mutant while overproduction of Ybp1 cannot (Gulshan *et al.* 2004). More recent work found that Ybp2/Ybh1 can associate with the kinetochore (Ohkuni *et al.* 2008) but its precise role in either oxidative stress or chromosome segregation remains unclear. Studies using a mutant allele of *YBP1* often found in laboratory yeast strains called *ybp1-1* (Veal *et al.* 2003; Okazaki *et al.* 2005) demonstrated that the peroxiredoxin Tsa1 is capable of folding Yap1, albeit less effectively than the Ybp1/Gpx3 system (Tachibana *et al.* 2009). It is interesting to note that *in vitro* folding experiments have provided evidence that Yap1 in the presence of Gpx3 alone can properly fold at some level (Okazaki *et al.* 2007). The role of Ybp1 might be to increase the efficiency of this process *in vivo* to more rapidly mount a defensive response to H_2O_2 challenge. A model summarizing our current picture of Yap1 trafficking during oxidative stress is shown in Figure 6.

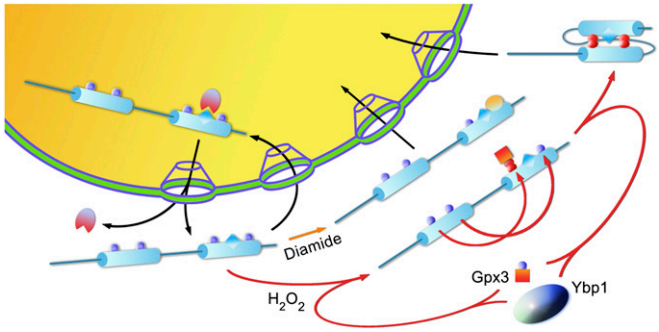

Figure 6 Yap1 folding and trafficking. A scheme for oxidant-specific folding and nuclear import of Yap1 is shown. The four key regulatory cysteine residues in their reduced conditions are indicated by the purple circles. Reduced Yap1 is imported into the nucleus at a basal rate but interacts with the exportin Crm1 and is returned to the cytoplasm in the absence of oxidative stress. In the presence of oxidants that directly act on Yap1 (like diamide), C-terminal cysteine residues are oxidized or modified (yellow circle) in a manner that prevents Crm1 from recognizing the nuclear export signal (blue triangle) in the Yap1 C terminus. Yap1 accumulates in the nucleus and activates gene expression. Finally, during challenge by peroxides that engage the Gpx3/Ybp1 folding pathway, Gpx3 is covalently linked to cysteine 598 of Yap1 by a disulfide bond (linked red circles). This modification, along with the participation of Ybp1, catalyzes an intramolecular folding reaction that leads to a dually disulfide bonded Yap1 form. This species also accumulates in the nucleus and can activate expression of genes required for the response to peroxide stress.

Yap1 homologs and redox stress: *S. cerevisiae* contains a family of proteins sharing sequence similarity with Yap1. These have been collectively referred to as the Yap family (Fernandes *et al.* 1997). While many of these factors have been well documented to play a role in stress responses (see (Rodrigues-Pousada *et al.* 2010 for a recent review), only Cad1/Yap2 has been implicated in tolerance to oxidative stress. Cad1 exhibits the highest degree of sequence conservation in two segments of its protein chain. The amino-terminal DNA binding domain is similar to that of Yap1, and the c-CRD region of Yap1 is also closely conserved with Cad1. Direct examination of the similarity of these domains in Yap1 and Cad1 suggested that these c-CRD domains share some degree of functional equivalency in the response to cadmium but not to H_2O_2 (Azevedo *et al.* 2007).

Interestingly, the alkylhydroperoxidase protein Ahp1 has been found to regulate the oxidative folding of Cad1 in a manner highly reminiscent of Yap1 and Gpx3 (Iwai *et al.* 2010). Biochemical experiments indicate that Ahp1 forms a covalent intermediate with Cad1 in the c-CRD region and catalyzes disulfide bond formation there. This folding reaction is less well characterized than that of Yap1 but apparently involves formation of Ahp1–Cad1 heterodimers followed by Cad1 disulfide-bonded homodimers.

Although good evidence for the Ahp1-catalyzed Cad1 folding exists, the functional contribution of Cad1 to oxidative stress phenotypes is minor (Iwai *et al.* 2010). In the absence of the *YAP1* gene, the role of Cad1 is more evident. Cad1 is absent from other species such as *C. glabrata* (data not shown), suggesting that the presence of this factor may be a specialized adaptation of *S. cerevisiae*. Overproduction of Cad1 has major phenotypic effects on a variety of drugs to which Yap1 also elicits tolerance (Bossier *et al.* 1993; Wu *et al.* 1993; Hirata *et al.* 1994). Oxidant resistance conferred by Cad1 does not appear to be the primary role of this transcription factor.

Skn7: Skn7 was originally isolated as a high-copy number suppressor of a strain defective in β-glucan synthesis (Brown *et al.* 1993). The identification of this gene piqued extensive interest in its study as Skn7 contained protein elements reminiscent of bacterial two-component regulatory systems as well as a DNA binding domain related to Hsf1 (Brown *et al.* 1993). Later experiments indicated that Skn7 acted downstream of a histidine kinase involved in the osmotic stress response called Sln1 (Li *et al.* 1998). Skn7 was linked to oxidative stress tolerance by a genetic screen searching for mutations that cause sensitivity to peroxide (Krems *et al.* 1996). One of these genes, originally called *POS9*, was found to be allelic with *SKN7*. Mutant strains lacking both Yap1 and Skn7 were no more sensitive to H_2O_2 than either single mutant (Krems *et al.* 1996; Morgan *et al.* 1997), suggesting that these two transcriptional regulators act in the same genetic pathway.

A likely explanation for this convergence of Yap1 and Skn7 function came from an analysis of transcriptional activation by these factors on promoters involved in thioredoxin homeostasis. *S. cerevisiae* contains three different genes encoding thioredoxins, an important antioxidant (Gan 1991; Muller 1991; Pedrajas *et al.* 1999). Transcriptional

activation of the thioredoxin-encoding *TRX2* gene by Yap1 is required for H_2O_2 resistance (Kuge and Jones 1994). Similarly, *TRX2* is also a target for Skn7 regulation and loss of either Yap1 or Skn7 alone is sufficient to prevent H_2O_2 induction of *TRX2* transcription (Morgan *et al.* 1997). A simple explanation for these data are that both Yap1 and Skn7 are required for H_2O_2-stimulated *TRX2* expression. This model was directly supported by demonstration that both Yap1 and Skn7 bound to the *TRX2* promoter at different sites (Morgan *et al.* 1997). Importantly, loss of the aspartate residue that serves as the ultimate acceptor for Sln1-mediated phosphorylation had no effect on Skn7-mediated oxidative stress resistance although this same mutation eliminated osmotic stress tolerance (Morgan *et al.* 1997; Ketela *et al.* 1998; Li *et al.* 1998).

The theme of Yap1 and Skn7 acting at a common promoter to induce oxidative stress tolerance is not restricted to genes influencing thioredoxin resistance. Global proteomic analyses demonstrated that Yap1 controlled the expression of a minimum of 32 different polypeptide chains (Lee *et al.* 1999a). Fifteen of these factors required the presence of both Yap1 and Skn7 to be induced by H_2O_2. The cognate target genes for these proteins include the two dismutase loci in *S. cerevisiae* (*SOD1* and *SOD2*), a peroxiredoxin (*TSA1*), and an alkyl hydroperoxide reductase (*AHP1*), among others. One caveat to these data are the lack of direct demonstration for an involvement of Yap1 in their regulation. A large number of genes are also regulated by Yap1 in a Skn7 independent fashion. An example of such a locus is argued to be *GSH1* encoding the γ-glutamylcysteine synthetase enzyme, the rate-limiting step in glutathione biosynthesis (Ohtake and Yabuuchi 1991). The case for lack of *GSH1* regulation by Skn7 is difficult to make since the data are negative. However, while the Yap1 control of *GSH1* is clear (Wu and Moye-Rowley 1994), the response of *GSH1* to Skn7 has not been reported (Dormer *et al.* 2002).

While much is known of the molecular basis of Yap1 regulation by oxidative stress, disappointingly little information is available detailing the control of Skn7 by oxidants. Skn7 is a constitutive nuclear protein and no evidence has been obtained documenting any changes in the expression of this factor in the presence of oxidants (Raitt *et al.* 2000). An intriguing observation linking Skn7 function with that of the heat shock transcription factor Hsf1 suggests a possible means of modulating Skn7 transcriptional activity (Raitt *et al.* 2000). Since Skn7 contains a DNA binding domain similar to that of Hsf1, cells lacking *SKN7* were tested for the ability to tolerate an acute heat shock. The *skn7*Δ strain was found to be more sensitive than an isogenic wild-type strain to this heat stress. Loss of Skn7 was also found to prevent the H_2O_2 induction of expression of the *SSA1* gene, a locus encoding a major Hsp70 protein. Strikingly, Skn7 was found to bind the Hsf1 recognition sequence as well as associate with Hsf1 itself.

Together, these data suggest that Skn7 associates with Hsf1 to mediate the H_2O_2-dependent induction of *SSA1* gene expression. Skn7 has also been argued to associate with an important transcriptional regulator of cell cycle progression called Mbp1, although the linkage of this interaction and oxidative stress remains obscure (Machado *et al.* 1997). The findings that two relatively unrelated transcription factors can both associate with Skn7 suggest that this might be a means by which the activity of Skn7 could be modulated at different promoters. Understanding the spectrum of protein:protein interactions in which Skn7 participates is an important future research question.

Several other features of Skn7 have been implicated in contributing to oxidative stress tolerance. Early work demonstrated that protein kinase A signaling was able to repress Skn7 function (Charizanis *et al.* 1999b). The mechanism of this inhibition is still uncertain. A genetic screen searching for factors required to support transcriptional activation of a Gal4–Skn7 fusion protein identified two proteins that satisfied this criterion. The first, Ccp1, is a mitochondrial cytochrome c peroxidase and appears to be involved in signaling the presence of mitochondrial oxidative stress to Skn7 (Charizanis *et al.* 1999a). The second, known as Fap7, is an essential protein and contains a nucleotide triphosphatase domain (Juhnke *et al.* 2000). Fap7 has also been implicated in ribosome subunit processing (Granneman *et al.* 2005) and arsenite resistance (Takahashi *et al.* 2010). Finally, recent data have defined phosphorylation sites in Skn7 that appear to be associated with the onset of oxidative stress (He *et al.* 2009).

The unique role of Skn7 in the oxidative stress response makes the limited understanding of this factor a critical gap in our knowledge. Skn7 is of singular importance in H_2O_2 tolerance, which cannot be bypassed by overproduction of Yap1 or use of a variety of constitutively active forms of this transcription factor (Coleman *et al.* 1999). One explanation for the unique contribution of Skn7 to oxidative stress resistance gene transcription might come from its ability to recruit different mediator components to key target promoters. Suggestive data consistent with this model have come from an analysis of the degradation of the transcription Mediator subunit cyclin C (also known as Srb11) (Krasley *et al.* 2006).

Cyclin C (CycC) is a subunit of the so-called Cdk8 subcomplex of the transcriptional Mediator (recently reviewed in Malik and Roeder 2010). Early evidence suggested primarily a negative role for the Cdk8 subcomplex (Holstege *et al.* 1998; Kuchin and Carlson 1998; Elmlund *et al.* 2006). More recent work has provided strong support for the idea that the Cdk8 subcomplex can also exert a positive influence on gene expression (Cooper and Strich 2002). CycC is rapidly degraded in response to oxidative stress and this degradation correlates with the induction of stress gene expression (Cooper *et al.* 1999; Vincent *et al.* 2001). Use of CycC as bait in a two-hybrid assay detected interaction with a protein referred to as Ask10 (activator of Skn7) (Page *et al.* 1996), previously argued to be a positive regulator of transcriptional activation by Skn7. Loss of *ASK10* elicits an

oxidant hypersensitive phenotype that can be suppressed by removal of the CycC gene. Biochemical experiments demonstrate that CycC degradation is blocked in the absence of Ask10 (Cohen *et al.* 2003).

A possible explanation of these data would come from Ask10 and Skn7 interacting to remove the repressive effect of CycC from oxidant responsive promoters. Skn7 might then be able to exert an additional effect on Mediator, which is required for induction of antioxidant gene expression. This central role of Mediator in permitting Skn7 function would fit well with what is known of the action of Yap1 during H_2O_2 induction of gene expression. Properly folded Yap1 can interact with the Mediator subunit Rox3 and this interaction is crucial for normal *TRX2* activation and H_2O_2 tolerance (Gulshan *et al.* 2005).

Further data linking Skn7 with Mediator emerged from a biochemical analysis of proteins associating with the Ccr4–Not complex, which is a global regulator of gene expression with a major role in stress resistance (Lenssen *et al.* 2002). This protein complex has been associated with activity of the general stress transcriptional regulators Msn2/4 (Lenssen *et al.* 2005) (discussed in the following section) but also interacts with Skn7. Use of Not1 in a two-hybrid screen detected interaction with Skn7 (Lenssen *et al.* 2007). Skn7 was also found to interact with another component of the Not module (Not5). Skn7:Not5 interaction was dependent on the presence of the Not4 protein, which is an E3 ubiquitin ligase (Mulder *et al.* 2007). Deletion of the *NOT4* gene led to increased occupancy of Skn7 on two different oxidative stress-responsive promoters (but not to a third) and also appeared to modestly elevate H_2O_2 resistance. Finally, purification of Skn7 detected the presence of the Cdk8 protein, a cyclin-dependent kinase associated with the Mediator complex (Lenssen *et al.* 2007). The presence of Cdk8 was found to be required for the increased expression of a Skn7 target gene (albeit a gene involved in osmotic stress resistance only) seen in a *not4*Δ strain.

These data support the view that Skn7 interacts with a range of general transcriptional regulators (Cdk8 and Ccr4–Not complex) that are known to influence oxidative stress tolerance. These interactions may explain the unique role of Skn7 in regulation of the response to oxidative challenge. Since these general transcription factors have not been demonstrated to interact with Yap1, their engagement by Skn7 could permit full oxidative stress response of relevant promoters. Solving the mechanistic puzzle of Skn7 input to oxidative stress resistance remains an important goal.

Msn2/4: Msn2 and Msn4 have already been considered above as these transcription factors are important participants in heat shock tolerance. However, these factors also play roles in resistance to oxidative stress. A mutant lacking both *MSN2* and *MSN4* is highly sensitive to oxidative stress (Martinez-Pastor *et al.* 1996). This observation, coupled with the large number of oxidative stress genes under Msn2/Msn4 control, suggests that these factors are responsive to oxidant challenge. Recent experiments have directly examined the localization of Msn2/4 to the nucleus upon oxidative stress (Boisnard *et al.* 2009). Interestingly, important differences with other stress-stimulated nuclear localization have been detected. First, PKA-dependent phosphorylation of Msn2/4 was not observed to change in response to H_2O_2 treatment. Second, the presence of the Trx1/2 thioredoxins was required for H_2O_2 to trigger Msn2/4 recruitment to the nucleus. A remarkable feature of H_2O_2-induced Msn2/4 nuclear localization is that only a fraction (21%) of the cells exhibited accumulation of these factors in the nucleus while nuclear localization was seen for ~100% of cells upon osmotic stress (Gorner *et al.* 1998; Boisnard *et al.* 2009). H_2O_2-dependent localization of Msn2/4 could be improved by removal of the *TRR1* gene encoding the thioredoxin reductase. This suggested the model that oxidized Trx1/2 were required to cause Msn2/4 nuclear localization (Figure 1). However, redox control of Msn2/4 localization does not appear to involve direct oxidative modification of Msn2/4 protein sequences as seen for the cysteine residues in Yap1 (see above). The precise mechanism of redox regulation of Msn2/4 function remains to be determined.

A potential complication in the analysis of oxidative stress control of Msn2/4 is illustrated by consideration of the role of a key target gene regulated by these factors. *CTT1* was one of the first genes demonstrated to be regulated by Msn2/4 and encodes the cytosolic catalase, an enzyme with obvious importance to the maintenance of redox balance. Strikingly, *CTT1* disruption mutants are not sensitive to H_2O_2 in exponentially growing cells (Izawa *et al.* 1996). This behavior is in marked contrast to *yap1*Δ mutant strains that are exquisitely sensitive to H_2O_2 challenge in log-phase cultures (Schnell *et al.* 1992; Kuge and Jones 1994). However, when late log-phase cultures are evaluated, this dependence is inverted (Izawa *et al.* 1996). Possibly, nuclear localization of Msn2/4 upon H_2O_2 stress is similarly dependent on growth phase of the cells. The most thorough study of Msn2/4 nuclear recruitment during H_2O_2 stress was done with log-phase cells, which leaves the tantalizing possibility that this process would be much more efficient in cells that have undergone the diauxic shift into late log growth.

A final feature of Msn2/4 regulation that seems likely to be of importance to the function of these factors during oxidative stress is their regulated degradation by the proteasome upon stress treatment. Ubiquitination of Msn2 was found to be triggered by phosphorylation mediated by the cyclin-dependent kinase Cdk8 (also known as Srb10) (Chi *et al.* 2001). Later experiments demonstrated that Msn2 was rapidly degraded when localized to the nucleus during heat shock and that this turnover required the function of the proteasome (Durchschlag *et al.* 2004; Lallet *et al.* 2004; Bose *et al.* 2005). These latter experiments did not investigate the stability of Msn2/4 after oxidative challenge but since both heat or oxidative stress elevates nuclear levels of these factors, it seems reasonable that Cdk8 may be involved in the

degradation of these transcription factors under either stress condition.

Antioxidant defenses

ROS are continuously produced in actively metabolizing cells. However, *S. cerevisiae*, like all organisms, contains effective antioxidant defense mechanisms, which detoxify ROS as they are generated and maintain the intracellular redox environment in a reduced state. An oxidative stress is said to occur when ROS overwhelm these defenses, resulting in genetic degeneration and physiological dysfunction, leading eventually to cell death (Figure 5). Antioxidant defenses include a number of protective enzymes that are present in different subcellular compartments and can be upregulated in response to ROS exposure (Table 3). Nonenzymic defenses typically consist of small molecules that can act as free radical scavengers; to date, only ascorbic acid and GSH have been extensively characterized in yeast.

Catalases: Catalases are ubiquitous heme-containing enzymes that catalyze the dismutation of H_2O_2 into H_2O and O_2 (Figure 4). Yeast has two such enzymes: the peroxisomal catalase A, encoded by *CTA1*, and the cytosolic catalase T, encoded by *CTT1*. *CTA1* expression is coordinated with peroxisomal fatty acid metabolism, suggesting that Cta1 may function in the detoxification of H_2O_2 generated from fatty acid β-oxidation (Hiltunen *et al.* 2003). Ctt1 is thought to play a more general role as an antioxidant during exposure to oxidative stress, since *CTT1* expression is induced by various stress conditions including heat, osmotic, starvation, and hydrogen peroxide stress (Martinez-Pastor *et al.* 1996). Surprisingly however, yeast mutants lacking both catalases are unaffected in hydrogen peroxide tolerance during exponential phase growth (Izawa *et al.* 1996). Redundancy in antioxidant defenses may account for this apparently minor role in hydrogen peroxide tolerance, since loss of catalases exacerbates the peroxide sensitivity of glutathione mutants and the glutathione system is hypersensitive to hydrogen peroxide in catalase mutants (Grant *et al.* 1998). However, catalases are important for the acquisition of peroxide resistance following pretreatment with low doses of H_2O_2 and upon entry into stationary phase, indicating a role during adaptive responses (Izawa *et al.* 1996).

Superoxide dismutases: Superoxide dismutases (SODs) convert the superoxide anion to hydrogen peroxide, which can then be reduced to water by catalases or peroxidases (Figure 4). SODs are ubiquitous antioxidants, which differ in their intracellular location and metal cofactor requirements between different organisms. Enzyme activity is dependent on redox cycling of the bound metal cofactor. Yeast contains a cytoplasmic Cu,Zn-SOD (Sod1) and a mitochondrial matrix Mn-SOD (Sod2), which appear to play distinct roles during oxidative stress conditions (Culotta *et al.* 2006). Cells deleted for *SOD1* are hypersensitive to superoxide-generating agents such as paraquat and display a number of oxidative stress-related phenotypes including vacuole damage and increased free iron concentrations (Culotta 2000). Additionally, aerobic inactivation of [4Fe-4S] cluster enzymes in *sod1* mutants results in auxotrophies for methionine and leucine (Slekar *et al.* 1996; Wallace *et al.* 2004). While Sod1 is predominantly cytosolic, it also localizes to the mitochondrial inner membrane space where it is thought to function in the detoxification of superoxide generated from respiration (Sturtz *et al.* 2001). Mutants deleted for *SOD2* are less affected in growth and stress sensitivity compared with *sod1* mutants but do show a reduced ability to grow under respiratory conditions (Van Loon *et al.* 1986). Sod2 is particularly required during stationary phase growth, which may be linked to superoxide generation from mitochondrial respiration (Longo *et al.* 1996). Cells deleted for *SOD2* can grow under conditions of hyperoxia as a result of mutations that disrupt the mitochondrial electron transport chain, confirming the role of respiration in ROS production in yeast (Guidot *et al.* 1993). Similarly, the viability of *sod1 sod2* mutants during long-term stationary phase incubation can be restored by similar mutations, further implicating the mitochondrial electron transport chain as the major source of ROS in yeast (Longo *et al.* 1996).

Methionine sulfoxide reductase: Amino acids are susceptible to oxidation by ROS (Stadtman and Levine 2003). Methionine residues are particularly susceptible, forming a racemic mixture of methionine-*S*-sulfoxide (Met-*S*-SO) and methionine-*R*-sulfoxide (Met-*R*-SO) in cells (Dean *et al.* 1997). Most organisms contain methionine sulfoxide reductases (MSRs), which protect against methionine oxidation by catalyzing thiol-dependent reduction of oxidized Met residues. This is particularly important because it means that methionine oxidation is readily reversible and can play an antioxidant role in scavenging ROS (Stadtman *et al.* 2003). Yeast contains three MSR enzymes that are required for resistance against oxidative stress (fRMsr/MsrA/MsrB) (Le *et al.* 2009). fRMsr is thought to be the main enzyme responsible for the reduction of free Met-*R*-SO, whereas MsrA and MsrB are active with Met-*S*-SO and Met-*R*-SO in proteins. A triple fRMsr/MsrA/MsrB mutant is viable on media containing methionine, but cannot grow if methionine is substituted with Met-SO (Le *et al.* 2009).

Thioredoxins: *S. cerevisiae*, like most eukaryotes, contains a cytoplasmic thioredoxin system, which functions in protection against oxidative stress (Figure 7). This comprises two thioredoxins (*TRX1* and *TRX2*) and a thioredoxin reductase (*TRR1*) (Gan 1991). Thioredoxin mutants are auxotrophic for sulfur amino acids, since thioredoxins are the sole hydrogen donors for PAPS reductase, the enzyme that converts 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to sulfite (Muller 1991). Mutants deleted for *TRX1* and *TRX2* are also affected in the cell cycle with a prolonged S phase and shortened G1 interval (Muller 1991). This correlates

Table 3 Protective enzymes that can be up-regulated in response to ROS exposure

Antioxidant	Gene	Location[a]	Activity
The thioredoxin system			
Thioredoxin	*TRX1*, *TRX2*	Cyt	Disulfide oxidoreductase activity
	TRX3	Mit	Mitochondrial disulphide oxidoreductase activity
Thioredoxin reductase	*TRR1*	Cyt	Reduces oxidized thioredoxins (Trx1, Trx2)
	TRR2	Mit	Reduces oxidized thioredoxin (Trx3)
Peroxiredoxin	*TSA1*, *TSA2*	Cyt	2-Cys Prx, thioredoxin peroxidase and chaperone activity
	AHP1	Cyt	2-Cys Prx, thioredoxin peroxidase particularly with alkyl hydroperoxides
	DOT5	Nuc	Nuclear 2-Cys Prx, functions in telomeric silencing
	PRX1	Mit	Mitochondrial 1-Cys Prx, thioredoxin peroxidase activity
The glutathione system			
GSH synthesis	*GSH1*, *GSH2*	Cyt	Catalyze two ATP-dependent steps in GSH biosynthesis
Glutathione reductase	*GLR1*	Cyt/Mit	Recycles oxidized GSSG to reduced GSH, co-localizes to Cyt and Mit
Glutathione transferase	*GTT1*	ER	Catalyze the conjugation of GSH to various electrophiles
	GTT2	Mit	
	GTO1	Per	Omega class glutathione transferase
	GTO2, *GTO3*	Cyt	Omega class glutathione transferase
Glutathione peroxidase	*GPX1*, *GPX2*	Cyt	Phospholipid hydroperoxide glutathione peroxidase
	GPX3	Cyt	Phospholipid hydroperoxide glutathione peroxidase, Yap1 signal transducer
Glutaredoxin	*GRX1*	Cyt	Glutathione disulfide oxidoreductase activity
GRX2	Cyt/Mit	Glutathione disulfide oxidoreductase activity, colocalizes to Cyt and Mit	
	GRX3, *GRX4*	Nuc	Monothiol glutaredoxin
	GRX5	Mit	Monothiol glutaredoxin, synthesis/assembly of iron-sulfur centers
	GRX6, *GRX7*	Gol	Cis-Golgi localized monothiol glutaredoxins
	GRX8	Cyt	Glutathione disulfide oxidoreductase activity
Superoxide dismutase	*SOD1*	Cyt/Nuc	Catalyze the dismutation of superoxide into oxygen and hydrogen peroxide
	SOD2	Mit	
Catalase	*CTT1*	Cyt	Catalyze the reduction of hydrogen peroxide to water and oxygen
	CTA1	Per	
Methionine sulphoxide	*MXR1* (*MSRA*)	Cyt	Catalyze thiol-dependent reduction of methionine (*S*)-sulfoxide
Reductase	*MXR2* (*MSRB*)	Mit	Catalyze thiol-dependent reduction of methionine (*R*)-sulfoxide
	fRMsr (YKL069W)	Cyt	Catalyze thiol-dependent reduction of free Met-*R*-SO
Erythroascorbate	*ALO1*	Mit	D-arabinono-1,4-lactone oxidase, final step of erythroascorbate synthesis

[a] Location is based on information provided at http://www.yeastgenome.org/.

with the role of cytoplasmic thioredoxins as the major reductants of ribonucleotide reductase during S phase (Koc *et al.* 2006; Camier *et al.* 2007). As in most organisms, yeast thioredoxins are active as antioxidants and play key roles in protection against oxidative stress induced by various ROS (Kuge and Jones 1994; Izawa *et al.* 1999). A major part of the antioxidant function of thioredoxins is mediated by peroxiredoxins (Prx's, see below). Oxidized thioredoxins (Trx1/Trx2) are rapidly observed (<15 sec) following exposure to hydrogen peroxide and are detected for >1 hr before returning to the reduced form (Okazaki *et al.* 2007). Trx2 appears to play the predominant role as an antioxidant, since mutants lacking *TRX2* are hypersensitive to hydroperoxides and mutants containing *TRX2*, in the absence of *TRX1*, show wild-type resistance to oxidative stress (Garrido and Grant 2002). However, Trx1 and Trx2 appear to be functionally redundant as antioxidants. This is emphasized by the similar redox midpoint potentials (E_m) of Trx1 and Trx2 (−275 and −265 mV, respectively), indicating the interchangeable nature of these proteins (Mason *et al.* 2006). The differential requirement for Trx1 and Trx2 appears to be related to differences in gene expression; *TRX2* expression is strongly upregulated in response to oxidative stress conditions, whereas *TRX1* may serve an ancillary or back-up role during conditions in which *TRX2* is insufficient to provide an antioxidant defense (Garrido and Grant 2002).

Yeast also contains a complete mitochondrial thioredoxin system, comprising a thioredoxin (Trx3) and a thioredoxin reductase (Trr2) (Figure 7) (Pedrajas *et al.* 1999). The redox states of the cytoplasmic and mitochondrial thioredoxin systems are independently maintained and cells can survive in the absence of both systems (Trotter and Grant 2005). The yeast mitochondrial thioredoxin system has been implicated in protection against oxidative stress generated during respiratory metabolism. However, the mitochondrial thioredoxin reductase was found to have an antioxidant role

independent of thioredoxin since mutants deleted for *TRR2* are sensitive to oxidative stress, compared with *trx3* mutants, which are unaffected in oxidant resistance (Pedrajas *et al.* 2000; Trotter and Grant 2005).

Peroxiredoxins: Peroxiredoxins (Prx) have multiple roles in stress protection, acting as antioxidants, molecular chaperones, and in the regulation of signal transduction (Wood *et al.* 2003). They use redox-active Cys residues to reduce peroxides and have been divided into two classes, the 1-Cys and 2-Cys Prx's, on the basis of the number of Cys residues directly involved in catalysis. Typical 2-Cys Prx's are active as a dimer and contain two redox-active Cys residues that are required for enzyme activity (Chae *et al.* 1994; Park *et al.* 2000). During catalysis, the peroxidatic cysteine residue of one subunit is oxidized to a sulfenic acid, which condenses with the resolving cysteine from the other subunit to form a disulfide that is reduced by thioredoxin (Figure 7). Three cytoplasmic 2-Cys Prx's (Tsa1, Tsa2, and Ahp1) have been described in yeast (Morgan and Veal 2007). All three display thioredoxin peroxidase activity, but appear to play distinct physiological roles. Tsa1 has best been characterized as an antioxidant in the detoxification of hydroperoxides (Garrido and Grant 2002; Wong *et al.* 2004), but has also been shown to act as a chaperone that promotes resistance to heat and reductive stresses (Jang *et al.* 2004; Rand and Grant 2006). Tsa2 is highly homologous to Tsa1 and possesses similar peroxidase and chaperone activities, but is expressed at significantly lower levels than Tsa1 (Jang *et al.* 2004). Ahp1 is active as an antioxidant, but in contrast to Tsa1, its catalytic efficiency is greater with alkyl hydroperoxides than with H_2O_2 (Lee *et al.* 1999b; Park *et al.* 2000). Differences in the cytoplasmic Prx's are further highlighted by the finding that in contrast to Tsa1 and Tsa2, overoxidation of Ahp1 to cysteine–sulfinic acid is not reversed by Srx1 (Biteau *et al.* 2003). Dot5 (nTPx) is a nuclear 2-Cys Prx, which is most active against alkyl hydroperoxides (Cha *et al.* 2003). However, it has been proposed to play a minor role as an antioxidant, predominantly functioning in telomeric silencing (Izawa *et al.* 2004).

Yeast Prx1 is a member of the 1-Cys family of Prx's and is active as a peroxidase (Pedrajas *et al.* 2000). 1-Cys Prx's contains a peroxidatic cysteine, but do not contain a resolving cysteine residue. Since 1-Cys Prx's cannot therefore form a disulfide, the cysteine–sulfenic acid generated by reaction with peroxides is thought to be reduced by a thiol-containing electron donor, but this reaction mechanism is poorly understood. The peroxidatic cysteine residue of Prx1 is oxidized to the sulfenic acid form by hydroperoxides (Greetham and Grant 2009). GSH efficiently attacks the sulfenic acid intermediate, resulting in the formation of glutathionylated Prx1 (Figure 7). Active Prx1 appears to be regenerated by reduction by Trr2 (Greetham and Grant 2009), or alternatively, by the Grx2 glutaredoxin (Pedrajas *et al.* 2010). This is an important finding since it suggests that there is a functional overlap between the GSH/glutaredoxin and

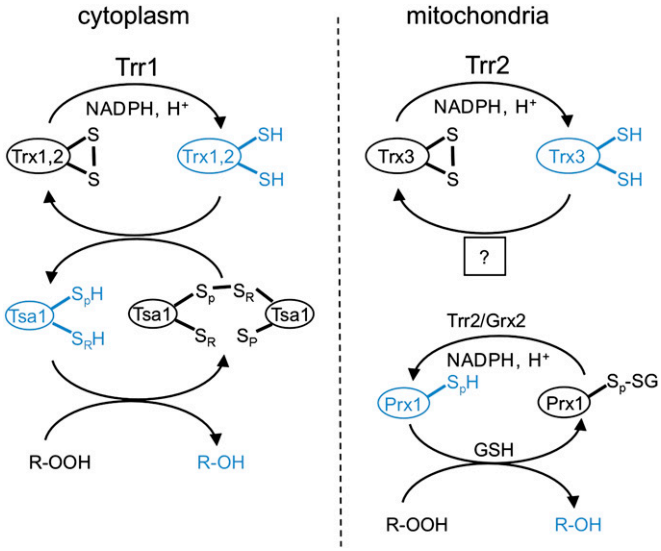

Figure 7 Comparison of cytoplasmic and mitochondrial thioredoxin systems. The yeast cytoplasmic thioredoxin system comprises two thioredoxins (Trx1-2) and a thioredoxin reductase (Trr1). The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase (Trr1). The yeast cytosol contains three typical 2-Cys Prx's (Tsa1, Tsa2, and Ahp1) but only Tsa1 is shown for simplicity. 2-Cys Prx's are active as a dimer and contain two redox active Cys residues that are directly involved in enzyme activity. During reduction of hydroperoxides, a disulphide bond is formed between the peroxidatic cysteine (S_P) of one subunit and the resolving cysteine (S_R) from the other subunit of the dimer. This disulphide is reduced by thioredoxin. Yeast contains a complete mitochondrial thioredoxin system including a thioredoxin (Trx3) and thioredoxin reductase (Trr2). The substrate(s) of Trx3 is currently unknown. Mitochondria contain a single 1-Cys Prx (Prx1). The peroxidatic Cys residue of Prx1 is oxidized to the sulphenic form by hydroperoxides. Oxidized Prx1 is glutathionylated and reduced by Grx2 or Trr2 to regenerate the active enzyme. Reduced components are shown in blue.

thioredoxin systems in mitochondrial antioxidant protection. Further studies are required to decipher the molecular regulation of the different yeast Prx isoforms to better understand the cellular functions of this family of proteins. This is important given the increasing evidence that human Prx's are implicated in various disease processes (Immenschuh and Baumgart-Vogt 2005; Kang *et al.* 2006). Importantly, a strain lacking all five yeast Prx's is viable but is hypersensitive to oxidative stress (Wong *et al.* 2004). It also displays an increased rate of spontaneous mutations, indicating that Prx's function to maintain genome stability in the presence of endogenous ROS.

The glutathione system: The oxidation of sulfhydryl groups is one of the earliest observable events during ROS-mediated damage. This underlies the importance of GSH (γ-glutamylcysteinylglycine) which is typically found as the most abundant low molecular-weight sulfhydryl compound (mM concentrations) in most organisms. Many roles have been proposed for GSH in a variety of cellular processes including amino acid transport; synthesis of nucleic acids and proteins; modulation of enzyme activity; and metabolism of carcinogens, xenobiotics, and ROS (Schafer and Buettner

2001). Not surprisingly therefore, GSH is an essential metabolite in eukaryotes, and for example, mice that are deficient in GSH biosynthesis die rapidly (Shi *et al.* 2000). Similarly, GSH is an essential metabolite in yeast where it appears to be required as a reductant during normal growth conditions (Grant *et al.* 1996b). Oxidative stress converts glutathione to its oxidized disulfide form (GSSG) (Figure 8). However, glutathione is predominantly present in its reduced GSH form in yeast and other eukaryotes due to the constitutive action of glutathione reductase (Glr1). Glr1 is an NADPH-dependent oxidoreductase, which converts GSSG to GSH using reducing power generated by the pentose phosphate pathway (López-Barea *et al.* 1990). Yeast *GLR1* is not essential for normal aerobic growth, but is required for viability during exposure to oxidative stress and following starvation conditions (Grant *et al.* 1996a,c).

GSH is synthesized via two ATP-dependent steps (Figure 8). γ-Glutamylcysteine synthetase (Gsh1) catalyzes the first and rate-limiting step where the dipeptide γ-Glu-Cys is formed from glutamate and cysteine (Lisowsky and Meister 1993). The second step is catalyzed by glutathione synthetase (Gsh2), which ligates γ-Glu-Cys with glycine (Grant *et al.* 1997). GSH biosynthesis is tightly regulated via two overlapping mechanisms. Gsh1 enzyme activity is feedback inhibited by GSH (Meister 1988) and *GSH1* expression is regulated by the cellular concentrations of GSH in parallel with sulfur amino acid biosynthesis (Wheeler *et al.* 2002, 2003). Mutants deleted for *GSH1* are unable to grow in the absence of exogenous GSH, but can undergo a limited number of cell divisions in the absence of GSH during which they utilize preaccumulated stores of GSH (Lee *et al.* 2001; Spector *et al.* 2001). This has enabled the *gsh1* mutant to be extensively used in studies aimed at determining the role of GSH during oxidative stress conditions, by examining the stress sensitivity of the *gsh1* mutant in the absence of exogenous GSH. These studies have shown that mutants lacking *GSH1* are generally sensitive to oxidants including peroxides, the superoxide anion, lipid hydroperoxides and their breakdown products, and heavy metals (Grant *et al.* 1996b; Evans *et al.* 1997; Turton *et al.* 1997; Thorsen *et al.* 2009).

Despite the apparent importance of GSH in protection against ROS, this may not explain the essential function of GSH, since the lethality of a *gsh1* mutant grown in the absence of GSH cannot be rescued by anaerobic growth conditions (Spector *et al.* 2001). GSH is important, however, during stress conditions in its role as a cofactor for stress defense enzymes, including glutathione transferase (GSTs) and glutathione peroxidases (Gpx's) as described below (Figure 8). The *gsh1* mutant has also been used to model the effects of GSH depletion, which can be caused by several processes, including conjugation to xenobiotics, excretion, and decreased synthesis and has been implicated in degenerative diseases, cell aging, and apoptosis (Jain *et al.* 1991; Hardings *et al.* 1997). GSH depletion in yeast causes a G1 cell cycle arrest and hence its essential function is unlikely to be associated with a lack of ribonucleotide reductase activity,

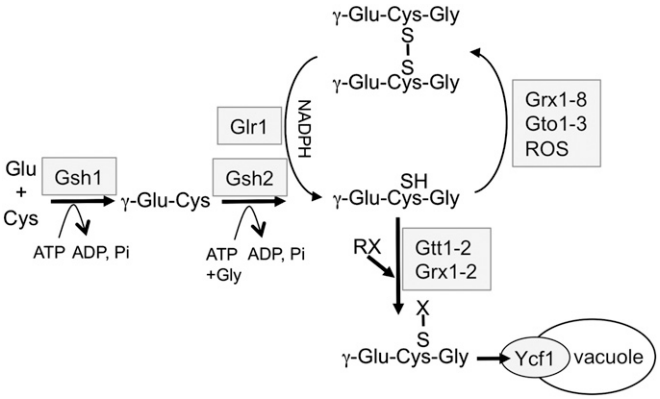

Figure 8 The glutathione system. GSH is synthesized from its constituent amino acids via two ATP-dependent steps. In the first step, Gsh1 (γ-glutamylcysteine synthetase) catalyses the formation of the dipeptide γ-glutamylcysteine (γ-Glu-Cys) from glutamic acid and cysteine. In the second step, Gsh2 (glutathione synthetase) catalyses the ligation of γ-Glu-Cys with glycine. GSH can be oxidized to GSSG by ROS or in reactions catalyzed by Grx1–8 and Gto1–3. Reduced GSH is regenerated in an NADPH-dependent reaction catalyzed by Glr1 (glutathione reductase). GSH can be conjugated to xenobiotics (RX) by GSTs, including Gtt1–2 and Grx1–2. GSH conjugates are transported to the vacuole by the Ycf1 GS-X pump.

which would be expected to affect the S phase of the cell cycle (Spector *et al.* 2001). The eukaryotic requirement for GSH is still uncertain but it may be explained due to its role in the synthesis of [4Fe-4S] clusters, which are essential for viability (Toledano *et al.* 2007).

Glutaredoxins: Glutaredoxins (Grx) are small heat-stable oxidoreductases, which were first discovered in *E. coli* as GSH-dependent hydrogen donors for ribonucleotide reductase (Holmgren 1989). Classical cellular glutaredoxins contain a conserved dithiol active site (Cys-Pro-Tyr-Cys) and form part of the glutaredoxin system, in which glutathione reductase transfers electrons from NADPH to glutaredoxins via GSH. They have proposed roles in many cellular processes including protein folding and regulation, reduction of dehydroascorbate, and protection against ROS and sulfur metabolism (Holmgren 1989). Two yeast genes encode classical dithiol glutaredoxins (*GRX1* and *GRX2*) (Luikenhuis *et al.* 1997). Grx1 and Grx2 are active as GSH-dependent oxidoreductases, but appear to have distinct cellular functions. Strains deleted for *GRX1* are sensitive to oxidative stress induced by the superoxide anion, whereas strains lacking *GRX2* are sensitive to hydrogen peroxide. This difference in oxidant sensitivity may reflect differences in the substrate proteins regulated by Grx1 and Grx2, or in their ability to detoxify ROS-mediated damage (Luikenhuis *et al.* 1997). Differences in the expression of *GRX1* and *GRX2* have also been described further indicating that the two glutaredoxin isoforms may play distinct roles during normal growth and stress conditions (Grant *et al.* 2000). Both genes are regulated in response to oxidative stress conditions via stress-responsive STRE elements, although the induction of *GRX2* is much more rapid and stronger compared with *GRX1*.

Six related glutaredoxins have also now been identified in yeast (Grx3–8). They are found in different subcellular compartments including nuclear (Grx3/4), the mitochondrial matrix (Grx5), and the early secretory pathway (Grx6/7) (Rodriguez-Manzaneque *et al.* 1999; Molina *et al.* 2004; Izquierdo *et al.* 2008; Mesecke *et al.* 2008; Eckers *et al.* 2009). Grx3/4 play an essential role in intracellular iron trafficking (Muhlenhoff *et al.* 2010) and Grx5 is required for mitochondrial [4Fe-4S] cluster assembly (Rodriguez-Manzaneque *et al.* 2002). Grx3–5 differ from dithiol glutaredoxins in that they contain a single cysteine residue at their putative active sites. They are important during the oxidative stress response since they function to regulate iron metabolism and availability. Grx6 and Grx7 are also monothiol glutaredoxins, which are thought to function in sulfhydryl regulation in the early secretory pathway during stress conditions (Izquierdo *et al.* 2008; Mesecke *et al.* 2008). Grx8 is a dithiol glutaredoxin but is not thought to function in the oxidative stress response (Eckers *et al.* 2009).

Glutathione peroxidases: Eukaryotic glutathione peroxidases (Gpx's) are thought to provide the major enzymatic defense against oxidative stress caused by hydroperoxides. They reduce hydrogen peroxide and other organic hydroperoxides, such as fatty acid hydroperoxides, to the corresponding alcohol, using reducing power provided by GSH (Michiels *et al.* 1994). Mammalian cells also contain phospholipid hydroperoxide Gpx's (PHGpx's), which are able to reduce membrane phospholipid hydroperoxides (Roveri *et al.* 1994). Interestingly, yeast does not contain any classical Gpx's, but expresses three PHGpx's encoded by *GPX1-3* (Inoue *et al.* 1999b; Avery and Avery 2001). These PHGpx enzymes have activity with phospholipid hydroperoxides as well as nonphospholipid hydroperoxides and are able to protect membrane lipids against peroxidation. Yeast mutants lacking PHGpx's are hypersensitive to hydroperoxides, including phospholipid hydroperoxides with Gpx3 appearing to account for the majority of activity (Avery and Avery 2001; Avery *et al.* 2004). However, the role of Gpx3 in oxidant tolerance is complicated, given its additional function as a peroxide sensor and activator of Yap1 (Delaunay *et al.* 2002). Furthermore, subsequent studies have shown that yeast PHGpx's are better classified as atypical 2-Cys peroxiredoxins, since they form an intramolecular disulfide bond as part of their catalytic cycle, which is cleaved by thioredoxin (Delaunay *et al.* 2002; Tanaka *et al.* 2005; Ohdate *et al.* 2010).

Glutathione transferases: Glutathione transferases (GSTs) are a major family of proteins, which are involved in the detoxification of many xenobiotic compounds (Sheehan *et al.* 2001). They catalyze the conjugation of electrophillic substrates to GSH prior to their removal from cells via glutathione conjugate pumps (Figure 8). Two genes encoding functional GSTs, designated *GTT1* and *GTT2*, have been identified in yeast (Choi *et al.* 1998). Purified recombinant Gtt1 and Gtt2 are active in a GST assay using 1-chloro-2,4-dinitrobenzene (CDNB) as a model substrate, but share limited sequence homology with each other and with GSTs from other species. Strains lacking *GTT1* and *GTT2* are viable and are unaffected in growth during normal aerobic conditions. In addition, the *gtt1*Δ *gtt2*Δ mutant does not show increased sensitivity to CDNB, which is surprising given that Gtt1 and Gtt2 would be expected to detoxify CDNB via conjugation (Choi *et al.* 1998) and the Ycf1 GS-X pump is required for CDNB resistance (Li *et al.* 1996). This may be explained by functional redundancy with glutaredoxins, since yeast glutaredoxins (Grx1 and Grx2) are active as GSTs with substrates such as CDNB (Collinson and Grant 2003). Mutant analysis has confirmed that Grx1 and Grx2 have an overlapping function with Gtt1 and Gtt2, since simultaneous loss of all four genes substantially reduces cellular GST activity and causes sensitivity to stress conditions, including exposure to xenobiotics, heat, and oxidants (Collinson *et al.* 2002; Collinson and Grant 2003). *S. cerevisiae* also contains three omega class GSTs encoded by *GTO1*, *GTO2*, and *GTO3* (Barreto *et al.* 2006; Garcera *et al.* 2006). These enzymes are induced in response oxidants under the control of Yap1 and STRE-responsive elements. However, Gto1-3 are not active as GSTs with CDNB, but show activity as thiol transferases (glutaredoxins) and as Gpx's. Mutants lacking *GTO1-3* display sensitivity to hydroperoxides and thiol oxidants and also show additive effects with *gtt* mutants including strong sensitivity to cadmium (Barreto *et al.* 2006; Garcera *et al.* 2006). The requirement for GSTs in *S. cerevisiae* appears complex and is provided by redundant gene families. Part of this redundancy may be explained by the differing locations of GSTs, including cytosolic (Grx1, Grx2, Gto2, and Gto3), ER (Gtt1), peroxisomal (Gto1), and mitochondrial (Gtt2 and Grx2).

Ascorbic acid: Ascorbic acid is a water soluble antioxidant, which commonly acts in a redox couple with glutathione in many eukaryotes (Winkler *et al.* 1994). However, the relevance of ascorbate to the yeast oxidative stress response is unclear since yeast contains a 5-carbon analog, erythroascorbate, which may have limited importance as an antioxidant. Strains deleted for *ALO1*, encoding D-arabinono-1,4-lactone oxidase, which catalyzes the final step in erythroascorbate biosynthesis, are sensitive to hydrogen peroxide and the superoxide anion (Huh *et al.* 1998). However, the extremely low levels of erythroascorbate detected in yeast make its functional role as an antioxidant questionable (Spickett *et al.* 2000). It has also been suggested that ascorbate may act as the physiological reductant for 1-Cys Prx's (Monteiro *et al.* 2007), although there does not appear to be an *in vivo* requirement for erythroascorbate to maintain the yeast 1-Cys peroxiredoxin (Greetham and Grant 2009).

Cellular responses to ROS

Yeast cells respond to ROS by altering the expression of genes encoding antioxidant defense mechanisms and genes

encoding enzymes, which repair and detoxify the resulting cellular damage. This forms the basis for inducible adaptive responses, where for example, cells treated with a low dose of oxidant can adapt to become resistant to a subsequent and otherwise lethal treatment. Adaptive responses have been extensively studied in *S. cerevisiae*, where it is now well established that there are a number of cellular responses that ensure the survival of the cell following exposure to oxidants such as hydrogen peroxide, or products of oxidative damage (Collinson and Dawes 1992; Jamieson 1992; Turton *et al.* 1997). While there has been considerable research on the cellular antioxidant defense systems, which protect against ROS, less is known about how adaptation occurs. Its nature depends on the treatment. For example, heat shock induces resistance to hydrogen peroxide, but hydrogen peroxide does not have the reverse effect. Cells adapted to hydrogen peroxide treatment become resistant to menadione (a superoxide generator), but not vice versa (Flattery-O'Brien *et al.* 1993). This hierarchical response to stress may indicate the existence of a number of different adaptation systems, which have overlapping components (Temple *et al.* 2005). *De novo* protein synthesis is known to be required for the induction of resistance to ROS, since it is abrogated by treatment with translation inhibitors such as cycloheximide (Collinson and Dawes 1992). Adaptation may therefore be explained by the pattern of proteins that are produced in response to different oxidant treatments. In agreement with this idea, extensive transcriptional and translational reprogramming is evident during adaptive treatments (Godon *et al.* 1998; Gasch *et al.* 2000; Shenton *et al.* 2006). It is also clear that yeast cells undergo extensive molecular changes during oxidant exposure that are summarized in the following sections.

Redox homeostasis and oxidative stress: Cells must respond to an oxidative stress by regulating their thiol systems to maintain the redox balance of the cell. There is also increasing evidence that ROS play important regulatory functions in cell signaling and thiol groups are key mediators of oxidative signal transduction (Rhee *et al.* 2005). However, there is a limited understanding at present of how cells protect against the deleterious consequences of ROS, while allowing their role in cell signaling (D'Autréaux and Toledano 2007; Veal *et al.* 2007). The composition and redox state of available thiols in protein and low molecular weight compounds is highly dynamic and varies depending on the growth conditions and local environment. Signal transduction pathways generally involve specific protein–protein interactions and increasing evidence indicates that oxidizing agents can control these interactions to bring about controlled signaling events. Cysteine residues are among the most easily oxidized residues in proteins, and most ROS-specific regulatory mechanisms are based on the oxidation of protein-cysteine residues. This is primarily due to the high reactivity of Cys residues and the ability of cysteine residues to cycle between different oxidation states (*e.g.*, disulfide, sulfenic acid, sulfinic acid, and sulfonic acid). An important precedent is provided by the Yap1 transcription factor, where Prx's act as peroxide sensors and activators. Cys residues react relatively slowly with ROS, but reactivity can be significantly enhanced by their ionization state, which is dependent on the local protein environment. This is emphasized by the finding that H_2O_2 stress in yeast causes protein-specific oxidation rather than general nonspecific oxidation of protein-SH groups (Le Moan *et al.* 2006).

All organisms contain complex regulatory machinery to maintain their redox homeostasis, primarily regulated by the GSH/glutaredoxin and thioredoxin systems. These systems are thermodynamically linked, since each uses NADPH as a source of reducing equivalents (Holmgren 1989). Extensive overlaps between the thioredoxin and GSH/glutaredoxin systems have been identified in yeast. For example, *GLR1* was identified in a genetic screen for mutations that confer a requirement for thioredoxins, providing the first *in vivo* evidence that thioredoxins and the GSH system have an overlapping redox function (Muller 1996). Subsequently, loss of *TRX1* and *TRX2* was shown to cause compensatory changes in glutathione levels and redox state confirming the tight regulatory link between the thioredoxin system and glutathione (Muller 1996; Garrido and Grant 2002). Extensive deletion analyses in yeast have further identified considerable genetic redundancy in these systems (Trotter and Grant 2003; Toledano *et al.* 2007; López-Mirabal and Winther 2008). The thioredoxin system has also been shown to function as an alternative reduction system for GSSG, although the physiological significance of this reduction reaction is not yet clear (Tan *et al.* 2010). The reason(s) for this apparent functional redundancy in highly conserved redox systems remains an important unanswered question. Extensive biochemical analyses suggest that the thioredoxin system, and not the glutathione system, is the predominant antioxidant system in yeast, since the main peroxidase enzymes including peroxiredoxins (Tsa1, Tsa2, Ahp1, and Dot5) and glutathione peroxidase-like enzymes (Gpx1-3) all appear to depend on the thioredoxin system for reduction (Toledano *et al.* 2007). However, one exception to this rule is now known, since mitochondrial Prx1 reduction requires glutathione (Greetham and Grant 2009).

Glutathione can form mixed disulfides with protein-SH groups (GSSP) in response to ROS exposure. Glutathionylation is a reversible post-translational modification, which is particularly important since it can both protect cysteine residues from irreversible oxidation and can regulate the activity of many target proteins. Eukaryotic glutaredoxins have long been thought to protect against oxidative stress by catalyzing the reduction of protein mixed disulfides with GSH (Cotgreave and Gerdes 1998). GSSP levels in yeast are maintained at low levels, but are increased following exposure to hydrogen peroxide (Grant *et al.* 1998). Additionally, GSSP levels are regulated in parallel with the growth cycle and are maximal during stationary phase growth (Greetham *et al.* 2010). However, unlike most eukaryotes examined to

date, thioredoxins, and not glutaredoxins, appear to be the major deglutathionylases in yeast, providing further evidence that the thioredoxin system and glutathione have extensive overlapping redox functions (Greetham *et al.* 2010). This does however leave open the question as to the function of yeast glutaredoxins. As mentioned previously, Grx1 and Grx2 are active with ribonucleotide reductase, but no other substrates have been confirmed *in vivo*. Glutaredoxins have been shown to reduce arsenate reductase (Acr2) *in vitro*, but it is unclear whether this is a physiological reaction (Mukhopadhyay *et al.* 2000). This is in contrast to plants such as *Arabidopsis*, where for example >100 putative redoxin targets have been identified (Meyer *et al.* 2008). Glutaredoxins are the ultimate electron donors for the glutathione system, but at present we have only a limited knowledge of their physiological targets in yeast. This is not a trivial problem since the identity and strength of any target interactions can change during growth conditions, which alter the cellular redox balance.

Translational regulation of gene expression: Global inhibition of protein synthesis is a common response to stress conditions. However, it is becoming increasingly recognized that differential translational control of specific mRNAs is required for survival during growth under stress conditions (Preiss *et al.* 2003; Smirnova *et al.* 2005). Inhibiting protein synthesis during oxidative stress conditions may prevent continued gene expression during potentially error-prone conditions as well as allow for the turnover of existing mRNAs and proteins while gene expression is reprogrammed to deal with the stress. Yeast cells respond to hydrogen peroxide stress with a rapid and reversible inhibition of protein synthesis with low adaptive concentrations of hydrogen peroxide inhibiting translation by >60% within 15 min (Godon *et al.* 1998; Shenton and Grant 2003; Shenton *et al.* 2006). The level of inhibition is dose dependent and is largely mediated by the Gcn2 protein kinase that phosphorylates the α-subunit of translation initiation factor 2 (eIF2) (Shenton *et al.* 2006). eIF2 is a guanine nucleotide binding factor, which in its GTP-bound form interacts with the initiator methionyl-tRNA (Met-tRNA$_i^{Met}$) to form a ternary complex that is competent for translation initiation. eIF2 is released from the ribosome as a binary complex with GDP, which is removed and replaced by GTP in a guanine-nucleotide exchange reaction catalyzed by eIF2B. Met-tRNA$_i^{Met}$ can only bind the eIF2/GTP complex, so translational control can be regulated by the activity of eIF2B. In yeast and mammalian cells, this is achieved by phosphorylation of eIF2α at a conserved serine (Ser51) residue (Pavitt *et al.* 1998; Harding *et al.* 2000). Phosphorylation converts eIF2 from a substrate to a competitive inhibitor of eIF2B and the resulting decrease in eIF2B activity leads to reduced ternary complex levels, which inhibits translation initiation (Figure 9) (Pavitt *et al.* 1998).

Gcn2 was first characterized for its activation in response to amino acid starvation (Hinnebusch 2005). Depletion of amino acids leads to an accumulation of uncharged tRNA, which activates Gcn2 via its HisRS-related domain. It is now known that Gcn2 can be activated in response to a variety of conditions including nutrient starvation (amino acids, purines, glucose) and exposure to sodium chloride, rapamycin, ethanol, and volatile anesthetics (Hinnebusch 2005; Palmer *et al.* 2005). It is likely that these other stress conditions also ultimately affect the levels of uncharged tRNA in the cell. For example, volatile anesthetics inhibit amino acid uptake (Palmer *et al.* 2005) and Gcn2 is activated by glucose starvation partly through an effect on vacuolar amino acid pools (Yang *et al.* 2000). Phosphorylation of eIF2α appears to be a general response to ROS, since Gcn2 can be activated by exposure to an organic hydroperoxide (cumene hydroperoxide), a thiol oxidant (diamide), and a heavy metal (cadmium) (Mascarenhas *et al.* 2008). Oxidative stress conditions appear to activate Gcn2 via an effect on uncharged tRNA levels since mutations in the HisRS-like domain abolish Gcn2-inhibition (Mascarenhas *et al.* 2008). The pleiotropic nature of oxidative stress means that it is currently not known how ROS cause an amino acid starvation and/or effect cellular uncharged tRNA levels. ROS may conceivably affect the transport and storage of amino acids within cells, similar to the effect of glucose starvation. Additionally, proteins and nucleic acids, which are required for tRNA-aminoacylation, may be susceptible to oxidation resulting in an accumulation of uncharged tRNA and activation of Gcn2.

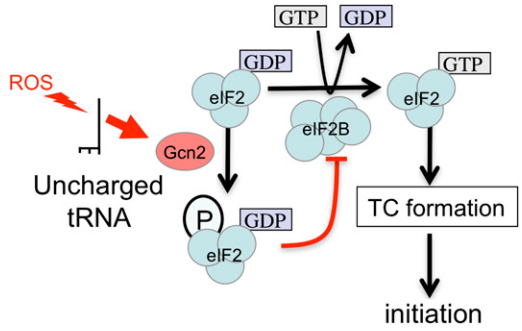

Figure 9 Control of translation initiation by Gcn2. Gcn2 is activated in response to diverse oxidative stress conditions that may occur via an accumulation of uncharged tRNA. Gcn2 phosphorylates eIF2, which converts it into a competitive inhibitor of the eIF2B guanine nucleotide exchange factor. Decreased eIF2B activity generates less eIF2 in the GTP-bound form, resulting in decreased ternary complex levels and inhibition of translation initiation.

It is well established that yeast cells alter global transcription patterns, including genes encoding antioxidants and other metabolic enzymes, in response to ROS (Gasch *et al.* 2000; Causton *et al.* 2001). However, given that oxidative stress results in a rapid and reversible inhibition of protein synthesis, it is unclear how changes in the gene expression program are translated into the cellular proteome. Oxidative stress causes complex changes in the pattern of protein synthesis. Although translation of most mRNAs is inhibited in response to oxidative stress conditions, certain mRNAs remain translationally active or are

strongly downregulated. For example, the *GCN4* mRNA is activated via a mechanism involving short upstream open reading frames (Hinnebusch 2005). Gcn4 is a transcription factor, which activates amino acid biosynthetic genes to overcome the imposed starvation which initially led to its translational control. Not only is Gcn4 upregulated in response to hydrogen peroxide, but it is specifically required for hydroperoxide resistance (Mascarenhas *et al.* 2008). Transcriptional profiling studies have shown that ~10% of the yeast genome is regulated by Gcn4 in response to amino acid starvation, suggesting that Gcn4 acts as a master regulator of gene expression (Natarajan *et al.* 2001). However, Gcn4 is required for a more limited set of genes in response to hydrogen peroxide stress and there is not a strong correlation with the amino acid starvation response at the genome-wide level, suggesting that other factors moderate the transcriptional output of Gcn4 during hydrogen peroxide stress conditions (Mascarenhas *et al.* 2008).

Adaptive concentrations of hydrogen peroxide increase the translation of several antioxidants and stress protective molecules. These include catalase (Ctt1) and glutathione peroxidase (Gpx2), which can reduce H_2O_2 directly, thioredoxin reductase (*TRR1*), which provides the reducing power for the thioredoxin system, and a GST (*GTT2*) and two GS-X pumps (*YCF1* and *YBT1*), which form part of the glutathione conjugation/removal system of cells (Shenton *et al.* 2006). A number of metabolic genes are also up- or downregulated in response to low concentrations of H_2O_2, consistent with significant metabolic reconfiguration occurring during oxidative stress conditions. In contrast to adaptive concentrations of hydrogen peroxide, high lethal concentrations of H_2O_2 do not appear to significantly affect the translation of antioxidants or other stress protective molecules (Shenton *et al.* 2006). Prominent upregulation of genes involved in ribosome biogenesis and rRNA processing was observed, which may indicate a requirement to replace ribosomal proteins and rRNA that become damaged by oxidative stress. In contrast, these genes are generally transcriptionally repressed as part of the ESR (Gasch and Werner-Washburne 2002). It is unclear why genes that are transcriptionally repressed by oxidants may be translationally activated but it may represent a means of "fine tuning" expression levels. Additionally, a significant number of the genes that are translationally downregulated in response high H_2O_2 concentrations are increased at the transcript level. These data indicate that certain genes are increased at the transcriptional level in response to H_2O_2, but remain poorly translated. Increasing transcript levels in the absence of active translation may provide a source of mRNAs that can become rapidly translated once the stress is removed (Shenton *et al.* 2006).

Oxidative stress must regulate translation via additional Gcn2-independent mechanism(s), since protein synthesis is still significantly inhibited in a *gcn2* mutant (Shenton *et al.* 2006). H_2O_2 causes a postinitiation inhibition of translation, increasing the average ribosomal transit time on mRNAs (Shenton *et al.* 2006). Regulation of mRNA expression via translation elongation is clearly a critical component of protein synthesis, but is relatively poorly understood. Oxidative stress in mammalian cells increases elongation factor 2 (eEF2) phosphorylation and oxidative modification, which is thought to contribute to translation inhibition (Patel *et al.* 2002). Similarly, the yeast mitogen-activated protein kinase, Rck2, which phosphorylates eEF2, may affect elongating ribosomes during stress (Swaminathan *et al.* 2006). Exposure to oxidative (*tert*-butyl hydroperoxide) or osmotic stress was found to cause a pronounced dissociation of polysomes in an *rck2*Δ mutant. Microarray analysis indicated that a number of weakly transcribed mRNAs associate more avidly with polysomes during stress conditions, consistent with a role for Rck2 in mRNA-polysome association.

While it is now becoming clearer that ribosome association and translational control of individual mRNAs can be highly regulated, much still remains to be investigated regarding alternative fates of mRNAs during oxidative stress conditions. For example, mRNA decay dynamics are significantly altered in response to oxidative stress conditions, which may contribute to downregulation of protein synthesis (Molina-Navarro *et al.* 2008). This may be explained by the targeting of mRNAs to stress granules, which are thought to act as storage factors that sort mRNAs for degradation or later translation following the relief of stress conditions (Teixeira *et al.* 2005). Storage or degradation of mRNAs in P bodies and stress granules may play an important role in regulating gene expression during oxidative stress conditions since it has been shown that the number of P bodies is significantly increased following UV and hydrogen peroxide stress (Teixeira *et al.* 2005; Mazzoni *et al.* 2007).

Metabolic reconfiguration is a rapid, regulated response to oxidative stress: It is well established that yeast cells adapt to oxidative stress conditions by altering global gene expression patterns, including transcription and translation of genes encoding antioxidants and other stress-protective defenses. However, it is now becoming increasingly recognized that post-translational changes are key regulators of stress responses. In fact, metabolic changes are detected within seconds of an oxidative stress, before slower (within minutes) changes in gene expression are measured (Chechik *et al.* 2008; Ralser *et al.* 2009). Key to these metabolic changes appears to be the reprogramming of carbohydrate metabolism, which is essential to maintain the redox balance of the cell during oxidative conditions. In particular, dynamic rerouting of the metabolic flux from glycolysis to the pentose phosphate pathway, with the concomitant generation of NADPH, appears to be a conserved response to oxidative stress (Ralser *et al.* 2007).

The pentose phosphate pathway is the source of cellular reducing power in the form of NADPH. NADPH is particularly important during exposure to oxidants, since it provides the reducing potential for most antioxidant and redox regulatory enzymes including the GSH/glutaredoxin

and thioredoxin systems. Glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) catalyze the first two steps of the pentose phosphate pathway and are the source of NADPH. G6PDH catalyzes the key NADPH-production step and is known to play a role in protection against oxidative stress (Slekar *et al.* 1996). Additionally, G6PDH and 6PGDH enzyme activities are maintained in yeast cells during oxidant exposure, confirming their role in the oxidative stress response (Izawa *et al.* 1998; Shenton and Grant 2003). The pentose phosphate pathway is directly connected to glycolysis via the oxidation of glucose 6-phosphate. Hence, any growth condition that influences glycolytic activity can potentially alter the flux of glucose equivalents through the pentose phosphate pathway leading to the generation of NADPH. This has been shown experimentally, since reducing the activity of glycolytic enzymes such as triosephosphate isomerase (TPI) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) confers resistance against oxidative stress conditions (Ralser *et al.* 2006, 2007). Quantitative metabolomic analysis has directly confirmed that reduced TPI or GAPDH activity redirects glucose equivalents to the pentose phosphate pathway, increasing the concentration of pentose phosphate pathway metabolites and shifting the NADPH/$NADP^+$ ratio to a more reduced state (Ralser *et al.* 2007).

An important question is how glycolytic enzyme activity is regulated during oxidative stress conditions. Increasing evidence suggests that post-translational modification of glycolytic enzymes is a common response to oxidative stress causing rapid and reversible changes in enzyme activity (Biswas *et al.* 2006). For example, GAPDH has frequently been identified as a target of oxidative modification in diverse cellular systems, leading to the suggestion that it may serve a regulatory role as a sensor of oxidative stress conditions (Chuang *et al.* 2005). The yeast Tdh2 and Tdh3 GAPDH isoenzymes share extensive sequence homology (98% similarity, 96% identity) but appear to play distinct roles in regulating glycolytic flux in response to oxidative stress conditions (Grant *et al.* 1999). The Tdh3 isoenzyme, but not the Tdh2 isoenzyme, is modified by glutathionylation following exposure to hydrogen peroxide. Glutathionylation modifies the active site cysteine residues of Tdh3 and correlates with an inhibition of GAPDH activity (Grant *et al.* 1999). Several glycolytic enzymes contain oxidized cysteine residues during normal growth conditions but only Tdh3 shows an increase in oxidation in response to hydrogen peroxide stress (Le Moan *et al.* 2006). Oxidation of Tdh3 appears to be a very rapid response, which can be detected within 1 min, before changes in gene expression occur. Irreversible oxidative damage may also inhibit glycolytic enzyme activity during oxidative stress conditions. Protein carbonyl formation is frequently used as a measure of protein oxidative damage and glycolytic enzymes including GAPDH, enolase, and TPI have been shown to be carbonylated in response to oxidative stress (Costa *et al.* 2002; Shanmuganathan *et al.* 2004). Additionally, the synthesis of a number of glycolytic enzymes is repressed following H_2O_2 exposure (Godon *et al.* 1998). Yeast cells therefore respond to an oxidative stress by inhibiting the activity of glycolytic enzymes and by switching off gene expression to prevent synthesis of any new enzymes. Combining regulation at the levels of protein modification and gene expression provides a rapid means of reversibly inhibiting the flux through glycolysis. Additionally, irreversible oxidative damage to glycolytic enzymes may add a further level of control.

Control of the cell division cycle vs. apoptotic cell death following oxidative stress: Yeast cells can respond to ROS exposure by delaying progression through the cell division cycle. This may enable them to repair any macromolecular damage without passing it on to their daughter cells (Shackelford *et al.* 2000). For example, hydrogen peroxide causes a RAD9-dependent G2 cell cycle arrest by activating the Rad53 checkpoint (Flattery-O'Brien and Dawes 1998). The nature of the cell cycle delay is specific to the particular ROS, since the superoxide anion was found to cause a G1 arrest independent of Rad9 function (Nunes and Siede 1996; Flattery-O'Brien and Dawes 1998). The superoxide-induced G1 arrest appears to be mediated via an inhibition of transcription of the *CLN1* and *CLN2* G1 cyclins (Lee *et al.* 1996). Similarly, LoaOOH exposure causes a G1 cell cycle delay, which is mediated by the Swi6 transcription factor (Alic *et al.* 2001; Fong *et al.* 2008). Swi6 is thought to act as a redox sensor that suppresses the expression of G1 cyclins in response to oxidation of a specific cysteine residue in Swi6 (Chiu *et al.* 2011). Swi6 therefore provides an example of an oxidant-specific mechanism for controlling the cell cycle. However, it is not yet clear whether other oxidant control mechanisms are generally unique to oxidative damage or whether they are more generally responses to DNA damage initiated by ROS exposure.

ROS exposure and the resulting oxidative stress can cause a form of programmed cell death (PCD) in yeast (Madeo *et al.* 2002). Yeast apoptosis was triggered in response to hydrogen peroxide exposure or depletion of GSH in *gsh1* mutants leading to the hypothesis that ROS generation is a key component of the apoptotic pathway (Madeo *et al.* 1999). Subsequently, numerous stress conditions have been shown to promote yeast apoptosis, many of which correlate with the production of ROS (Pereira *et al.* 2008; Perrone *et al.* 2008). For many of these stress conditions, it is not clear whether ROS production is the factor that triggers the apoptotic response or whether ROS generation is a byproduct of the cell death pathway. Clear examples are provided by mutants in *CDC48*, which normally functions in retrotranslocation of ubiquitinated proteins from the ER into the cytosol for degradation by the proteasome and in yeast expressing the mammalian proapoptotic protein Bax. Apoptosis has been shown to involve an accumulation of ROS in both cases (Madeo *et al.* 1999). Importantly, ROS scavengers and anaerobic growth conditions were found to suppress PCD, confirming that at least under

these conditions ROS generation is an important intermediate in the apoptotic pathway. Yeast contains a metacaspase (Yca1), which mediates cell death in response to various stimuli (Madeo *et al.* 2002; Khan *et al.* 2005). In the absence of Yca1, apoptosis is suppressed and peroxide exposure damages yeast cells, causing an accumulation of oxidized proteins and activation of proteasome activity (Khan *et al.* 2005). Yca1 is known to induce apoptosis in response to oxidative stress caused by hydrogen peroxide exposure, and *yca1* mutants are resistant to hydrogen peroxide, consistent with apoptosis accounting for cell death during ROS exposure (Madeo *et al.* 2002; Khan *et al.* 2005). Yeast also contain a homolog of the mammalian apoptosis-inducing factor (AIF), which regulates apoptosis in yeast (Wissing *et al.* 2004). Aif1 also appears to play a role in ROS-induced apoptosis, since *aif1*Δ mutants are resistant to hydrogen peroxide and overexpression of *AIF1* strongly stimulates peroxide-induced apoptosis.

More work is required to decipher the mechanisms that dictate whether yeast cells undergo a cell cycle division delay *vs.* PCD. This decision may be related to the particular ROS that yeast cells are exposed to as well as to the dosage and exposure time. Delaying the cell division cycle may enable yeast cells to attempt to detoxify and repair ROS-mediated damage, enabling them to overcome and adapt to the stress condition. Apoptosis provides a mechanism for the selective death of a subset of the yeast population, which can be beneficial in a unicellular organism under certain stress conditions (Gourlay *et al.* 2006).

Higher concentrations of oxidants are likely to overwhelm yeast cell antioxidant defenses, causing extensive cellular damage and ultimately necrotic cell death of the population.

Future directions

What is the future of research into the yeast HSR? With genomic and proteomic technologies providing the bird's-eye view of the transcriptional changes and protein–protein interactions, respectively, the volume of information obtained in the last decade has been tremendous. The primary task over the next decade will be to sift through these mountains of data to validate the large-scale results and continue making connections among the various functional nodes identified. As with all biological systems, a laudable but challenging goal is to achieve predictive confidence in the outcomes of experimental manipulations, which requires full understanding of the primary, secondary, tertiary, etc., effects of a stress stimulus. Now that the response is better understood, the focus can be applied to the products of the HSR—the stress proteins—to decipher their precise roles in stress tolerance and adaptation. The driving force behind these investigations will likely remain the parallels between the stressed yeast cell and the pathological human cell. For example, understanding how Hsp90 regulates the activity of a number of clients required for stress survival in yeast should shed light on how the chaperone does the same thing in a tumor. Moreover, pharmacologic and genetic manipulation in the former scenario will provide insight that will support therapeutic intervention in the latter.

As described above for the study of heat stress resistance, our understanding of the basic components and processes involved in supporting tolerance to oxidative stress has expanded greatly in recent years. However, important questions remain. Clear data have been provided demonstrating that expression of a variety of genes is strongly induced upon stress with oxidants but the precise nature of the signal(s) that triggers this response remains elusive. Global analyses have assembled a parts list of proteins and genes involved in oxidative stress resistance, but reconstructing these individual constituents into their cellular context is a central challenge. A cell experiences oxidative stress as the sum total of the pathways considered above and must integrate their functions into a cogent response. This integration seems likely to be a natural application for systems biology as research moves forward. It is also important that future research examines yeast cells under different growth conditions. Most studies to date have examined cells grown under fermentative conditions using glucose as a carbon source. These studied should be extended to include respiratory growth conditions, which would be expected to generate intracellular ROS via the mitochondrial electron transport chain. Respiratory growth conditions are also particularly relevant to mammalian cell growth. Additionally, many antioxidant genes are subject to carbon catabolite repression (*e.g.*, *SOD2*) and are induced under respiratory conditions presumably as a response to endogenously generated oxidative stress.

Oxidative stress is often thought of as an externally imposed challenge but every eukaryotic cell contains oxidative environments such as the endoplasmic reticulum and mitochondria within its cytoplasm. Details underlying the communication between and coordination of the oxidative contents of these organelles and the reductive nature of the rest of the cell are obscure. Key questions remain regarding how cells maintain compartment-specific redox regulation while protecting against oxidative damage to other components of the cell. The discovery of apoptotic machinery in *S. cerevisiae* provides the ability to employ the facile genetic analysis of the organism to explore this fundamental eukaryotic process. The yeast system should provide a model to identify the functional links between ROS and fungal apoptosis. Given the past record of successful investigations in *S. cerevisiae*, we are confident that future studies of oxidative stress tolerance will prove fruitful in expanding the understanding of these and other areas of eukaryotic cell biology.

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