Molecular Cell
Article

Life Span Extension and H_2O_2 Resistance Elicited by Caloric Restriction Require the Peroxiredoxin Tsa1 in *Saccharomyces cerevisiae*

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DOI 10.1016/j.molcel.2011.07.027

SUMMARY

Caloric restriction (CR) extends the life span of organisms ranging from yeast to primates. Here, we show that the thiol-dependent peroxiredoxin Tsa1 and its partner sulfiredoxin, Srx1, are required for CR to extend the replicative life span of yeast cells. Tsa1 becomes hyperoxidized/inactive during aging, and CR mitigates such oxidation by elevating the levels of Srx1, which is required to reduce/reactivate hyperoxidized Tsa1. CR, by lowering cAMP-PKA activity, enhances Gcn2-dependent *SRX1* translation, resulting in increased resistance to H_2O_2 and life span extension. Moreover, an extra copy of the *SRX1* gene is sufficient to extend the life span of cells grown in high glucose concentrations by 20% in a Tsa1-dependent and Sir2-independent manner. The data demonstrate that Tsa1 is required to ensure yeast longevity and that CR extends yeast life span, in part, by counteracting age-induced hyperoxidation of this peroxiredoxin.

INTRODUCTION

Caloric restriction (CR; McCay et al., 1935) is the only known intervention that prolongs life span in as taxonomically diverse organisms as yeast, worms, flies, fish, and mammals. While the mechanism(s) underlying the effects of CR on life span is still obscure, in many organisms CR is associated with reduced signaling through nutrient-responsive pathways leading to a global reprogramming away from growth and reproduction toward maintenance (Kenyon, 2010). CR-induced life span extension has, for example, been linked to decreased activity in (1) the nutrient-responsive target-of-rapamycin (TOR) pathway, (2) insulin/insulin-growth factor-like (IGF-1) signaling (Kenyon, 2010), and (3) the cAMP-dependent protein kinase A (PKA) pathway (Enns et al., 2009; Lin et al., 2000).

Apart from reducing signaling through nutrient-responsive pathways, many organisms studied respond to CR by decreasing the production of reactive oxygen species (ROS) (Sohal and Weindruch, 1996), increasing the resistance to oxidative insults, and reducing oxidative damage (Levine, 2002). For example, the beneficial effects of CR on brain aging are suggested to result from a cellular stress response enhancing resistance to oxidative insults (Fontan-Lozano et al., 2008). Similarly, the antiaging effects of the sirtuin Sirt1, one proposed mediator of CR in mammals, have been linked to oxidative stress resistance (Hsu et al., 2008).

In yeast, life span extension by CR commonly involves reducing the glucose concentration from 2% to 0.5% (or lower) (Kaeberlein, 2010). A phenocopy of CR can be achieved by genetic interventions, for example by reducing signaling through the cAMP-PKA pathway (Kaeberlein, 2010; Lin et al., 2000). Whereas CR is intimately associated with oxidative stress resistance in mammals, oxidative stress resistance has been suggested to be unaffected by CR, and genetic CR mimetics, in yeast (Lin et al., 2002). Instead, CR, by increasing respiration and the NAD^+/NADH ratio, was proposed to activate the NAD-dependent histone deacetylase Sir2 reducing the production of toxic rDNA circles (Lin et al., 2000; Lin et al., 2002). However, CR has been reported to increase the life span of respiratory-deficient as well as Sir2-deficient cells in which the number of toxic rDNA circles is kept low by a *FOB1* deletion (Kaeberlein et al., 2005a; Kaeberlein et al., 2004). In addition, different views have been presented on how reduced TOR signaling increases replicative life span in response to CR. One model posits that reduced TOR activity increases the degradation of the Sir2 inhibitor nicotinamide through activating the transcription factors Msn2/Msn4 and their target *PNC1*, thus boosting Sir2 activity (Medvedik et al., 2007). Another model points to a TOR-dependent decreased production of ribosomal 60S leading to activation of the Gcn4 transcription factor, which was reported to be partially required for life span extension by CR (Steffen et al., 2008). Thus, the mechanisms by which CR extends life span through the cAMP-PKA and TOR pathways are not fully understood, nor have the key downstream targets regulating life span been identified. Moreover, it may be difficult to find such targets, since it has been argued that life span extension by CR depends on the combined activity of many gene products responding to CR (Kenyon, 2010).

In view of the fact that bacteria, filamentous fungi, flies, worms, fish, and mammals respond to CR by elevating their resistance to oxidants, it is somewhat surprising that budding yeast appears

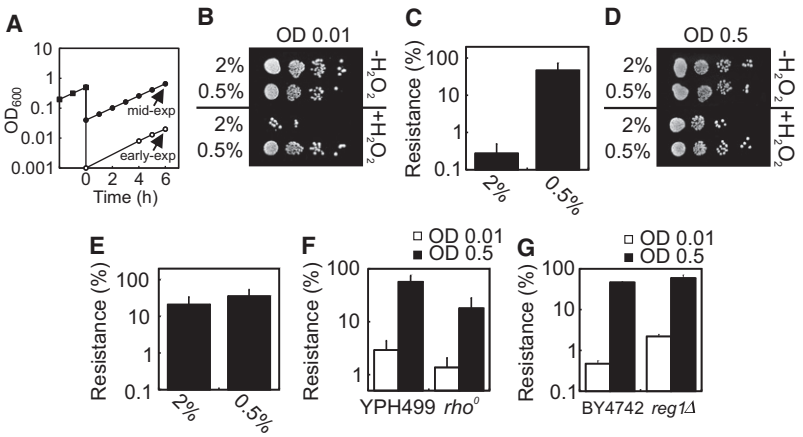

Figure 1. CR Elicits Increased H_2O_2 Resistance via a Respiration-Independent Mechanism at Low Cell Densities

(A) Cultures of strain YPH98 were grown exponentially in 2% or 0.5% glucose to different cell densities. H_2O_2 resistance was tested when the diluted cultures had reached an OD_{600} of 0.5 or 0.01.

(B and C) H_2O_2 resistance of early exponential phase cells (OD_{600} = 0.01) grown in 2% or 0.5% glucose as indicated. Resistance (1.75 mM) was quantified (n = 3, average values ± SD) as described in the Supplemental Experimental Procedures.

(D and E) H_2O_2 resistance of midexponential phase cells (OD_{600} = 0.5) grown in 2% or 0.5% glucose as in (B) and (C) (n = 2).

(F) H_2O_2 resistance of the YPH499 wild-type strain and an isogenic strain lacking mitochondrial DNA (*rho*^{0}) grown to early (OD_{600} = 0.01) and midexponential (OD_{600} = 0.5) phase in synthetic glucose medium as in (B) and (C) (n = 3). Cells were spotted on YPD plates with or without 2 mM H_2O_2.

(G) H_2O_2 resistance of the BY4742 wild-type strain and an isogenic *reg1Δ* strain grown to early (OD_{600} = 0.01) or midexponential (OD_{600} = 0.5) phase in synthetic glucose medium as in (B) and (C) (n = 2). Cells were spotted on YPD plates with and without 1.25 mM H_2O_2.

to lack such a response. Here, we demonstrate that such a correlation exists but has been missed previously because yeast cells cultivated in high glucose (2%) liquid medium display high cAMP-PKA activity during exponential growth only at low optical densities. Taking this into consideration, we demonstrate that (1) CR provokes a robust resistance to H_2O_2; (2) this effect of CR is mediated by a reduction of cAMP-PKA, but not TOR, activity; (3) the thiol-dependent peroxiredoxin Tsa1 is required for development of the cAMP-PKA-dependent H_2O_2 resistance; (4) low PKA activity stimulates Tsa1 peroxidase activity by enhancing translation of the *SRX1* transcript, encoding the sulfiredoxin Srx1 that reactivates hyperoxidized (sulfinylated) Tsa1; (5) Tsa1 becomes hyperoxidized and inactivated as cells age; (6) Srx1 overexpression mitigates such age-dependent hyperoxidation of Tsa1 and prolongs the replicative life span of cells; and (7) both Srx1 and Tsa1 are necessary for CR to elicit a full life span extension.

RESULTS

CR Induces H_2O_2 Resistance by a Respiration-Independent Mechanism

The effect of CR on H_2O_2 resistance was tested by growing cells in either 2% or 0.5% glucose. Resistance was scored at different optical (cell) densities, demonstrating that CR cells (0.5% glucose) were more resistant than cells grown at 2% glucose in samples taken at low optical densities (e.g., OD_{600} = 0.01, ~2 × 10^5 cells/ml; Figures 1A–1C). However, there was no effect of CR when the culture had reached an OD_{600} = 0.5 (~1 × 10^7 cells/ml; Figures 1A, 1D, and 1E). In other words, at this cell density, the cells were H_2O_2 resistant in both 2% and 0.5% glucose. Thus, there is metabolic/regulatory shift in cells cultivated in 2% glucose occurring when the cells reach midexponential phase (between an OD_{600} of 0.25 and 0.5; see Figures S1A–S1C available online), leading to the development of H_2O_2 resistance.

Since cells cultivated in 2% glucose have been reported to shift from predominantly fermentative growth to respiratory growth upon reaching higher cell densities (Zaman et al., 2008), we tested if such alterations in respiratory activity might be underlying the development of H_2O_2 resistance. However, the rate of oxygen consumption was not altered during the development of H_2O_2 resistance in cells grown to an optical density of 0.5 (Table S1). In addition, a mutant (*rho*^{0}) lacking mitochondrial DNA and respiratory activity (Table S1) still developed H_2O_2 resistance upon reaching an OD_{600} of 0.5 (Figure 1F), as did a *reg1Δ* mutant (Figure 1G), which displays constitutively elevated respiratory activity regardless of cell density (Table S1). To elucidate if the development of H_2O_2 resistance might be due to reduced H_2O_2 permeability at higher cell densities, we tested the H_2O_2 sensitivity of *erg6Δ* mutant cells, which exhibit constitutively increased H_2O_2 permeability due to a deficiency in ergosterol biosynthesis (Branco et al., 2004). The *erg6*Δ mutation did not affect the acquisition of H_2O_2 resistance in midexponential phase cells (Figure S1D), suggesting that altered H_2O_2 permeability is not the cause of H_2O_2 resistance at high cell densities.

Reduction in cAMP-PKA Signaling Elicits Elevated H_2O_2 Tolerance

We next approached the question of which signaling pathway is involved in the development of H_2O_2 resistance upon growth to midexponential phase and whether the same pathway is involved in CR-induced H_2O_2 tolerance. Since CR is known to reduce cAMP-PKA (protein kinase A) activity (Lin et al., 2000; Zaman et al., 2008; Figure 2A) and both cAMP-PKA and TOR signaling affect stress resistance and CR-induced life span extension (Kaeberlein et al., 2005b; Lin et al., 2000), we tested a series of mutants altered in these two signaling pathways. As seen in Figure 2B, constitutive high cAMP-PKA activity achieved by deleting *PDE2*, encoding a high-affinity cAMP phosphodiesterase, prevented any significant increase ($p = 0.25$) in the H_2O_2 resistance in midexponential phase. Reciprocally, reducing cAMP-PKA signaling (as demonstrated in Figures S2A–S2D) either by overexpressing *PDE2* (Figure 2C), deleting the activator of adenylate cyclase, *RAS2* (Figure 2B), or expressing single hypomorphic PKA catalytic subunit alleles (Figure 2D;

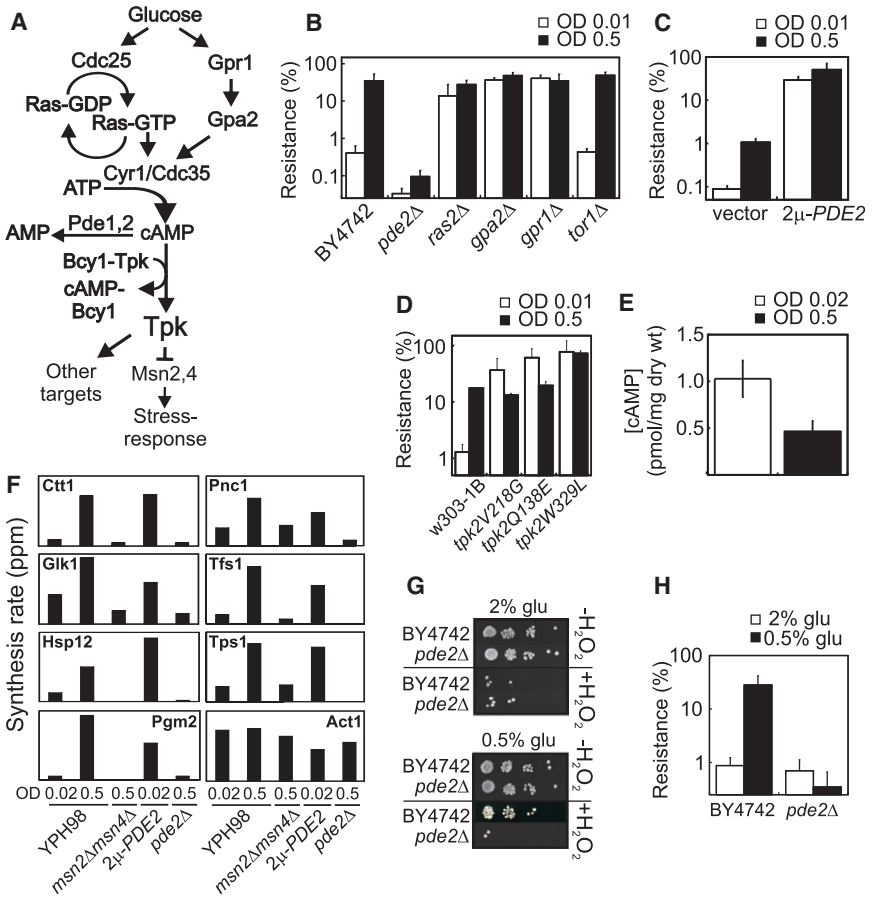

Figure 2. H_2O_2 Resistance Induced by CR and Growth to Midexponential Phase Requires Reduced PKA Activity

(A) Overview of the PKA signaling pathway (adapted from Zaman et al., 2008).

(B) H_2O_2 resistance of the BY4742 wild-type strain and the indicated isogenic deletion strains grown to early and midexponential phase in synthetic glucose medium as in Figures 1B and 1C (n = 2–4). Cells were spotted on YPD plates with or without 1.5 mM H_2O_2.

(C) H_2O_2 resistance of the YPH98 wild-type strain carrying the yEP352 vector or overexpressing *PDE2* (2μ-*PDE2*, plasmid pGR103) as in Figures 1B and 1C (n = 2). Cells were grown to early or midexponential phase in selective synthetic glucose medium before being spotted onto YPD plates with or without 2 mM H_2O_2.

(D) H_2O_2 resistance of the w303-1B wild-type strain and the *tpk1Δtpk3Δbcy1Δ* deletion strains with the indicated hypomorphic *tpk2* alleles as in Figures 1B and 1C (n = 2). Cells were grown to early and midexponential phase in synthetic glucose medium before being spotted onto YPD plates with or without 1 mM H_2O_2.

(E) cAMP levels in BY4742 wild-type cells grown to early and midexponential phase in synthetic glucose medium (n = 4, average values ± SEM).

(F) Rates of synthesis of selected proteins in the YPH98 wild-type strain and the indicated mutant strains. Cells were grown in synthetic glucose medium to early (OD_{600} 0.02) or midexponential (OD_{600} 0.5) phase, pulse-labeled with ^{35}S-methionine for 20 min, and protein synthesis rates analyzed by 2D-PAGE (see also Figure S2). The bars represent radioactivity in the indicated protein spots normalized to the total radioactivity in the gels.

(G and H) H_2O_2 resistance of the BY4742 wild-type and the *pde2Δ* strain in early exponential phase (OD_{600} 0.01) as in Figures 1B and 1C (n = 2–3). Cells were grown in YP medium supplemented with the indicated glucose concentrations and spotted on YPD plates with or without 1.4 mM H_2O_2.

tpk2V218G, *tpk2Q138E*, and *tpk2W329L*; Zaman et al., 2008) resulted in H_2O_2 resistance already at low OD without subjecting the cells to CR. Similarly, deleting either *GPR1* or *GPA2*, encoding the G protein glucose receptor Gpr1 and its G protein α subunit Gpa2 (Figure 2A), respectively, made cells constitutively H_2O_2 resistant (Figure 2B). In contrast, reducing TOR signaling, either by deleting *TOR1* (Figure 2B), treating cells with rapamycin to inhibit TORC1 (Figure S2E), or genetically reducing TORC2 activity (*tor2-21* allele, Figure S2F), did not appreciably affect the gain of H_2O_2 resistance. Thus, we conclude that a reduction in cAMP-PKA, rather than TOR, activity is required for cell density-dependent acquisition of H_2O_2 resistance. In line with this notion, we found that cAMP levels were reduced as cells reached midexponential phase (Figure 2E), and the rates of synthesis of several Msn2/Msn4 targets were significantly higher in midexponential phase compared to early exponential phase cells (Figure 2F). The activities of the Msn2/Msn4 transcription factors are negatively regulated by cAMP-PKA (Figures S2A–S2D), and increased expression of their target genes indicates reduced cAMP-PKA activity. Confirming this, the elevated expression of the Msn2/4-target genes at an OD_{600} of 0.5 was counteracted by deleting *PDE2* (Figure 2F). Conversely, the expression of Msn2/4 target genes could be elevated already at low OD by overexpressing *PDE2* (Figure 2F) or by expressing a single hypomorphic PKA catalytic subunit (*tpk2W329L* allele, Figures S2C and S2D).

To examine if CR, like growth to midexponential phase, also required reduced cAMP signaling to elicit H_2O_2 resistance, we tested the resistance of *pde2Δ* mutant cells grown in 2% and 0.5% glucose. Indeed, as seen in Figures 2G and 2H, CR failed to elicit H_2O_2 resistance in the *pde2Δ* mutant. Thus, the likely candidate genes for H_2O_2 resistance elicited by both CR and growth to midexponential phase are under negative control by cAMP-PKA. However, it appears that these candidates do not belong to the Msn2/4 regulon per se, since simultaneous inactivation of Msn2 and Msn4 only weakly affected the development of H_2O_2 resistance in midexponential phase (Figure S2G).

Tsa1 Is Required for Reduced Signaling through cAMP-PKA to Elicit H_2O_2 Resistance

In contrast to Msn2 and Msn4, both the Yap1 and Skn7 transcription factors, which control large, and overlapping, oxidative stress responsive regulons (Lee et al., 1999), were required for the development of full H_2O_2 resistance elicited by low PKA

activity (Figures S2G and S2H). We hypothesized that the key players eliciting H_2O_2 resistance upon a reduction in cAMP-PKA signaling might be identified by looking for differences in the proteome of cells with high and low PKA activity subjected to H_2O_2 exposure. As expected, exposure of cells to H_2O_2 led to increased production of several targets (e.g., Trr1 and Trx2) of the Yap1-Skn7 transcription factors (Figure 3A). However, the induction of some specific Yap1-Skn7 targets (e.g., the peroxiredoxins Tsa1 and Tsa2) was either weak or totally absent in early exponential phase cells (OD_{600} of 0.02; Figure 3A). This failure of early exponential phase cells to induce Tsa1/Tsa2 was completely suppressed by ectopically reducing cAMP-PKA activity through Pde2 overproduction (Figure 3A; 2μ-*PDE2*). Cells displaying high PKA activity (OD 0.02) produced *TSA1* mRNA to the same level as cells displaying low PKA activity (cells at OD 0.5 or cells overproducing Pde2 [2μ-*PDE2*] at 0.02; Figure S3A), indicating that PKA inhibits Tsa1 de novo synthesis posttranscriptionally.

Moreover, we found that Tsa1 formed different isoforms during H_2O_2 exposure depending on glucose levels and the PKA activity in cells. Tsa1 is the major peroxiredoxin of yeast and belongs to the thioredoxin-dependent 2-Cys subgroup of the thiol-based peroxidases, the catalytic cycle of which is outlined in Figure 3B. During each catalytic cycle, a proportion of Tsa1 is inactivated by further oxidation of the peroxidatic cysteine to the sulfinic (Cys–SOOH) or sulfonic acid (Cys–SO_3H) forms (Wood et al., 2003). We found that an acidic isoform of Tsa1 accumulated upon H_2O_2 exposure in cells with high cAMP-PKA activity (either cells at OD_{600} of 0.02 or *pde2Δ* cells at OD_{600} 0.5), but not in cells with low PKA activity (CR cells, mid-exponential phase cells, or early exponential phase cells overproducing Pde2; Figure 3C, Figure S3B). Mass spectrometry confirmed that this acidic isoform was indeed the sulfinylated, hyperoxidized isoform of Tsa1 (Figure 3D, Figures S3C–S3H, Table S2). To further elucidate the role of cAMP-PKA signaling in reducing hyperoxidized, sulfinylated Tsa1, we used 4′acetamido-2,2′-disulphonic acid (AMS) that adds ~0.5 kDa per reduced cysteine (Cys–SH) in proteins but does not alkylate Cys-SOOH groups, to test the efficiency of cells with high and low PKA activity to reduce sulfinylated Tsa1 upon exposure to H_2O_2. As seen in Figure 3E, Tsa1 remained hyperoxidized by sulfinylation in cells with high PKA activity throughout the experiment, whereas cells with low PKA activity had fully reduced all sulfinylated Tsa1 by 60 min. The total Tsa1 levels did not differ between unstressed cells displaying different PKA activities (Figure S3J and S3K).

As both the production and oxidation of Tsa1 upon H_2O_2 exposure were dependent on the status of the cAMP-PKA signaling pathway, we next tested whether Tsa1 was important for the acquisition of H_2O_2 resistance during CR and treatments mimicking CR. Indeed, deletion of *TSA1* abolished H_2O_2 resistance induced by (1) CR (Figures 3F and 3I), (2) growth to mid-exponential phase (Figure S3L), (3) genetically reducing glucose signaling (*gpr1*Δ, *gpa2*Δ; Figure 3G), and (4) genetically reducing PKA activity (Pde2 overproduction; Figure 3H). The peroxidase activity of Tsa1 requires both the catalytic cysteine, C48, and the resolving cysteine, C171 (see Figure 3B). Tsa1 can also act as a chaperone to inhibit protein aggregation, but this function does not require C171 (Jang et al., 2004). We found that cells expressing Tsa1 carrying serine substitutions in either C48 or C171 failed to acquire H_2O_2 resistance in response to CR (Figure 3I), suggesting that it is the Tsa1 peroxidase rather than chaperone activity that is required for H_2O_2 resistance. Consistently, cells lacking either thioredoxin reductase (*trr1Δ*) or both cytosolic thioredoxins (*trx1Δtrx2Δ*), which reduce 2-Cys Prxs including Tsa1, also failed to develop H_2O_2 resistance (Figure S3M). In contrast, deletion of genes encoding the catalases Ctt1 and Cta1 or either of the peroxiredoxins Ahp1 or Tsa2 did not abolish the acquisition of H_2O_2 resistance (Figures S2G, S3O, and S3P). Tsa1 mutant cells were slightly more resistant to H_2O_2 than wild-type cells during conditions generating high PKA activity (Figures 3F–3I). This resistance is probably due to a compensatory response increasing the levels of the peroxiredoxins Ahp1 (Figures S3N and S3P, see also Figures S2A and S2B) and possibly Tsa2 (Iraqui et al., 2009).

The sulfiredoxins (Srx1 in yeast) are enzymes that reduce the hyperoxidized, sulfinic acid form of 2-Cys Prxs (Biteau et al., 2003; Woo et al., 2005). Therefore we tested the effect of deleting *SRX1* and found that this prevented any significant increases in H_2O_2 resistance triggered by CR (Figure 3I) and CR mimetics (Figures S3L and S3Q). Srx1 was also necessary for the reduction of sulfinylated Tsa1 achieved by reducing PKA signaling (Figure S3I). In addition, the decrease in Tsa1 sulfinylation upon diminishing PKA activity was independent of increased Tsa1 de novo synthesis (Figure 3A), since rapamycin strongly reduced Tsa1 synthesis (Figure S3R and S3S) but did not inhibit Tsa1 desulfinylation (Figure S3B).

SRX1 Is Regulated by PKA at the Posttranscriptional Level through the eIF2 Kinase Gcn2

Since H_2O_2 resistance and reduction of hyperoxidized Tsa1 required Srx1 upon reduced PKA signaling, we monitored the effect of altered PKA signaling on changes in Srx1 protein and mRNA levels. In agreement with the extent of Tsa1 sulfinylation in cells with different cAMP-PKA activities (Figure 3C, Figure S3B), cells with a high cAMP-PKA activity (OD_{600} of 0.02 or *pde2Δ* mutant cells) exhibited a reduced ability to accumulate Srx1 upon H_2O_2 exposure, while cells with ectopically reduced cAMP-PKA activity overproduced Srx1 (Pde2 overproduction; Figures 4A–4D). Similarly, CR cells or cells grown to an OD_{600} of 0.5 accumulated Srx1 to higher levels than cells in high glucose at an OD_{600} of 0.02 (Figures S4A and S4B). While cells displaying a high cAMP-PKA activity failed to proficiently elevate Srx1 protein levels upon peroxide stress, Yap1/Skn7-dependent *SRX1* transcription was induced to the same extent as in wild-type cells (Figures 4E and 4F), indicating that the *SRX1* transcripts made under conditions of high PKA activity are not efficiently translated. Indeed, we found that in cells with high PKA activity (*pde2Δ*) only 10% of *SRX1* mRNAs were associated with polysomes following H_2O_2 stress, whereas in cells with low PKA activity (2μ-*PDE2*) 50% or more of these transcripts eluted with the polysome fractions (Figure 4G). Wild-type cells displayed an intermediate polysome association (25%) of *SRX1* mRNAs (Figure S4C). No difference was observed in unstressed cells (Figures S4D–S4F). The control *ACT1* mRNA was largely polysomal regardless of PKA activity (Figure 4G). Lowering

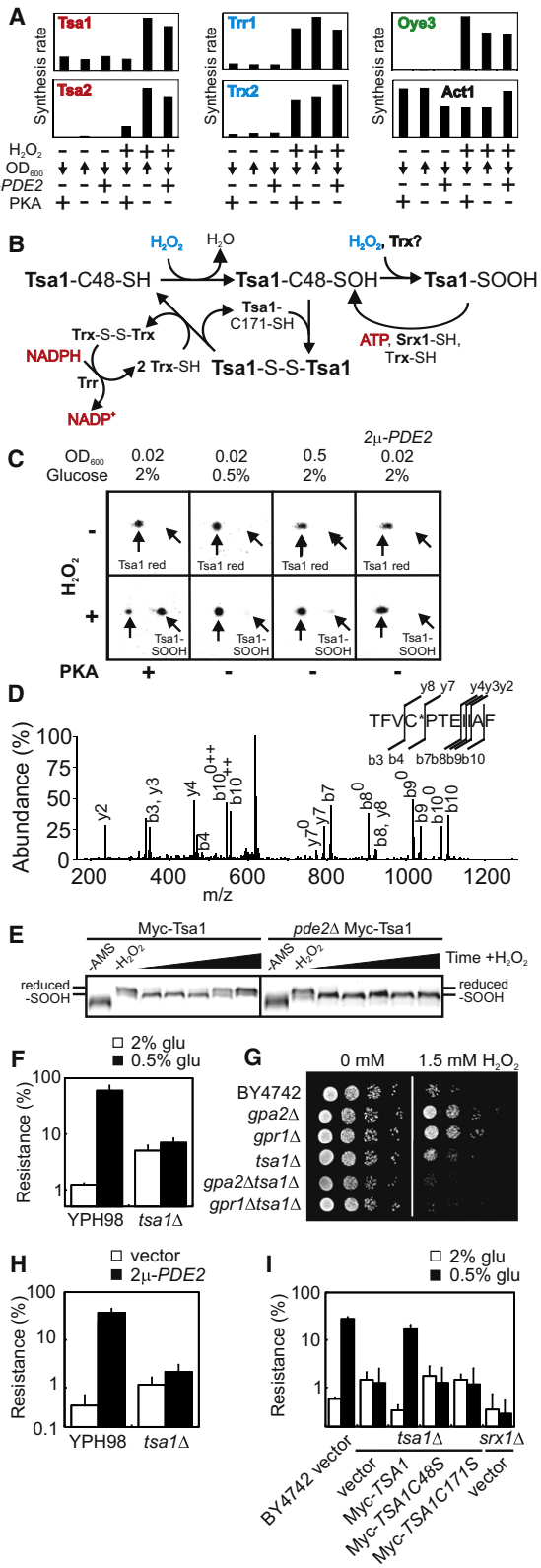

Figure 3. Tsa1 Peroxidase Activity Is Required for Resistance Induced by CR and Low PKA Activity

(A) Rates of synthesis of selected proteins upon H_2O_2 exposure of the YPH98 wild-type strain and an isogenic strain overexpressing *PDE2*. Cells were grown in synthetic glucose medium (for 2μ-*PDE2* [plasmid pGR103] with uracil selection) to early (OD_{600} 0.02, OD_{600} "↓") or midexponential (OD_{600} 0.5, OD_{600} "↑") phase. Midexponential phase cells were diluted to OD_{600} 0.02 before cells were treated with 0.4 mM H_2O_2 for 20 min and pulse-labeled with ^{35}S-methionine for 20 min. Proteins extracted were separated by 2D-PAGE. The bars represent radioactivity in specific proteins normalized to total radioactivity in gels. Examples of shared Yap1/Skn7 targets (Lee et al., 1999) that were repressed (protein name in red) or unaffected (protein name in blue) by high PKA activity are indicated. Oye3, a target of Yap1 only, is indicated in green. The vector (yEP352) did not influence the protein synthesis rates presented or Tsa1 sulfinylation (see C, data not shown). "PKA +" denotes high PKA activity (wild-type strain grown in 2% glucose and harvested at an OD_{600} of 0.02) and "PKA –" low PKA activity achieved either by growth to an OD_{600} of 0.5 or overproduction of Pde2 (2μ-*PDE2*).

(B) Overview of catalysis in typical 2-Cys peroxiredoxins. H_2O_2 is reduced by the peroxidatic cysteine that is oxidized to a sulfenic acid intermediate (Tsa1-C48-SOH in Tsa1). The nascent R-SOH then condenses with the resolving cysteine (Tsa1-C171-SH in Tsa1) of another peroxiredoxin molecule to produce a disulfide-linked dimer, subsequently reduced by thioredoxin (Trx), thus completing the catalytic cycle. A small proportion of Cys-SOH molecules are further oxidized to the sulfinic acid form (Cys–SOOH) in each catalytic cycle, which inactivates peroxidase activity (Wood et al., 2003). Sulfiredoxins (Srx1 in *S. cerevisiae*) reduce the sulfinylated forms of 2-Cys Prxs (Biteau et al., 2003; Woo et al., 2005). Trr, thioredoxin reductase.

(C) Sulfinylation of Tsa1 upon H_2O_2 exposure (0.4 mM) of cells displaying different PKA activities. The YPH98 wild-type strain was grown in either 2% or 0.5% glucose to different cell densities (OD_{600} of 0.02 or 0.5) generating different PKA activities ("+" or "−") as indicated. Cells at an OD_{600} of 0.5 were diluted to an OD_{600} of 0.02 before H_2O_2 was added. In addition, overexpression of *PDE2* was used to counteract the high PKA activity in cells grown in 2% glucose to an OD_{600} 0.02. Proteins were subjected to 2D-PAGE analysis, and total protein was detected by Coomassie staining. Reduced Tsa1 and hyperoxidized Tsa1 (Tsa1-SOOH) are indicated.

(D) Identification of the acidic Tsa1 isoform as Tsa1-Cys48-SOOH. An annotated MS/MS spectrum for the fragmentation of a peptide spanning the sulfinylated catalytic cysteine (Cys48-SOOH) obtained by LC-MS/MS analysis is shown. For more details, see Figures S3C–S3H and Table S2.

(E) Effect of high PKA activity on the reduction of hyperoxidized Tsa1 after H_2O_2 exposure using immunoblot analysis of Myc-Tsa1. Cells of strains YPH98 *tsa1*Δ or YMM103 (*pde2*Δ*tsa1*Δ) transformed with the plasmid pRS316-Myc-*TSA1* were grown to midexponential phase (OD_{600} 0.5) in selective synthetic glucose medium and left untreated (−AMS and $-H_2O_2$ lanes) or treated with 0.4 mM H_2O_2 for 5, 10, 20, 40, or 60 min (Time $+H_2O_2$ lanes). Proteins extracted from samples were first reacted with dithiotreitol (DTT) and subsequently alkylated either using N-ethylmaleimide (−AMS) or AMS adding an extra ~0.5 kDa per Cys-SH before SDS-PAGE analysis.

(F) H_2O_2 resistance of the YPH98 wild-type strain and the isogenic *tsa1Δ* strain grown to early exponential phase (OD_{600} 0.01) in YP medium supplemented with the indicated amounts of glucose as in Figures 1B and 1C (n = 2). Cells were spotted on YPD plates with or without 1.75 mM H_2O_2.

(G) H_2O_2 resistance of the BY4742 wild-type strain and the indicated mutants grown to early exponential phase (OD_{600} 0.01) in YPD medium as in Figures 1B and 1C (n = 2). Cells were spotted on YPD plates ±1.5 mM H_2O_2.

(H) H_2O_2 resistance of the YPH98 wild-type strain and the *tsa1Δ* strain carrying the yEP352 vector or overexpressing *PDE2* (pGR103 plasmid) as in Figures 1B and 1C (n = 2). Cells were grown to early exponential phase (OD_{600} 0.01) in selective synthetic glucose medium before being spotted on YPD plates ±1.5 mM H_2O_2.

(I) H_2O_2 resistance of the BY4742 wild-type strain, *tsa1Δ*, and *srx1Δ* cells carrying the pRS316 vector or expressing the indicated Myc-*TSA1* alleles as in Figures 1B and 1C (n = 2). Cells were grown in YP medium supplemented with the indicated glucose amounts to early exponential phase (OD_{600} 0.01) before being spotted on YPD plates with or without 1.2 mM H_2O_2.

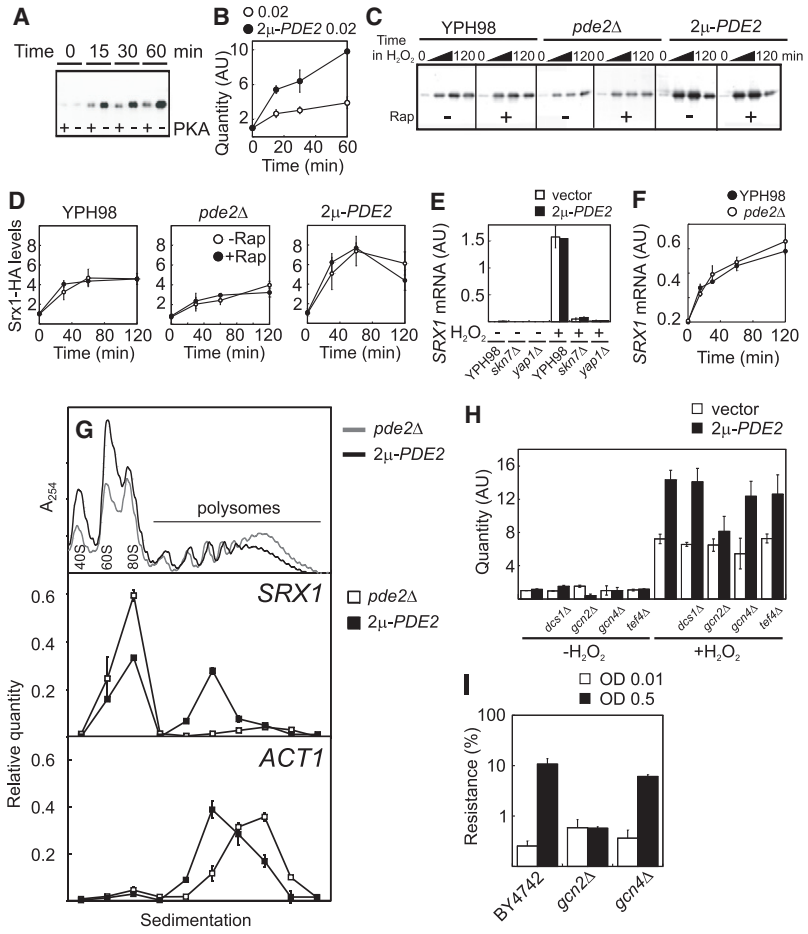

Figure 4. Translation of the *SRX1* Transcript Following H_2O_2 Exposure Requires Reduced PKA Activity and the eIF2 Kinase Gcn2

(A and B) Srx1 levels after H_2O_2 exposure in cells with high or low PKA activity. Srx1-HA levels were analyzed using immunoblotting of YPH98 *srx1Δ* cells transformed with pRS315-*SRX1-HA* and carrying either the vector (yEP352, "PKA +") or overexpressing *PDE2* (2μ-*PDE2*, plasmid pGR103, "PKA −") (n = 2, average values ± SEM). Cells were grown in selective synthetic glucose medium to early exponential phase (OD_{600} 0.02) and exposed to 0.4 mM H_2O_2.

(C and D) Srx1 levels after H_2O_2 exposure in cells with different PKA and TOR activity. Srx1-HA levels were determined as in (A) and (B). The YPH98 wild-type and the indicated mutant strains were grown in selective synthetic glucose medium to exponential phase (OD_{600} 0.25). Cultures were treated with 220 nM rapamycin (+Rap) or drug vehicle (−Rap) for 2 hr, after which cells were treated with 0.8 mM H_2O_2. Samples for protein analysis were withdrawn at the indicated time points.

(E) Quantitative RT-PCR analysis of *SRX1* transcript levels in the YPH98 wild-type strain and indicated deletion mutants with either the yEP352 vector (white bars) or overexpressing *PDE2* (black bars, pGR103 plasmid). Cells were grown in selective synthetic glucose medium to early exponential phase (OD_{600} 0.02) and left untreated or stressed with 0.4 mM H_2O_2 for 30 min. Values presented were normalized to the values of *ACT1* (n = 2, average values ± SD).

(F) Quantitative RT-PCR analysis of *SRX1* transcript levels in the YPH98 wild-type and *pde2Δ* strain grown to mid-exponential phase (OD_{600} 0.5) and stressed with 0.8 mM H_2O_2. Values were normalized to the values of *ACT1* (n = 2, average values ± SD).

(G) Polysome analysis of extracts from strains with high PKA (*pde2Δ* with the yEP352 vector) and low PKA (YPH98 with pGR103 [2μ-*PDE2*]) activity grown to midexponential phase (OD_{600} 0.5) and treated with 0.8 mM H_2O_2 for 15 min. (Top panel) Polysome UV profile recorded during extract elution following ultracentrifuge separation in 10%–50% sucrose gradients. (Bottom two panels) Quantitative RT-PCR determination of *SRX1* or *ACT1* mRNA in the sucrose gradient fractions collected (n = 2, average values ± SD).

(H) Effect of reduced PKA activity on Srx1-HA levels in H_2O_2-stressed strains deficient for the indicated translational components. Strains yMM105 (*srx1Δ*), yMM109 (*dcs1Δsrx1Δ*), yMM110 (*gcn2Δsrx1Δ*), yMM111 (*gcn4Δsrx1Δ*), or yMM112 (*tef4Δsrx1Δ*) transformed with the plasmids pRS315-*SRX1-HA* and either yEP352 (vector) or pGR103 (2μ-*PDE2*) were grown to early exponential phase (OD_{600} 0.02). Protein samples were withdrawn 20 min following the addition of H_2O_2 (0.6 mM). Quantifications of Srx1-HA levels (n = 2, average values ± SEM) were normalized to those of the untreated ($-H_2O_2$) strain YMM105 (*srx1Δ*) carrying the vector (unlabeled bars).

(I) H_2O_2 resistance of the BY4742 wild-type and indicated isogenic deletion mutant strains grown to early and midexponential phase in synthetic glucose medium and spotted on YPD plates with or without 1 mM H_2O_2 as in Figures 1B and 1C (n = 2).

TOR activity did not affect Srx1 levels (Figures 4C and 4D), and the Srx1 protein half-life was unaffected by altering cellular PKA activity (Figures S4G and S4H).

We next measured Srx1 protein levels following H_2O_2 stress at high (OD_{600} 0.02) and low PKA (*PDE2* overexpression) activity in mutants deleted for genes (e.g., *DCS1*, *GCN2*, or *TEF4*) previously associated with PKA-dependent effects on translation (Figure 4H, see also Figure S4I). The absence of Gcn2, which regulates translational initiation factor 2 (eIF2) in response to, e.g., glucose starvation (Hinnebusch, 2005), markedly reduced the cells' ability to elevate Srx1 levels upon reduced PKA activity. Consistently, *GCN2*-deficient cells were unable to develop H_2O_2 resistance upon a reduction in PKA activity (Figure 4I, Figure S4I). Gcn2 stimulates the translation of the transcription factor *GCN4* (Hinnebusch, 2005), which has been implicated in the response to peroxide stress (Mascarenhas et al., 2008) and TOR-dependent life span extension (Steffen et al., 2008). However, we found that *GCN4* was dispensable for Srx1 production and H_2O_2 resistance (Figures 4H and 4I).

Overexpression of *SRX1* Prolongs Life Span in a Tsa1-Dependent Manner

Considering that 2-Cys peroxiredoxins have a unique ability to react with low levels of endogenously generated peroxides (Fourquet et al., 2008), it is possible that Tsa1 is required for the longevity of cells under normal, nonstress conditions. Indeed, we found that cells lacking Tsa1 aged more rapidly than wild-type cells (Figure 5A), in agreement with the premature aging of cells carrying a Tsa1 point mutation conferring H_2O_2 sensitivity (Timmermann et al., 2010). The effect of lacking

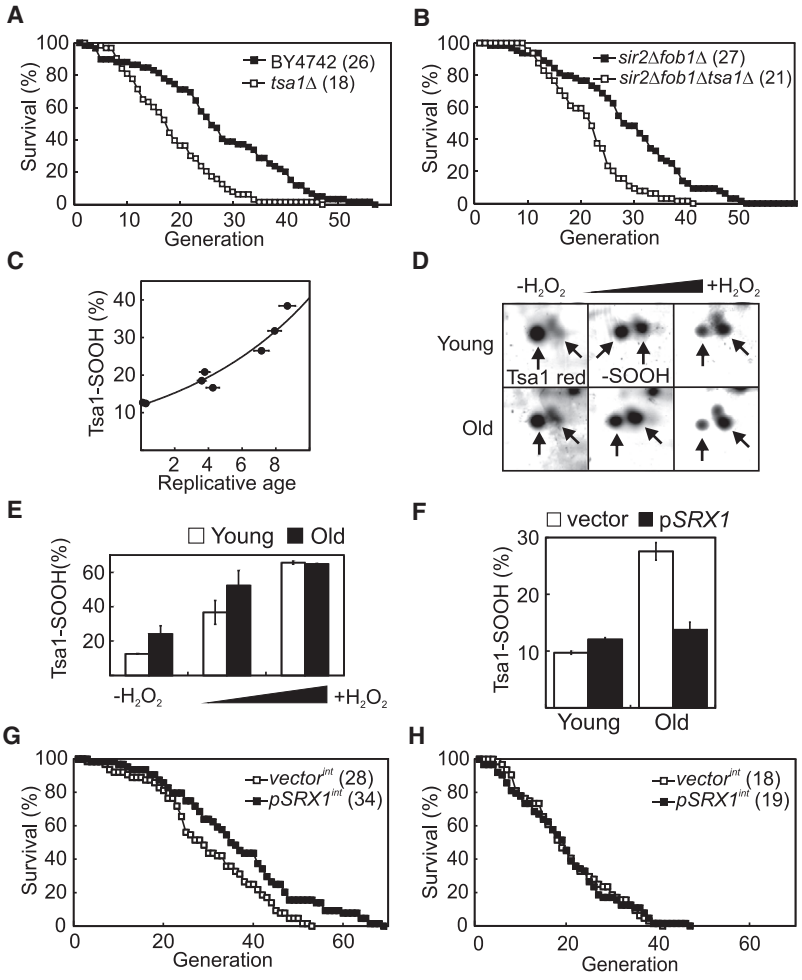

Figure 5. Srx1-Mediated Reduction of Age-Induced Tsa1 Hyperoxidation Extends Replicative Life Span

(A) Life span of the BY4742 wild-type and *tsa1Δ* strains in YPD medium. Median life span for each strain is indicated in brackets.

(B) Life span of the BY4741 *sir2Δfob1Δ* and *sir2Δfob1Δtsa1Δ* (YMM118) strains in YPD medium.

(C) Tsa1 sulfinylation as a function of replicative age. Tsa1-SOOH was quantified by 2D-PAGE (see the Supplemental Experimental Procedures) in mother cells of various replicative ages isolated by the biotin-streptavidin method (n = 4) or by elutriation (n = 4). Tsa1-SOOH levels are expressed as the fraction of total Tsa1. The purity of the age fractions and the average age were estimated by Calcofluor-staining and bud-scar counting (±SEM, n = 100 each). The fraction of Tsa1-SOOH was plotted against average replicative age and fitted to an exponential curve ($y = k_1 * e^{k2*x}$, $k_1 = k_2 = 0.12$, $R = 0.97$).

(D and E) Tsa1 sulfinylation in young and old cells upon addition of exogenous H_2O_2. BY4742 wild-type cells grown in synthetic glucose medium were separated by elutriation to isolate young and old cells. Cells were treated with 0.8 or 1 mM H_2O_2, and after 40 min samples were withdrawn, proteins extracted, separated by 2D-PAGE analysis and Tsa1-SOOH quantified as in (C) (n = 2, average values ± SD). Young cells had on average 0.19 ± 0.035 bud scars and old cells 8.4 ± 0.36 (±SEM, n = 200 and 300, respectively).

(F) Tsa1 sulfinylation in old cells carrying an extra copy of the *SRX1* gene. YMM122 (*sir2Δfob1Δ*) cells carrying plasmids pRS316 (vector) or pRS316-*SRX1-HA* (*pSRX1*) were grown in selective synthetic glucose medium and separated by the biotin-streptavidin method to isolate young and old cells. Proteins were extracted and separated by 2D-PAGE analysis using silver staining to detect proteins, and Tsa1-SOOH was quantified as in (C) (n = 2, average values ± SD). Young cells carrying the vector or *pSRX1* displayed on average 0.46 ± 0.096 (n = 201, ±SEM) and 0.47 ± 0.097 (n = 201) bud scars, respectively, whereas old cells had 9.3 ± 0.38 (n = 200) and 8.8 ± 0.32 (n = 200) bud scars, respectively.

(G) Effect of a genomic-integrated extra copy of the *SRX1* gene on the life span of cells grown at a high concentration of glucose. Life span analysis of strain YMM126 (*sir2Δfob1Δ vector*^{int}) and YMM127 (*sir2Δfob1Δ pSRX1*^{int}) as in (A).

(H) Effect of a genomic-integrated extra copy of the *SRX1* gene on the life span of cells lacking Tsa1. Life span analysis of strains YMM128 (*sir2Δfob1Δtsa1Δ vector*^{int}) and YMM129 (*sir2Δfob1Δtsa1Δ pSRX1*^{int}) as in (A).

TSA1 was maintained in cells deleted for *SIR2* and *FOB1*, in which ERC levels are kept at bay (Kaeberlein et al., 2004), demonstrating that the accelerated aging in *TSA1* mutants is independent of Sir2 activity and ERCs (Figure 5B). In addition, we found that Tsa1 became increasingly hyperoxidized (sulfinylated) as a function of the replicative age of the mother cell (Figures 5C–5E), indicating that aged cells have a limited ability to perform Srx1-dependent reduction of sulfinylated Tsa1. Exogenous H_2O_2 forced most of Tsa1 into its sulfinylated form in both young and old cells (Figures 5D and 5E). In support of Srx1 being limited in aging cells, providing the cells with an extra copy of the *SRX1* gene reduced age-induced Tsa1 sulfinylation (Figure 5F) and extended life span by 20% in a *TSA1*-dependent manner (Figures 5G and 5H, Figures S5A and S5B). This life span extension was observed both in *SIR2/FOB1*-proficient and -deficient cells, demonstrating that Srx1-dependent life span extension is ERC/Sir2 independent (Figure S5C, Figure 5G).

Tsa1 and Srx1 Are Required for Full Life Span Extension by CR

In view of the requirement for *TSA1* in yeast longevity and CR-induced H_2O_2 resistance, we next tested whether life span extension by CR also depended on *TSA1* and its sulfiredoxin *SRX1*. In cells lacking Sir2 and Fob1, CR extended the life span of *TSA1*- and *SRX1*-proficient cells by 36% ± 3.6% (Figure 6A). This effect of CR was markedly reduced in the absence of *TSA1* (7.1% ± 3.4% increase, Figure 6A) or *SRX1* (4.3% ± 11% increase, Figure 6B). Similarly, in *SIR2*- and *FOB1*-proficient cells, CR required both *TSA1* and *SRX1* to fully extend life span (Figures S6A–S6C). In addition, CR did not further extend the life span of cells carrying an extra copy of *SRX1* (Figure 6C, Figure S6D). A conditional mutation in *CDC25*, a genetic CR mimetic shown to prolong yeast life span (*cdc25-10*, Lin et al., 2000), counteracted age-induced Tsa1 hyperoxidation (Figure 6D) and required *TSA1* both for increased H_2O_2 resistance (Figure 6E) and full life span

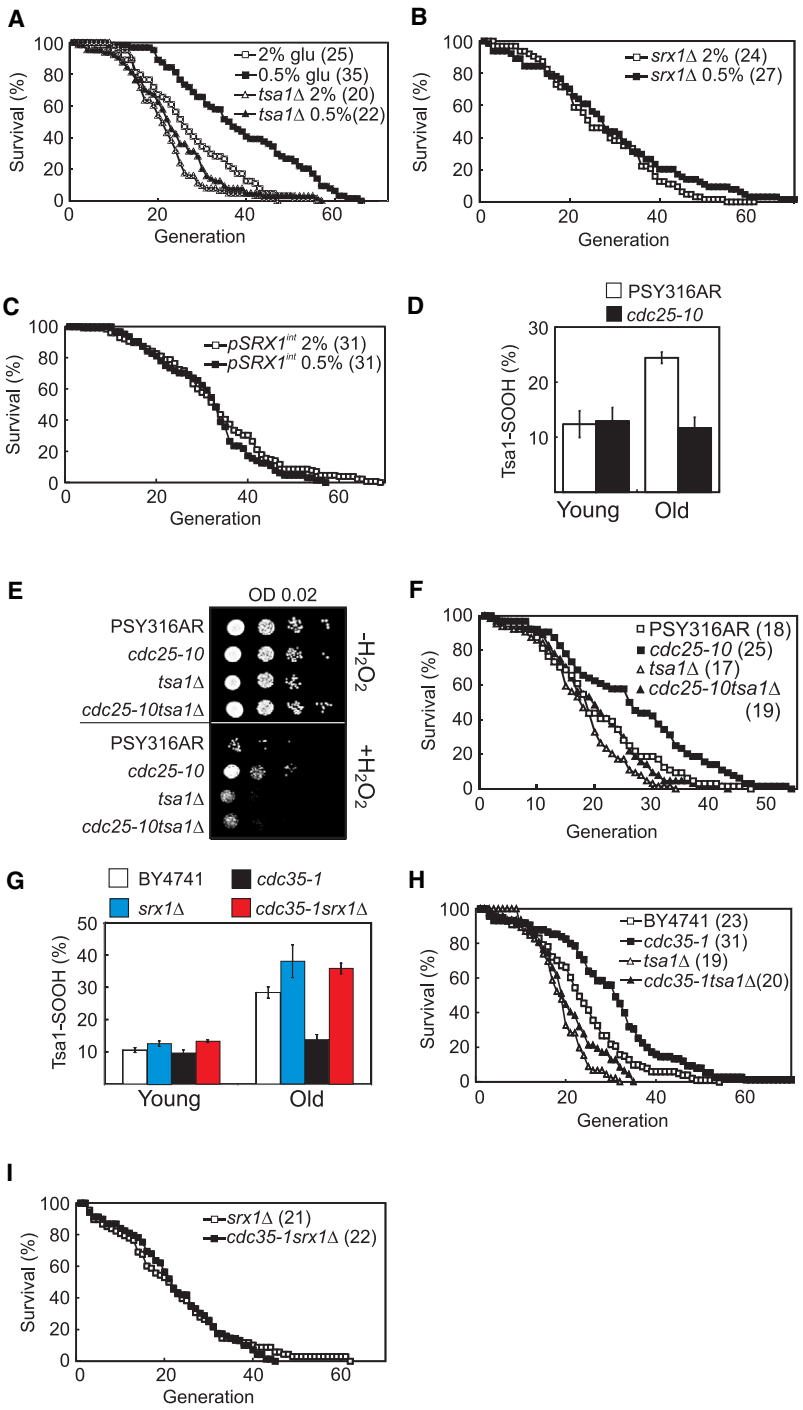

Figure 6. Srx1-Mediated Reduction of Tsa1 Hyperoxidation Is Required for CR-Induced Life Span Extension

(A) Life span analysis of strains BY4741 (*sir2Δfob1Δ*) and YMM118 (*sir2Δfob1Δtsa1Δ*) in YP medium supplemented with either 2% or 0.5% glucose.

(B) Life span analysis of strain YMM119 (*sir2Δfob1Δsrx1Δ*) in YP medium containing 2% or 0.5% glucose.

(C) Effect of CR on the life span of cells carrying an extra copy of *SRX1*. Life span analysis of strain YMM127 (*sir2Δfob1Δ pSRX1*^{int}) in YP medium containing 2% or 0.5% glucose.

(D) Effect of lowering PKA activity on Tsa1 sulfinylation in old cells. Wild-type PSY316AR and *cdc25-10* cells grown in synthetic glucose medium were separated by the biotin-streptavidin method to isolate young and old cells. Proteins were extracted and separated by 2D-PAGE analysis with silver-staining to detect proteins, and Tsa1-SOOH was quantified as in Figure 5C (n = 2, average values ± SD). Young PSY316AR and *cdc25-10* cells had on average 1.2 ± 0.12 (n = 362, ±SEM) and 1.7 ± 0.17 (n = 360) bud scars, respectively, whereas old cells had 7.8 ± 0.28 (n = 283) and 10.1 ± 0.41 (n = 255) bud scars, respectively.

(E) H_2O_2 resistance of PSY316AR (wild-type) and the indicated mutant strains following growth to early exponential phase in YPD medium.

(F) Life span of PSY316AR (wild-type) and indicated mutant strains in YPD medium.

(G) Effects of a CR mimetic and Srx1 deficiency on the hyperoxidation of Tsa1 in old cells. The wild-type BY4741 and the indicated mutant strains grown in YPD medium were separated by the biotin-streptavidin method to isolate young and old cells. Proteins were extracted and separated by 2D-PAGE analysis with silver staining to detect total protein, and Tsa1-SOOH was quantified as in Figure 5C. Young BY4741, *srx1Δ*, *cdc35-1*, and *cdc35-1srx1Δ* cells had on average 3.0 ± 0.41, 2.2 ± 0.30, 1.9 ± 0.33, and 2.4 ± 0.37 (±SEM) bud scars, respectively (n = 225, 200, 220, and 220, respectively), whereas old cells had on average 13.5 ± 0.37, 13.5 ± 0.41, 13.0 ± 0.51, and 12.8 ± 0.42 (n = 200, 200, 194, and 203, respectively).

(H and I) Effect of the CR mimetic *cdc35-1* on the life span of cells lacking Tsa1 (H) or Srx1 (I). The relevant genotypes of the strains analyzed are indicated in the figure.

extension (35% ± 2.5% increase in *TSA1* cells compared to 15% ± 5.6% increase in *Δtsa1* cells, Figure 6F). The conditional *cdc25-10* mutation is in the yeast PSY316AR genetic background. However, another CR-mimetic mutation in the adenylate cyclase gene (*cdc35-1*, Figure S6A) in the BY background also reduced age-induced Tsa1 hyperoxidation in an *SRX1*-dependent manner (Figure 6G). In addition, the *cdc35-1* allele also extended life span in a manner dependent on both *TSA1* and *SRX1* (Figures 6H and 6I). Thus, both bona fide CR and genetic mimetics of CR causing reduced PKA activity require a functional, reduced peroxiredoxin Tsa1 to fully retard aging.

DISCUSSION

In this work, we demonstrate that the antioxidant enzyme Tsa1, and its partner Srx1, are key proteins conferring increased resistance to H_2O_2 elicited by CR. Tsa1, like other eukaryotic 2-Cys Prxs, has the unusual property of undergoing H_2O_2-mediated inactivation by hyperoxidation. A small proportion of 2-Cys Prxs is inactivated in each catalytic cycle, and this inactivation can be reversed by sulfiredoxins (Srx1 in yeast) through ATP-dependent reduction (Biteau et al., 2003). We show that Srx1 is a crucial factor in CR-induced H_2O_2 resistance, as CR, via the translation-initiation-factor-2

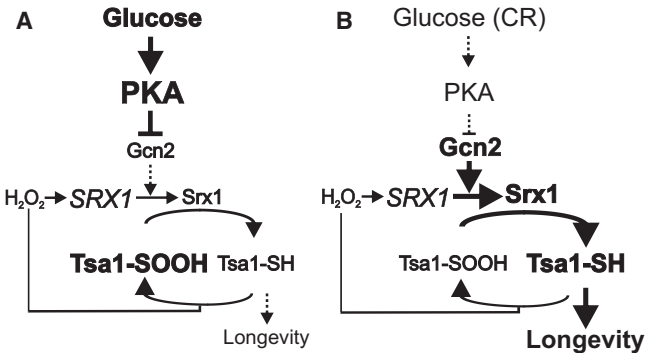

Figure 7. Model for How CR Elicits Tsa1/Srx1-Dependent H_2O_2 Resistance and Life Span Extension

(A) At a high concentration of glucose, leading to a high cAMP-PKA activity, H_2O_2 stress activates Yap1/Skn7-dependent transcription of the *SRX1* mRNA, but its translation is inhibited by PKA. As a consequence, Srx1 production is attenuated and Tsa1 hyperoxidized and inactivated.

(B) During CR, PKA activity is reduced, and this relieves the translational block of the *SRX1* mRNA in a Gcn2-dependent manner to provide more Srx1 protein and, as a consequence, more reduced, peroxidase-active Tsa1.

(eIF2) kinase Gcn2, relieves a cAMP-PKA-dependent inhibition in the translation of the *SRX1* gene (Figure 7).

In addition to being required for CR-induced H_2O_2 resistance, we show that Tsa1 and Srx1 are both necessary for a full life span extension by CR. Interestingly, we found that Tsa1 becomes hyperoxidized (sulfinylated) during aging, indicating that aged yeast cells have a limited ability to perform Srx1-dependent reduction of hyperoxidized and sulfinylated Tsa1. In support of this notion, providing the cells with an extra copy of the *SRX1* gene counteracted age-related hyperoxidation of Tsa1 and extended replicative life span by 15%–20% (Figure 5G, Figures S5A and S5C) in a *TSA1*-dependent manner (Figure 5H, Figure S5B). This life span extension by an extra copy of the *SRX1* gene is comparable to that achieved by a rapamycin-dependent reduction of TOR signaling (15.4%; Medvedik et al., 2007). Hyperoxidation of the mitochondrial PrxIII enzyme has been reported also in the liver of aged rats, indicating that Prx inactivation may be a common phenotype in aging organisms (Musicco et al., 2009).

Deleting the yeast *TSA1* causes a dramatic genome instability with gross chromosomal rearrangements and synthetic lethality in combination with deficiencies in Rad51-, Rad52-, and Rad6-mediated DNA repair (Huang and Kolodner, 2005). In addition, mice lacking the Tsa1 homolog Prdxl display increased genomic instability and an increased incidence of malignant tumors, and they age prematurely (Neumann et al., 2003). Similar results have been demonstrated for worms lacking the 2-Cys peroxiredoxin PRDX-2 (Olahova et al., 2008). The role of Tsa1 in protecting the genome and the fact that Tsa1 becomes hyperoxidized in aging cells is interesting in view of the fact that replicative aging of yeast mother cells encompasses a progressive decline in the maintenance of the genome. This decline includes accumulation of extrachromosomal rDNA circles (ERCs; Sinclair and Guarente, 1997) and a switch to a hyperrecombinational state (McMurray and Gottschling, 2003). McMurray and Gottschling (2004) suggested that the accumulation of damaged, oxidized, and aggregated proteins in aging cells (Erjavec et al., 2007; Liu et al., 2010) might lead to the loss of function of gene products critical for maintaining genome integrity. Our data are in line with this idea and suggest that Tsa1 might be one such gene product of special importance for the maintenance of the genome which itself, in addition to its partner Srx1, is inactivated during aging.

It has been difficult to establish if the cAMP-PKA or the TOR pathway predominates in regulating cellular responses to nutritional cues, including CR, because of the highly interconnected nature of these pathways (Zaman et al., 2008). Therefore, our observation of the distinct role of PKA and TOR in H_2O_2 resistance elicited by CR is somewhat surprising. As shown herein, reducing TOR signaling did not affect the ratio of reduced/oxidized Tsa1 (or Srx1 levels or H_2O_2 resistance) in cells with high PKA activity, whereas reducing PKA effectively increased this ratio regardless of TOR activity (Figure S3B, Figures 4C and 4D, Figure S2E). Moreover, TOR and PKA signaling affected Tsa1 synthesis in an opposite manner (Figures S3R and S3S; Figure 3A). Thus, with respect to stress-induced Tsa1 synthesis upon CR, the TOR and PKA pathways are antagonizing each other with the development of H_2O_2 tolerance being due to the reduction in PKA-dependent signaling. These results points to the importance of clarifying to what extent the beneficial effects of CR in yeast primarily act through PKA or TOR.

Prxs themselves have recently been shown to be involved in nutrient signaling affecting longevity. Specifically, a neuronal *Drosophila* Prx was identified as a downstream effecter for life span regulation and oxidative stress resistance of the insulin signaling-regulated transcription factor FOXO (Lee et al., 2009). In view of these results and the fact that *TSA1* displays such an important role for CR to extend yeast life span, we believe it might be warranted to elucidate the possible importance of Prx enzymes as a potential public, evolutionary conserved mechanism for life span extension by CR.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions

The *Saccharomyces cerevisiae* strains used were grown and manipulated at 30°C using standard techniques and yeast media (Molin et al., 2007; for details, see the Supplemental Experimental Procedures). H_2O_2 resistance assays were performed as described in the Supplemental Experimental Procedures. Where indicated, rapamycin (Sigma, catalog number R0395-1MG) stock solution (1 mg/ml in 95% ethanol) or ethanol alone was added to the cultures.

Plasmids

Plasmids used are found in the Supplemental Experimental Procedures.

cAMP Measurement

cAMP was extracted from cells by a TCA extraction method as described in the Supplemental Experimental Procedures and measured using the LANCE cAMP 384 kit (Perkin-Elmer, cat# AD0262) as recommended by the supplier.

Radiolabeling and 2D-PAGE Analysis

For protein synthesis rate determinations, cells were labeled with 200 μCi ^{35}S-methionine for 20 min at 30°C. Cell harvesting, protein extraction, and 2D-PAGE were performed as described (Maillet et al., 1996).

Immunoblotting

Immunoblot analysis of Srx1-HA levels and Myc-Tsa1 sulfinylation was done as described (Biteau et al., 2003) with small modifications as described in the Supplemental Experimental Procedures.

LC-MS/MS Analysis

NanoLC-MS/MS was performed at the Proteomics Core Facility, Sahlgrenska Academy, University of Gothenburg on in-gel chymotrypsinated samples cut out of Coomassie-stained gels separating 800 ug of total protein (see the Supplemental Experimental Procedures).

RT-PCR Analysis

Transcript levels were analyzed as described in the Supplemental Experimental Procedures.

Polysome Analysis

Polysome analysis was performed essentially as described previously (Swaminathan et al., 2006) with small modifications as described in the Supplemental Experimental Procedures.

Life Span Analysis

Life span survival experiments were performed as described (Erjavec et al., 2007) on 39–128 virgin cells per curve as indicated in the Supplemental Experimental Procedures. Increases in median life span presented are average relative effects from two experiments (pairs of life span curves) ± SD.

Isolation of Old Cells

Isolation of old cells was done using magnetic sorting or centrifugal elutriation as described previously (Erjavec et al., 2007).

Reproducibility and Statistical Analyses

All experiments presented in this paper were repeated at least twice to confirm reproducibility. Values in figures with error bars are averages from two to four experiments, and error bars indicate SD or in some cases SEM (Figures 2E, 4B, and 4D). In addition, statistical analyses used to back up all claims made can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2011.07.027.

ACKNOWLEDGMENTS

Gilles Lagniel and Ellinor Pettersson are gratefully acknowledged for 2D-PAGE analysis. We thank Michael Hall, James Broach, Pierre Thuriaux, Charlie Boone, and Su-Ju Lin for supplying strains, and Laurence Nachin and Jonas Warringer for comments on the manuscript. The work was funded by the Swedish Research Council (VR), the Knut and Alice Wallenberg Foundation (Wallenberg Scholar Grant), and an ERC Advanced Research Grant (QualiAge) to T.N.; the platform for quantitative biology (Gothenburg University) to M.M.; the EU grant ACE to J.L. and M.B.T.; and a fund program (Equipe labellisée ligue 2009) from la Ligue Nationale Francaise contre le Cancer to M.B.T.

Received: September 20, 2010
Revised: January 3, 2011
Accepted: July 9, 2011
Published: September 1, 2011

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