Research Article

Identification of Potential Calorie Restriction-Mimicking Yeast Mutants with Increased Mitochondrial Respiratory Chain and Nitric Oxide Levels

Bin Li,[1] Craig Skinner,[1] Pablo R. Castello,[2] Michiko Kato,[1] Erin Easlon,[1] Li Xie,[1] Tianlin Li,[1] Shu-Ping Lu,[1] Chen Wang,[1] Felicia Tsang,[1] Robert O. Poyton,[2] and Su-Ju Lin[1]

[1] *Department of Microbiology, College of Biological Sciences, University of California at Davis, Davis, CA 95616, USA*
[2] *The Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309, USA*

Correspondence should be addressed to Su-Ju Lin, slin@ucdavis.edu

Calorie restriction (CR) induces a metabolic shift towards mitochondrial respiration; however, molecular mechanisms underlying CR remain unclear. Recent studies suggest that CR-induced mitochondrial activity is associated with nitric oxide (NO) production. To understand the role of mitochondria in CR, we identify and study *Saccharomyces cerevisiae* mutants with increased NO levels as potential CR mimics. Analysis of the top 17 mutants demonstrates a correlation between increased NO, mitochondrial respiration, and longevity. Interestingly, treating yeast with NO donors such as GSNO (*S*-nitrosoglutathione) is sufficient to partially mimic CR to extend lifespan. CR-increased NO is largely dependent on mitochondrial electron transport and cytochrome c oxidase (COX). Although COX normally produces NO under hypoxic conditions, CR-treated yeast cells are able to produce NO under normoxic conditions. Our results suggest that CR may derepress some hypoxic genes for mitochondrial proteins that function to promote the production of NO and the extension of lifespan.

1. Introduction

Calorie restriction (CR) extends lifespan in a variety of organisms and has also been shown to ameliorate many age-associated diseases such as diabetes and cancers [1–3]. However, the molecular mechanisms underlying CR-induced beneficial effects are still not fully understood. Owing to a short lifespan and well-established molecular genetic techniques, the budding yeast *Saccharomyces cerevisiae* represents a powerful system to identify new components in CR signaling pathways and to study these factors at the molecular/genetic level. Yeast lifespan can be studied in two distinct ways: replicative lifespan (RLS) and chronological lifespan (CLS). RLS measures the number of cell divisions an individual yeast cell undergoes before senescence (division potential), whereas CLS measures the length of time cells remain viable at a nondividing state (postmitotic survival). RLS may serve as a model to understand the mechanisms of replicative senescence such as in stem cells, while CLS may be more relevant to postmitotic cell senescence in adult animals [4, 5].

Moderate CR can be imposed in yeast by reducing the glucose concentration from 2% to 0.5% in rich media [6–9], which extends both RLS and CLS. In yeast, CR is suggested to function through reducing the activities of conserved nutrient-sensing pathways. Decreasing the activities of the Ras-cAMP/PKA (cyclic AMP-activated protein kinase A) pathway, Sch9 (homolog of mammalian S6K kinases) and Tor1 kinases have been shown to mimic CR and extend lifespan [6, 10, 11]. The recent identification of additional CR-specific longevity genes provides further insight into the molecular mechanisms underlying CR and the resulting metabolic alterations [7, 12–17]. The Sir2 family proteins (sirtuins) are among identified CR downstream targets; they are conserved longevity factors that were originally discovered and studied in yeast [3]. Sirtuins are NAD⁺-dependent

protein deacetylases that are responsive to metabolic changes and stress and have been shown to play important roles in several CR models [3, 18, 19].

Mitochondria have also been shown to play important roles in CR. In yeast, CR induces a shunting of carbon metabolism from fermentation to the mitochondrial TCA cycle [12]. This metabolic shift to respiration is necessary and sufficient for the activation of Sir2-mediated lifespan extension in certain yeast strains [12]. A link between CR and increased mitochondrial metabolism has also been reported in higher eukaryotes including mammals [13, 14, 20–22]. Notably, the age-dependent decline in expression of genes encoding components of the mitochondrial respiratory chain has been reported in several species [22–24]. Since mitochondria are the major sites of energy production in eukaryotic cells, these findings highlight the crucial role of energy metabolism in CR. Since CR regimens involve the reduction of nutrient input, it is believed that a global change in nutrient sensing and regulatory pathways as well as changes in the mitochondrial respiratory chain are translated to physiological responses to counteract age-induced effects [25–27].

The role of the mitochondrial respiratory chain in CR is still unclear. In yeast, it has been suggested that CR activates mitochondrial respiration to prevent the accumulation of toxic metabolites [28–30]. Although the mitochondrial electron transport chain is also the primary site for reactive oxygen species (ROS) generation in the eukaryotic cell, increased mitochondrial electron flow during CR would be expected to reduce ROS levels [3, 31–33]. Recently, cytochrome c oxidase (COX) of the mitochondrial respiratory chain has been shown to catalyze the reduction of nitrite to nitric oxide (NO) [34]. When considered with the findings that the respiratory chain is involved in CR and that NO has been implicated in CR, it is of interest to ask if the NO involved is produced by mitochondrial COX.

In order to begin to elucidate how CR modulates complex genetic and metabolic networks to alter stress resistance, genomic stability, and lifespan, it is essential to uncover additional factors in the CR pathway. Towards this end, we have explored the relationship between NO and CR and have identified new genes in CR.

2. Materials and Methods

2.1. Yeast Strains and Media. Yeast strains BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* and the genome-wide gene deletion collections (nonessential genes) were acquired from Open Biosystems. Medium used for replicative lifespan (RLS) analysis was YEP (2% bacto peptone, 1% yeast extract, 1.5% agar) supplemented with filter-sterilized glucose at a final concentration of 2% or 0.5%. Medium used for chronological lifespan (CLS) analysis was minimal synthetic SD (6.7 g/L yeast nitrogen base) supplemented with 4x auxotrophic amino acids (leucine, histine, uracil, and lysine) and glucose to a final concentration of 2% or 0.5%. Gene deletions were made by replacing the wild-type genes with the reusable Kan^r marker as described in [16] and verified

by Polymerase Chain Reaction (PCR) using oligonucleotides flanking the genes of interest.

2.2. GSNO Treatments. Different concentrations of GSNO were added to yeast cells during chronological lifespan assays at different time points. GSNO was made as follow: 200 μL of 0.5 M GSH in 0.5 M HCL was added to 200 μL of 0.5 M NaNO$_2$ and then incubated at 4°C in the dark. After 30 minutes, 400 μL of 0.25 M NaOH was added to neutralize the solution. This reaction generated GSNO at final concentration of ~125 mM. Inactivated GSNO was generated by exposing GSNO to UV illumination for 30 min (photolysis) [35].

2.3. Measurement of Relative NO Levels Using the DAF-FM DA Fluorescence Dye. Diaminofluorescein-FM diacetate (Kamiya Biomedical) was diluted to 1 μM in reaction buffer (0.2 M phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose) immediately prior to the assay. Cells at different growth stages were harvested, washed twice, and then resuspended into 120 μL of reaction buffer (0.2 M potassium phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose). 100 μL of cell suspension was added to 100 μL reaction buffer with 1 μM of DAF-FM DA in 96-well fluorescence assay plates ($\sim 5 \times 10^6$ cells were analyzed in each well). After 2 hours of incubation (30°C), fluorescence signals were detected using a plate reader with excitation at 485 nm and emission monitored at emission at 535 nm [36, 37].

2.4. Nitric Oxide (NO) Measurement by an NO-Specific Electrode in Intact Cells. Cells were grown in YPD media containing either 0.5% or 2% glucose on a shaker (200 rpm) at 28°C and harvested in mid-exponential phase. Cells were washed twice with cold distilled water, pelleted, and kept on ice until use. Nitrite-dependent NO synthesis was measured essentially as described in [34, 38]. Briefly, cells were resuspended in 2 mL PBS (pH 6.5) in an NO chamber and bubbled 5 min with nitrogen. Then the chamber was closed, and NO production was measured at 28°C after the addition of 1 mM sodium nitrite using an NO electrode (amiNO-700, Innovative Instrument) as described in [34, 38].

2.5. Polarographic Measurement of NO Production and O$_2$ Consumption by Mitochondria. Cells were grown as above and harvested in mid-exponential phase. Mitochondria were prepared as described in [39]. NO production was measured with an amino-700 nitric oxide sensor connected to an inNOII nitric oxide measuring system (Innovative Instruments, Inc). Simultaneous measurement of O$_2$ concentration in the chamber was performed by a 2 mm Clark-type electrode Oxy-2 (Innovative Instruments, Inc) connected to the inNOII. Except where noted, all solutions were nitrite-free. Measurements were performed at 28°C in a final reaction volume of 2 mL, in a thermostated chamber with a close-fitting lid containing ports for the electrodes and a Hamilton syringe. Assays for NO production by the mitochondrial respiratory chain were performed in NO Assay Medium (6 mM succinate, 650 mM mannitol, 10 mM

K_2HPO_4 [pH 6.5], 0.1 mM EDTA, and 10 mM KCl). Except where noted, the NO assay medium was prebubbled with air in order to be saturated with oxygen ($200\,\mu M$) prior to addition of $NaNO_2$, which was added to a final concentration of 1 mM, and the measurement of NO production.

2.6. Screens for Genetic Mimics of CR Base on Relative Levels of NO. Each mutant strain from the deletion collection (Open Biosystems) was inoculated into SD in 96-well plates and allowed to grow for 2 days. $4\,\mu L$ saturated culture was then added into $400\,\mu L$ fresh SD and allowed to grow for 12 hr. To prepare for NO measurement, cells were spun down in 96-well plates at 3000 RCF, washed, and then resuspended into $120\,\mu L$ reaction buffer (0.2 M phosphate buffer, pH 7, 1 mM EDTA, 0.1% glucose). $100\,\mu L$ cell suspension was added to $100\,\mu L$ reaction buffer with $1\,\mu M$ of DAF-FM DA (Kamiya Biomedical) prealiquoted in 96-well fluorescence assay plates. Multiple wild-type (WT) controls grown under both normal (2% glucose) and CR (0.5% glucose) conditions were included on each 96-well plate as internal controls. Average NO levels of WT controls grown under normal condition (after normalized to cell number) were set to 1. NO levels of the mutants were normalized to the WT controls on the same plate. We have determined the variations among different plates and different experiments to be ~20% (data not shown), which was used as the standard deviation for the initial screen.

2.7. Chronological Lifespan (CLS). Three to four single colonies derived from each strain were analyzed in each experiment as described in [4] with a few modifications. Cells were grown in 10 mL SD (at a starting OD_{600} of 0.1) in 50 mL tubes on a roller drum set at 250 rpm to ensure proper aerations. Cell viability (~2×10^8 cells were analyzed in each sample) was monitored every 2-3 days by plating a fraction of the culture onto fresh media to determine the colony-forming units (CFU). Cell survival rates were calculated by normalizing the CFU of each time point to the CFU of day 0 (48 hr after starting CLS, when cells just entered stationary phase). Numbers on the *x*-axes of CLS graphs denote the number of days after entering stationary phase (nonmitotic state). GSNO-treated cells were grown in the dark for the first 24 hr.

2.8. Replicative Lifespan (RLS). All RLS analyses were carried out on YEP agar plates supplemented with glucose at a final concentration of 2% or 0.5% with 50 cells per strain per experiment [16] using a micromanipulator under a microscope. All RLS assays were repeated at least twice by two different individuals.

2.9. Oxygen Consumption. Oxygen consumption was monitored using a Clark-type electrode as previously described in [16].

2.10. GSNO and NO Metabolizing Activities in Cell Lysates. About 300 OD_{600} units of yeast cells grown to different stages were harvested and homogenized in 1.5 mL lysis buffer containing 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma), and protease inhibitor tablet (Roche) using a beads beater. The GSNO reductase (GSNOR) activity of Sfa1 was determined by measuring GSNO-dependent NADH consumption using fluorescence (340/460 nm) as described in [40] in $200\,\mu L$ reaction buffer ($200\,\mu M$ NADH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA) with $15\,\mu g$ (total proteins) cell lysate and 0 or $200\,\mu M$ GSNO at room temperature. The NO metabolizing activity of Yhb1 was determined by measuring NO-dependent NADH consumption using fluorescence (340/460 nm) as described in [41] in a $200\,\mu L$ reaction mix containing $200\,\mu M$ NADH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, $15\,\mu g$ lysate, and 0 or $300\,\mu M$ MAHMA NONOate (Cayman Chemical) at room temperature. The NADPH-dependent NO-metabolizing activity was measured by MAHMA NONOate-dependent consumption of NADPH using fluorescence (340/460 nm) as described in [42] in a $200\,\mu L$ reaction mix containing $250\,\mu M$ NADPH, 20 mM Tris-HCl, pH 8, 0.5 mM EDTA, $15\,\mu g$ lysate, and 0 or $300\,\mu M$ MAHMA NONOate at room temperature.

2.11. CYC7 Reporter Assay. Approximately 1 kb of the *CYC7* promoter was cloned into the *URA3+*, pYES2.1 vector (Invitrogen) using the BamHI site immediately upstream of the β-galactosidase gene. For the liquid β-galactosidase assay, 5 OD_{600} units of yeast cells grown in SD w/o uracil for 18 hrs (starting OD_{600} = 0.1) under normoxic condition were washed, lysed by bead beating in breaking buffer (100 mM Tris-HCl pH 8, 20% glycerol, 1 mM DTT, 0.02% SDS, 8 mM PMSF), and separated from debris by centrifugation. $50\,\mu L$ of lysate was then added to $450\,\mu L$ of Z buffer (0.1 M sodium phosphate buffer pH 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), preincubated at 30°C for 5 min, and then $100\,\mu L$ 4 mg/mL ONPG in Z buffer was added. After 1 hr incubation at 30°C, the reaction was stopped with $250\,\mu L$ of 1 M $NaCO_3$ and read at 420 nm. This reading was then normalized to protein concentration of the lysate and finally normalized to the WT 2% glucose sample.

2.12. L-NAME Treatment for NO Quantitation. Cells were first inoculated in SD (staring OD_{600} = 0.1) and allowed to grow for 12 hrs. The arginine analogue and NOS inhibitor L-NAME (Sigma) was then added to these cells at 200 mM final concentration as previously described in [43]. After 2 hr incubation at 30°C, cells were washed and then incubated with the DAF-FM DA fluorescence dye to determine relative NO levels as described previously.

2.13. Hydrogen Peroxide and GSNO Toxicity Tests. For hydrogen peroxide treatments, cells were pregrown in SD with 2% or 0.5% glucose (CR) for 20 hrs (staring OD_{600} = 0.1) then were treated with 0.5% H_2O_2 for 1 hr. Following this treatment, cells were plated on YPD in 5-fold serial dilutions. For GSNO toxicity test, cells were pregrown in SD with 2% or 0.5% glucose (CR) or pretreated with $25\,\mu M$ GSNO for 18 hr. GSNO was then added to cells at a final concentration of 1.5 mM. After 24 hr treatment, cells were removed from

the GSNO-containing SD, plated on YPD in 5-fold serial dilutions, and then allowed to grow for 2 days at 30°C.

2.14. Statistical Analyses. Statistical analysis of CLS was carried out using the AUC method (area under the curve) [44, 45]. *P* values were calculated for each pair of lifespans as shown in Supplemental Table 2 (see Supplementary Material available online at doi: 10.4061/2011/673185). Statistical analysis of RLS was carried out using the JMP statistics software (SAS), and the Wilcoxon rank sums tests *P* values were calculated for each pair of lifespans as shown in Supplemental Table 3. All other *P* values were calculated using Student's *t*-test (two-tailed).

3. Results

3.1. Calorie Restriction Induces a Significant Increase in NO Production. Moderate CR in yeast results in a metabolic shift from fermentation to mitochondrial respiration [12]. In mice, CR-induced mitochondrial biogenesis was attributed to increased expression of endothelial nitric oxide synthase (eNOS) [20]. To further understand the role of NO in CR, we determined whether CR increased NO levels in yeast using an NO-sensitive dye, diaminofluorescein-FM diacetate (DAF-FM DA). Although DAF-FM DA is fairly specific to NO and NO oxidation products [36, 37], we further confirmed that it did not cross-react with other reactive oxygen species (such as superoxide and hydrogen peroxide) *in vivo* under our assay conditions (Supplemental Figure 1). As shown in Figure 1(a), CR significantly increased NO-related signals during early growth stages, while cells were actively dividing. The long-lived *tor1Δ* and *hxk2Δ* mutants [6, 11] also showed increased NO signals (Figure 1(b)). In addition, CR, *tor1Δ*, and *hxk2Δ* also conferred resistance towards nitrosative stress (treatments of a physiological NO donor *S*-nitrosoglutathione, GSNO) (Figure 1(c)). We next determined whether the activities of major NO detoxification enzymes were increased by CR. Yeast flavohemoglobin (Yhb1), which catalyzes the conversion of NO to nitrite or nitrous oxide, and GSNO reductase (GSNOR, Sfa1) are the two major enzymes that protect yeast cells from nitrosative stress [40, 41, 46]. Interestingly, Yhb1 activity determined in the crude cell lysate [41] was increased by CR (Figure 1(d)), whereas Sfa1 activity [40] was not increased by CR, suggesting that Yhb1 may play an important role in CR. In addition, CR also slightly induced NADPH-dependent NO-metabolizing activity (Figure 1(d)), which has been reported in mammalian cells [42]. Together, these results demonstrate that CR-induced increases in NO are not due to decreased NO degradation.

3.2. A Genetic Screen for High NO Mutants as Potential CR Genetic Mimics. To identify additional components in the CR pathway, we screened the yeast deletion collection (~4500 strains) for mutants with elevated NO levels similar to CR. About 157 high-NO mutants were identified and ranked by NO levels, growth rate, and resistance to GSNO (Supplemental Table 1). CR increases both replicative and

chronological lifespan (RLS and CLS). Therefore, we used these criteria to classify potential "CR genetic mimics." Among the top 15 hits, 5 mutants (*hxt17Δ, pkh2Δ, gup1Δ, hhf1Δ, and soy1Δ*) showed extended CLS and RLS (Table 1). Hxt17 is a high-affinity hexose transporter, which may mimic CR by decreasing the activity of the glucose sensing pathways [6]. Pkh2 is a Ser/Thr protein kinase that activates Sch9 [47], and *pkh2Δ* is likely to behave similar to *sch9Δ*, which has been shown to mimic CR [11]. Recently, *pkh2Δ* was identified as an RLS-extending mutant [48], providing further validation for our screen. Gup1 is an ER membrane-localized O-acyltransferase involved in remodeling the fatty acid moiety of the GPI anchor [49]. *HHF1* encodes one of two identical histone H4 proteins. Depleting histone H4 has been shown to alter the expression levels of specific yeast genes including many genes that regulate energy homeostasis [50, 51]. Soy1/Aim4 is suggested to participate in mitochondrial genome maintenance [52]; however, its molecular function remains unclear.

We next determined whether these long-lived mutants function in the same pathway as CR to extend lifespan. As expected, CR did not significantly alter CLS (Figures 2(a)–2(d)) or RLS (Figures 3(a)–3(d)) of most of these long-lived mutants, suggesting that these mutations might be in genes that function in the same (or overlapping) pathway to extend CLS and/or RLS (detailed statistical analysis of CLS and RLS is shown in Supplemental Tables 2 and 3, resp.). Two notable exceptions were observed in our studies: the *gup1Δ* (Figures 2(c) and 3(c)) and the *soy1Δ* (Figure 3(b)) mutants. CR further increased *gup1Δ* RLS (*gup1Δ* versus *gup1Δ*, CR; *P* = .038) and vice versa (CR versus *gup1Δ*, CR; *P* = .004) (Figure 3(c)), suggesting a positive interaction between *gup1Δ* and CR in RLS extension. In addition, although CR-induced CLS was not further increased by *gup1Δ* (CR versus *gup1Δ*, CR; *P* = .073), the *gup1Δ* mutant exhibited longer CLS under CR (*gup1Δ* versus *gup1Δ*, CR; *P* = .035). Interestingly, CR appeared to decrease *soy1Δ*-induced RLS (*soy1Δ* versus *soy1Δ*, CR; *P* = .008) (Figure 3(b)), suggesting a negative interaction between *soy1Δ* and CR. However, *soy1Δ* did not significantly change CR-induced RLS (CR versus *soy1Δ*, CR; *P* = .49). A potential positive interaction was also observed between *hhf1Δ* and CR. CR appeared to extend RLS more significantly when combined with *hhf1Δ* (CR versus *hhf1Δ*, CR; *P* = .001). However, CR did not significantly change *hhf1Δ*-induced RLS (Figure 3(d)) (*hhf1Δ* versus *hhf1Δ*, CR; *P* = .19).

3.3. CR-Induced Nitric Oxide Production Is Most Likely Generated by Enhanced Mitochondrial Respiration and the Cytochrome c Oxidase. We next determined whether these long-lived, high-NO mutants displayed an increase in mitochondrial respiration similar to CR cells. As shown in Figure 4(a), all of these mutants showed increased oxygen consumption, demonstrating a correlation between increased mitochondrial respiration and NO production. In addition, a functional electron transport chain was required for CR-induced lifespan. CR failed to extend RLS [7, 12] and CLS (Figure 4(b)) in the respiratory-deficient *cyt1Δ* mutant
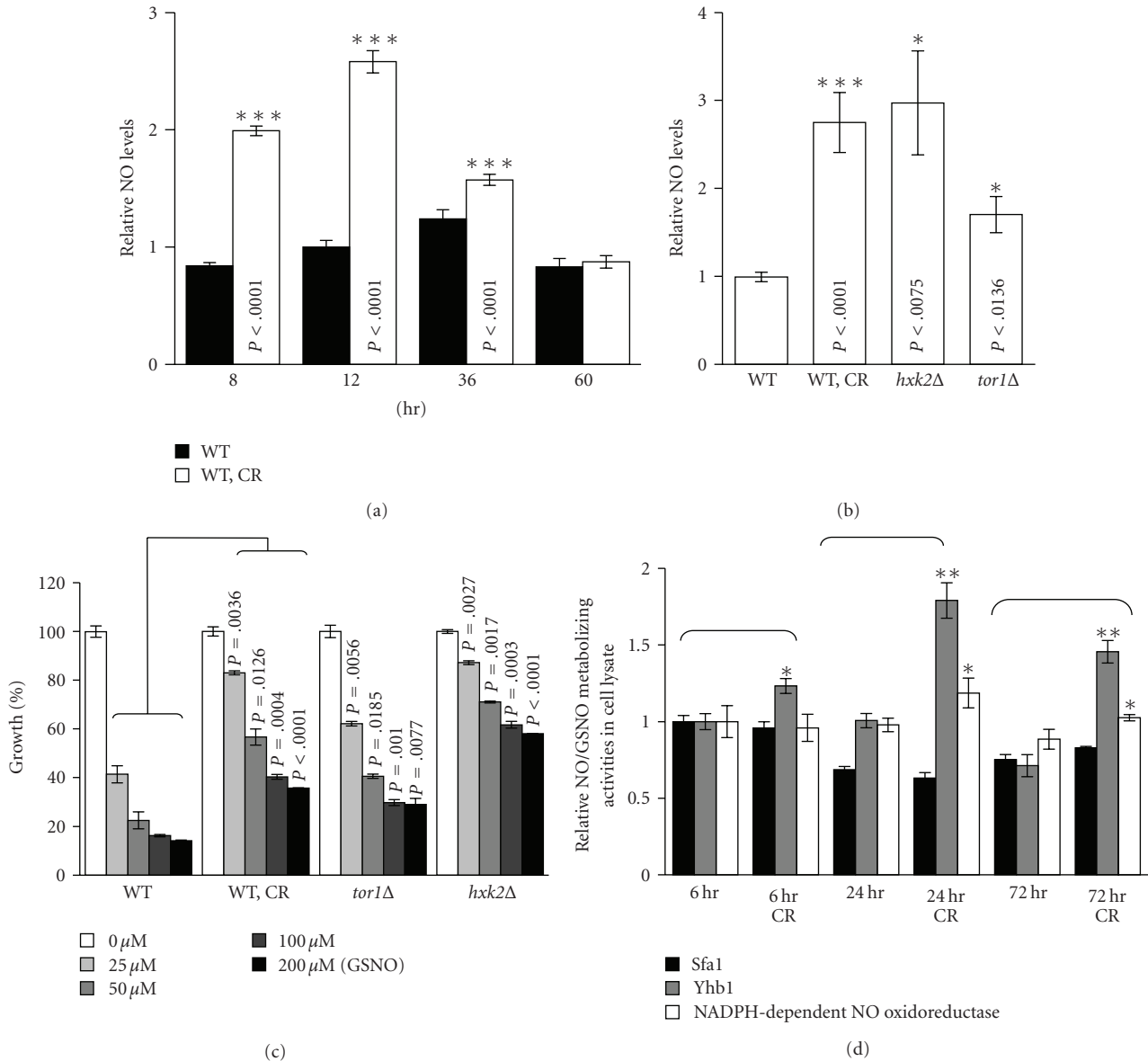
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FIGURE 1: Calorie restriction (CR) and CR genetic mimics increase nitric oxide (NO) levels and confer resistance to nitrosative stress in yeast cell. (a) CR increases NO-related signals during early growth stage. (b) Long-lived $tor1\Delta$ and $hxk2\Delta$ mutants also show increased NO-related signals. (c) CR and CR mimics confer resistance to S-nitrosoglutathione- (GSNO-) induced toxicity. Results show percentages (%) of growth normalized to no treatment controls after treated with GSNO at indicated concentrations for 20 hr. (d) Yeast cells grown in CR show increased NO metabolizing activities determined in cell lysates. P values are calculated comparing CR cells and the $tor1\Delta$ or $hxk2\Delta$ mutants to WT control. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. One representative set of three independent experiments is shown. Error bars denote standard deviations.

lacking cytochrome c1. Deletion of *CYT1* also eliminated the long RLS (Figure 4(c)) and CLS (B. Li and S.-J. Lin, unpublished) of potential CR mimics. We then further examined how CR increased NO production. Although there is biochemical evidence that NOS homologs are present in yeast [43], the existence of nitric oxide synthases in yeast remains controversial since no NOS sequence homolog has been identified. In contrast, cytochrome c oxidase (COX) has been shown to produce NO in a nitrite- ($NO_2{}^-$-) dependent manner under hypoxic conditions [34]. Therefore, the mitochondrial electron transport chain is a potential source

of NO production in CR cells. Our results (Figure 4(d)) showed that CR- and CR mimics-induced NO increases were largely abolished in cells lacking *CYT1*. In addition, CR-induced NO production was not inhibited by the NOS inhibitor (arginine analog) L-NAME (Figure 4(e)).

Since the COX complex was reported to produce NO under hypoxic conditions [34], we examined whether calorie-restricted yeast cells could synthesize NO under normal (normoxic) conditions. Using an NO-specific electrode, we verified that calorie-restricted BY4742 WT (ρ^+) cells could produce NO when grown under normoxic
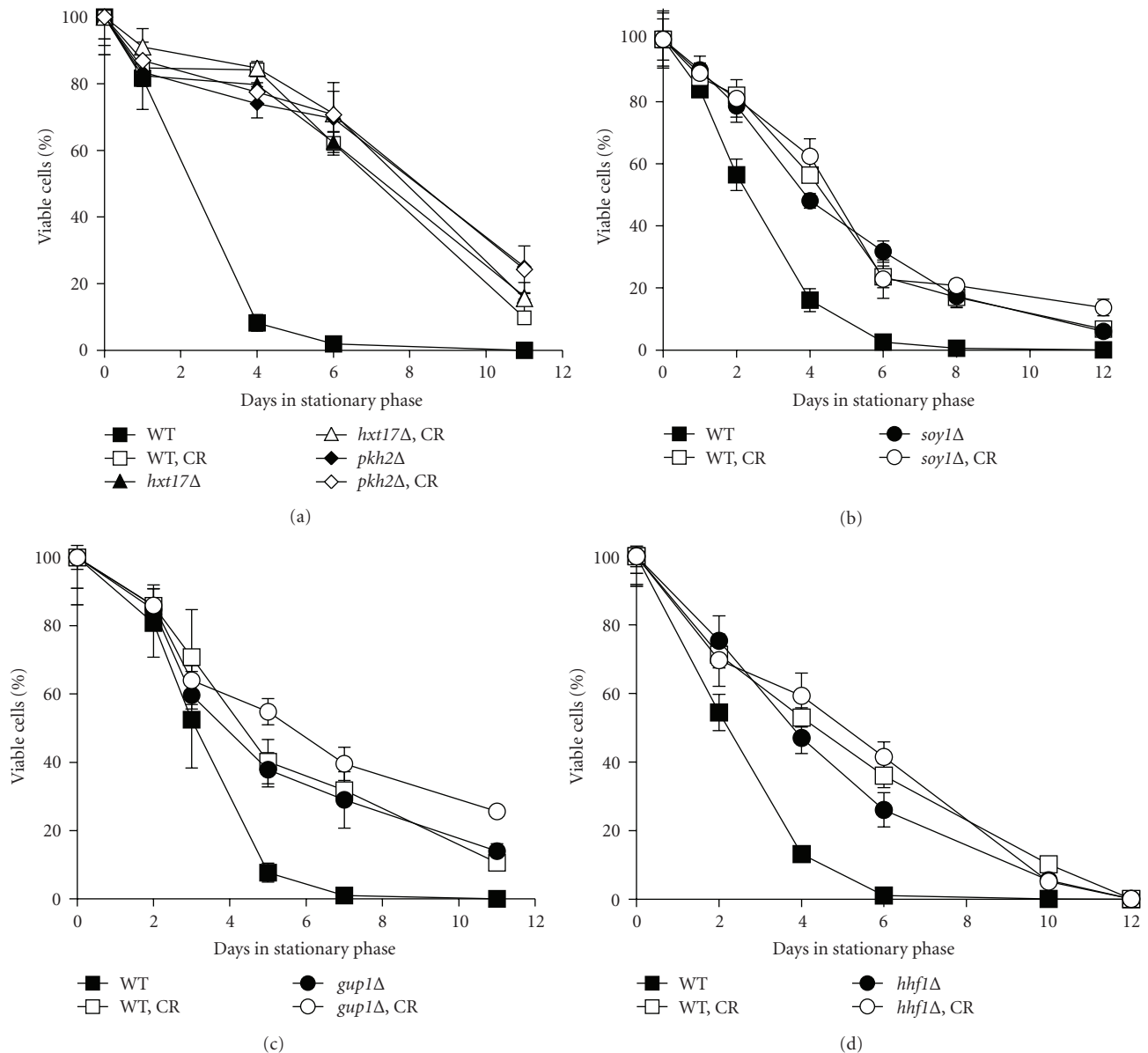
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FIGURE 2: Chronological lifespan (CLS) analysis of potential CR genetic mimics. (a)–(d) Potential CR-mimicking high-NO mutants show extended CLS, and most do not significantly further increase the CLS of cells grown in CR. (c) Although CR further increases $gup1\Delta$-induced CLS ($gup1\Delta$ versus $gup1\Delta$, CR; $P = .035$), $gup1\Delta$ does not further increase CR-induced CLS (CR versus $gup1\Delta$, CR; $P = .073$). (d) Although CR further increases $hhf1\Delta$-induced CLS ($hhf1\Delta$ versus $hhf1\Delta$, CR; $P = .02$), $hhf1\Delta$ does not further increase CR-induced CLS (CR versus $hhf1\Delta$, CR; $P = .28$). One representative set of three independent experiments, each conducted in quadruplicate, is shown. Error bars denote standard deviations. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. Numbers on the x-axes denote the number of days after entering stationary phase (nonmitotic state). Pairwise statistical analysis of CLS is shown in Supplemental Table 2.

conditions (Figure 4(f)). In contrast, calorie restriction failed to induce NO production either in the BY4742 (ρ^0), a rho^0 strain lacking mitochondrial DNA, or in the respiratory-deficient $cyt1\Delta$ mutant under normoxic conditions. These findings further support the conclusion that the NO synthesis in calorie-restricted yeast cells requires a functional mitochondrial respiratory chain. To explore this further, we examined the oxygen sensitivity of nitrite-dependent NO synthesis by mitochondria isolated from WT cells. As shown in Figure 4(g), oxygen sensitivity reported previously

for nitrite-dependent mitochondrial NO synthesis [34] was reduced in mitochondria isolated from WT cells grown under CR. These results demonstrated that yeast mitochondria are capable of NO synthesis at oxygen concentrations that are in the normoxic range experienced by yeast cells grown in colonies or in liquid culture.

Eukaryotic cells harbor both aerobic and hypoxic isoforms for several components of the electron transport chain to ensure proper electron transfer in response to different oxygen concentrations [32, 55–57]. It has been reported that,
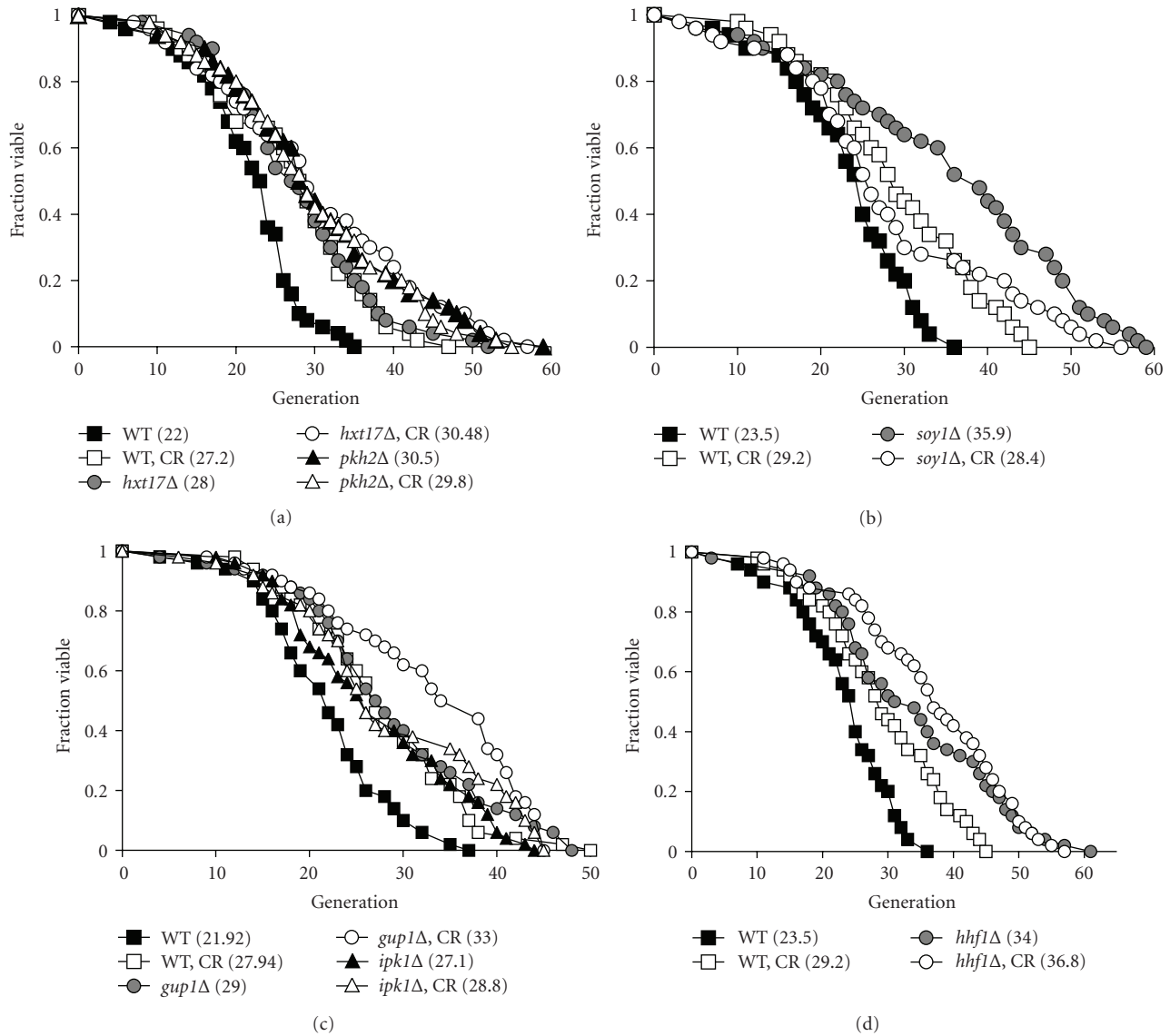
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FIGURE 3: Replicative lifespan (RLS) analysis of potential CR genetic mimics. (a) Potential CR mimicking high-NO mutants show extended RLS and do not significantly further increase the RLS of cells grown in CR. (b) CR decreases $soy1\Delta$-induced RLS ($soy1\Delta$ versus $soy1\Delta$, CR; $P = .008$); however, $soy1\Delta$ does not significantly change CR-induced RLS (CR versus $soy1\Delta$, CR; $P = .49$). (c) CR further increases $gup1\Delta$-induced RLS ($gup1\Delta$ versus $gup1\Delta$, CR; $P = .038$). $gup1\Delta$ also further increases CR-induced RLS (CR versus $gup1\Delta$, CR; $P = .004$). (d) Although $hhf1\Delta$ further increases CR-induced RLS (CR versus $hhf1\Delta$, CR; $P = .001$), CR does not further increase $hhf1\Delta$-induced RLS ($hhf1\Delta$ versus $hhf1\Delta$, CR; $P = .19$). One representative set of three independent experiments is shown. WT: BY4742 wild-type control; CR: 0.5% glucose; Δ: gene deletion. Pairwise statistical analysis of RLS is shown in Supplemental Table 3.

although COX-specific NO synthesis is strongly inhibited by oxygen when COX contains the aerobic isoform of subunit V (Va), the oxygen inhibition is relieved when COX contains subunit Vb, the hypoxic subunit V isoform [38]. Interestingly, CR increased the expression of the hypoxia inducible cytochrome c *CYC7* (Figure 4(h)) as well as *COX5b*, which encodes COX hypoxic subunit Vb (P. Castello and R. Poyton, unpublished). Therefore, CR might induce NO production in part by derepressing expression of the hypoxic isoforms of mitochondrial electron transport complexes and perhaps additional hypoxia responsive genes.

3.4. NO Detoxification Enzymes Are Partially Required for CR-Induced Lifespan Extension. Next, we examined the roles of NO detoxification enzymes Yhb1 and Sfa1 in CR. CR failed to extend RLS in the $sfa1\Delta yhb1\Delta$ mutant (Figure 5(a)), indicating that Yhb1 and Sfa1 are required for optimal NO homeostasis during CR-induced RLS. Deleting *YHB1* partially abolished CR-induced RLS ($P = .03$, CR versus CR, $yhb1\Delta$), whereas deleting *SFA1* alone had no significant effect. Therefore, it appeared that Yhb1 and Sfa1 play redundant roles under our CR/RLS assay condition. The lifespan of potential CR mimics *hxt17Δ, pkh2Δ, and*
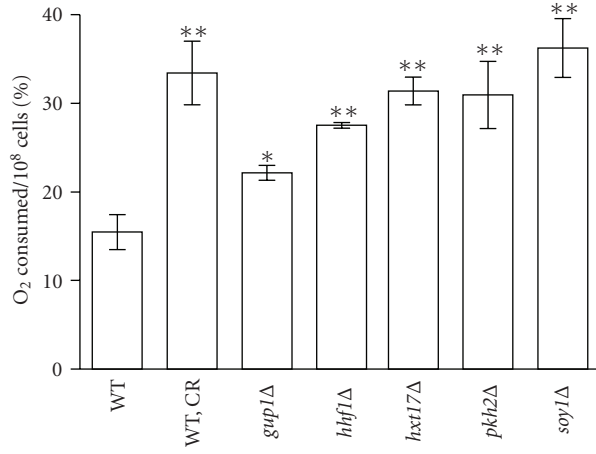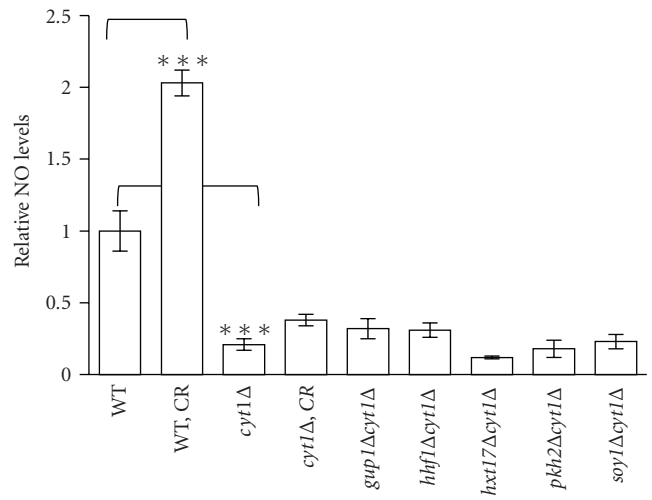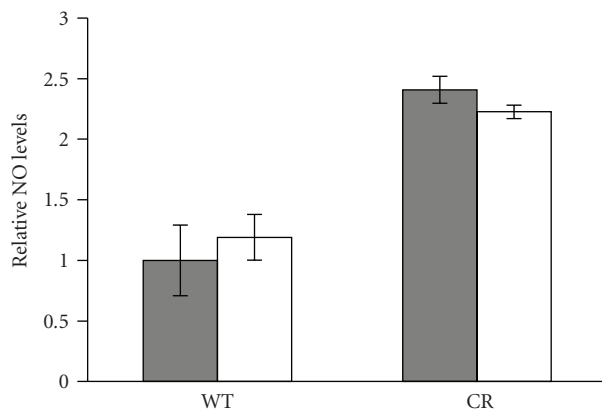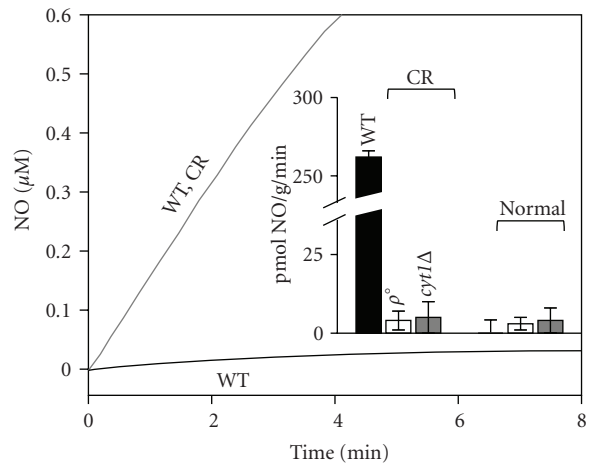
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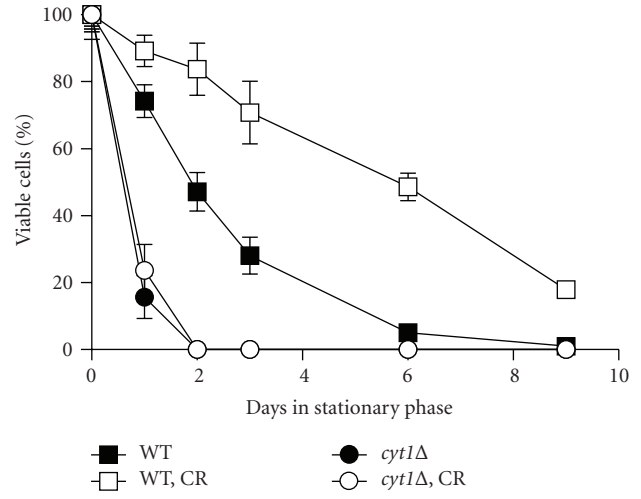
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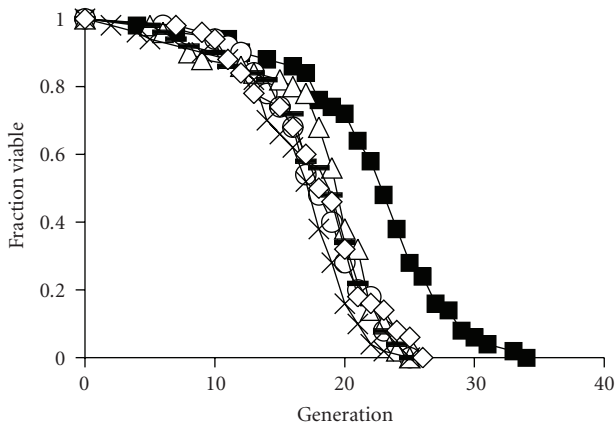
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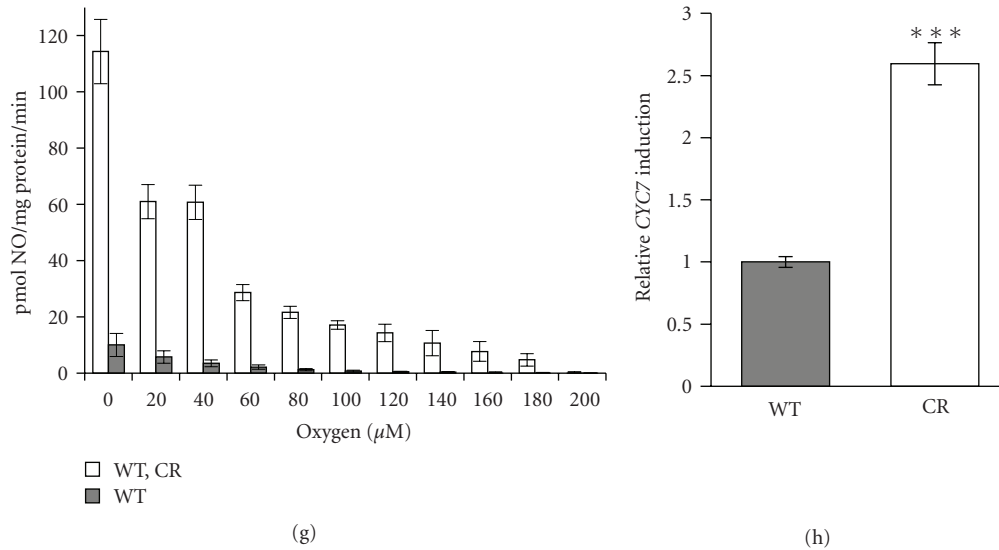

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(f)

Figure 4: Continued.

(g)

(h)

FIGURE 4: Mitochondrial respiration and NO production play important roles in CR-induced lifespan. (a) All potential CR mimic mutants show increased oxygen consumption. (b) CR-induced CLS is prevented in the $cyt1\Delta$ mutant. Numbers on x-axes denote the number of days after entering stationary phase (nonmitotic state). (c) CR mimic mutants-induced RLS is prevented in the $cyt1\Delta$ mutant. (d) Increases in NO by CR or CR mimic mutants are largely abolished in the $cyt1\Delta$ mutant. (e) CR-induced NO production is not inhibited by L-NAME, an NOS inhibitor. (f) Involvement of the respiratory chain in cellular NO production. Cells (160 mg) were suspended in 2 mL of PBS and prebubbled for 5 min with N_2 to create anoxic conditions. After 5 min of prebubbling, $NaNO_2$ was added to a final concentration of 1 mM, and NO production was measured with an NO polarographic electrode. Insert: NO production rates for BY4742 ρ^+ (black), BY4742 ρ^0 (white), and $cyt1\Delta$ (grey) for cells that were grown to log phase in 2% glucose (normal) or 0.5% glucose (CR). (g) Effects of oxygen concentrations on mitochondrial NO production. Isolated mitochondria from cells grown under normal and CR conditions were assayed for NO production in assay medium as described in the Materials and Methods Section. (h) CR increases $CYC7$ gene expression using a β-galactosidase reporter-based promoter activity assay. Original values: 39 ± 2 nmoles ONPG converted/min/mg protein for WT; 105 ± 4 nmoles ONPG converted/min/mg protein for CR. WT: BY4742 and BY4742 (ρ^+) wild-type control; ρ^0: cells lacking mitochondrial DNA; CR: 0.5% glucose. One representative set of experiment is shown. Error bars denote standard deviations. P values are calculated using Student's t-test ($^*P < .05$; $^{**}P < .01$; $^{***}P < .005$).

$hhf1\Delta$ was also reduced to WT levels in the $yhb1\Delta sfa1\Delta$ mutant (Figure 5(b)). Deleting $YHB1/SFA1$ was not likely to prevent CR-induced RLS solely by reducing mitochondrial activity. As shown in Figure 5(c), CR-induced increases in oxygen consumption and NO production were only partially reduced in the $yhb1\Delta sfa1\Delta$ mutant. Interestingly, $yhb1\Delta sfa1\Delta$ did not abolish CR-induced CLS (Figure 5(d)), suggesting that additional NO-detoxification enzymes and stress response factors might compensate for the loss of Yhb1/Sfa1 for CR-induced CLS. In line with our results, it has been reported that the stress response factors such as Rim15 and superoxide dismutases play important roles in CR-induced CLS [9, 58].

3.5. Treatment with GSNO Extends Yeast CLS. We then examined whether treating cells with NO donors was sufficient to mimic CR to extend lifespan. Interestingly, GSNO appeared to be the most effective NO donor for lifespan extension among the different NO donors tested (B. Li and S.-J. Lin, unpublished). As shown in Figures 6(a) and 6(b), both a single treatment of 25 μM S-nitrosoglutathione (GSNO) (which caused mild growth inhibition, Figure 1(c))

and multiple treatments of 5 μM GSNO (no growth inhibition observed) extended CLS to a level similar to CR. GSNO did not further increase CR-induced (Figures 6(a) and 6(b)) or CR mimics-induced (Figure 6(c)) CLS suggesting that GSNO and CR may function in the same pathway to extend CLS. Interestingly, GSNO treatments partially rescued the short CLS of the respiratory-deficient $cyt1\Delta$ mutant (Figure 6(d)), suggesting that it may partially compensate for the loss of respiration or may also confer some respiration-independent beneficial effects. For example, GSNO may extend lifespan by promoting specific protein modifications [59]. However, GSNO treatment (25 μM) did not appear to have an impact upon RLS (B. Li and S.-J. Lin, unpublished); this could be an effect of GSNO instability in the plate-based RLS assay. It is also likely that GSNO only confers partial beneficial effects of CR. Since CR confers resistance to various stresses, we examined whether GSNO might mimic CR by increasing stress resistance. As shown in Figure 6(e), cells pretreated with a low dose of GSNO were more resistant to GSNO-induced toxicity. Pretreatment of GSNO also slightly increased the resistance to hydrogen peroxide compared to calorie-restricted cells (Figure 6(f)). Therefore, GSNO may extend CLS in part by increasing stress responses,

TABLE 1: List of potential genetic mimics of CR.

Deletion	NO[a]	CLS[b]	RLS[c]	O$_2$[d]	Biological function
ypk1Δ	4.1 ± 1.4	E (0.006)	N (21.5[wt]; 23.2[mt]; 0.15)	0.8 ± 0.03	Ser/Thr protein kinase; sphingolipid signaling
tps1Δ	3.9 ± 0.9	N (0.18)	N (22[wt]; 21.4[mt]; 0.65)	1.1 ± 0.04	Trehalose biosynthesis; response to stress
reg1Δ	2.9 ± 0.4	E[e]	S (21.5[wt]; 17.6[mt]; 0.0014)	1.2 ± 0.1	Negative regulation of glucose-repressible genes
soy1Δ	2.9 ± 0.4	E (0.0024)	E (23.5[wt]; 35.9[mt]; 0.0001)	2.4 ± 0.2	Function unknown; associates with nuclear pore complex
hxk2Δ	2.5 ± 0.8	N[f]	E[g]	~3[h]	Hexokinase; glycolysis
erg4Δ	2.1 ± 0.3	E (0.005)	N (21.5[wt]; 23.9[mt]; 0.54)	1 ± 0.03	Ergosterol biosynthesis
fyv5Δ	1.8 ± 0.5	E (0.004)	N (22.3[wt]; 21.9[mt]; 0.25)	1.5 ± 0.02	Ion homeostasis
ipk1Δ	1.8 ± 0.7	N (0.07)	E (21.9[wt]; 27.1[mt]; 0.0065)	2.3 ± 0.2	Inositol phosphate biosynthesis
gup1Δ	1.7 ± 0.1	E (0.026)	E (21.9[wt]; 29[mt]; 0.0001)	1.4 ± 0.05	Remodeling the GPI anchors; glycerol transport
lip5Δ	1.7 ± 0.3	N (0.86)	E (22.5[wt]; 28.4[mt]; 0.01)	0.7 ± 0.05	Biosynthesis of lipoic acid
hhf1Δ	1.7 ± 0.4	E (0.005)	E (21.3[wt]; 27.7[mt]; 0.0001)	1.8 ± 0.02	Histone H4 proteins; chromatin assembly
dse2Δ	1.7 ± 0.1	E (0.0005)	N/A[i]	1.6 ± 0.02	Required for cell separation
avl9Δ	1.6 ± 0.1	N (0.091)	N (23.4[wt]; 26.02[mt]; 0.2)	0.9 ± 0.01	Conserved protein involved in exocytic transport
utr1Δ	1.6 ± 0.2	E (0.001)	N (22.5[wt]; 24.48[mt]; 0.52)	1 ± 0.07	Cytosolic NAD kinase
zap1Δ	1.5 ± 0.1	E (0.02)	E[j] (22.5[wt]; 29.8[mt]; 0.0124)	1.6 ± 0.1	Zinc-regulated transcription factor
pkh2Δ	1.4 ± 0.2	E (0.0035)	E (22[wt]; 30.5[mt]; 0.0001)	2 ± 0.25	Ser/Thr protein kinase; sphingolipid signaling
hxt17Δ	1.3 ± 0.1	E (0.0004)	E (22[wt]; 28[mt]; 0.0014)	2 ± 0.1	Hexose transporter

This table lists top 15 hits from our screen and the pkh2Δ and hxt17Δ mutants. Mutants listed here were first selected by their "growth" (at least 75% of WT; no severe growth defects) and by "resistance to GSNO" (at least 150% of WT) and then were ranked by NO levels.
[a]Numbers show average relative intracellular NO levels $\pm$ standard deviation (normalized to wild-type control) (for details, see Section 2). [b]CLS: chronological lifespan, E: extended; N: normal; P values are calculated using the AUC method (area under the curve) (see Section 2). Results of one set of representative experiment (each conducted in triplicate or quadruplicate, $n = 3$ or 4 independent colonies) are shown. About 2×10^8 cells were analyzed in each sample. [c]RLS: replicative lifespan; E: extended; N: normal; S: short; N/A: not determined. Numbers show (average wild-type RLS; average mutant RLS; P values). P values are calculated using the Wilcoxon rank sums tests. Results of one set of representative experiment are shown. 50 cells ($n = 50$) were analyzed for each strain in each experiment. [d]O$_2$: relative average oxygen consumption rate $\pm$ standard deviation (normalized to wild-type control). [e]Reported [53]. [f]Reported [54]. [g]Reported [6]. [h]Reported [12]. [i]N/A: not available. Cells are sticky, unable to separate. [j]Exhibits early cell death.

possibly via inducing hormesis effect. Interestingly, not all of the high-respiration activity CR genetic mimics examined showed increased resistance towards hydrogen peroxide (Figure 6(f)), although these mutants all showed increased resistance towards GSNO (Supplemental Table 1). It is possible that different mutants only partially mimic CR by activating a different subset of stress response pathway. It is also very likely that additional mechanisms play important roles in these mutants.

4. Discussion

Calorie restriction (CR) extends longevity and ameliorates age-related diseases in many organisms. Understanding how CR induces these beneficial effects may therefore provide insights into the molecular mechanisms underlying age-associated diseases. Considerable progress has been made in the discovery of genes/proteins and metabolites that affect longevity. However, many molecular aspects of CR-induced events remain unclear. Uncovering additional long-lived CR-mimicking mutants will likely contribute to the understanding of the molecular mechanisms of CR. To

identify new components in the CR pathway, we developed a nitric oxide- (NO-) based screen for mutants that showed higher intracellular levels of NO as potential CR mimics.

Nine of the top 17 high-NO mutants summarized in Table 1 show a positive correlation between increased mitochondrial respiration and NO production. There is also a positive correlation between increased respiration and longevity. These nine mutants showed increased oxygen consumption (9 out of 17) and increased CLS and/or RLS. In addition, mutants that showed both extended RLS and CLS (5 out of 17) all showed increased oxygen consumption as well as NO levels (Table 1). Two mutants (avl9Δ and tps1Δ) that did not show either extended CLS or RLS also did not show increased oxygen consumption. However, it is noteworthy that 5 long-lived mutants (with extended RLS or CLS) did not possess increased oxygen consumption.

Although CR cells have increased NO levels, a higher intracellular NO concentration is not sufficient to extend lifespan under all growth conditions and/or genetic backgrounds. For example, the avl9Δ and tps1Δ mutants have higher NO levels but do not show increased lifespan (Table 1). It is possible that increases in the stress response
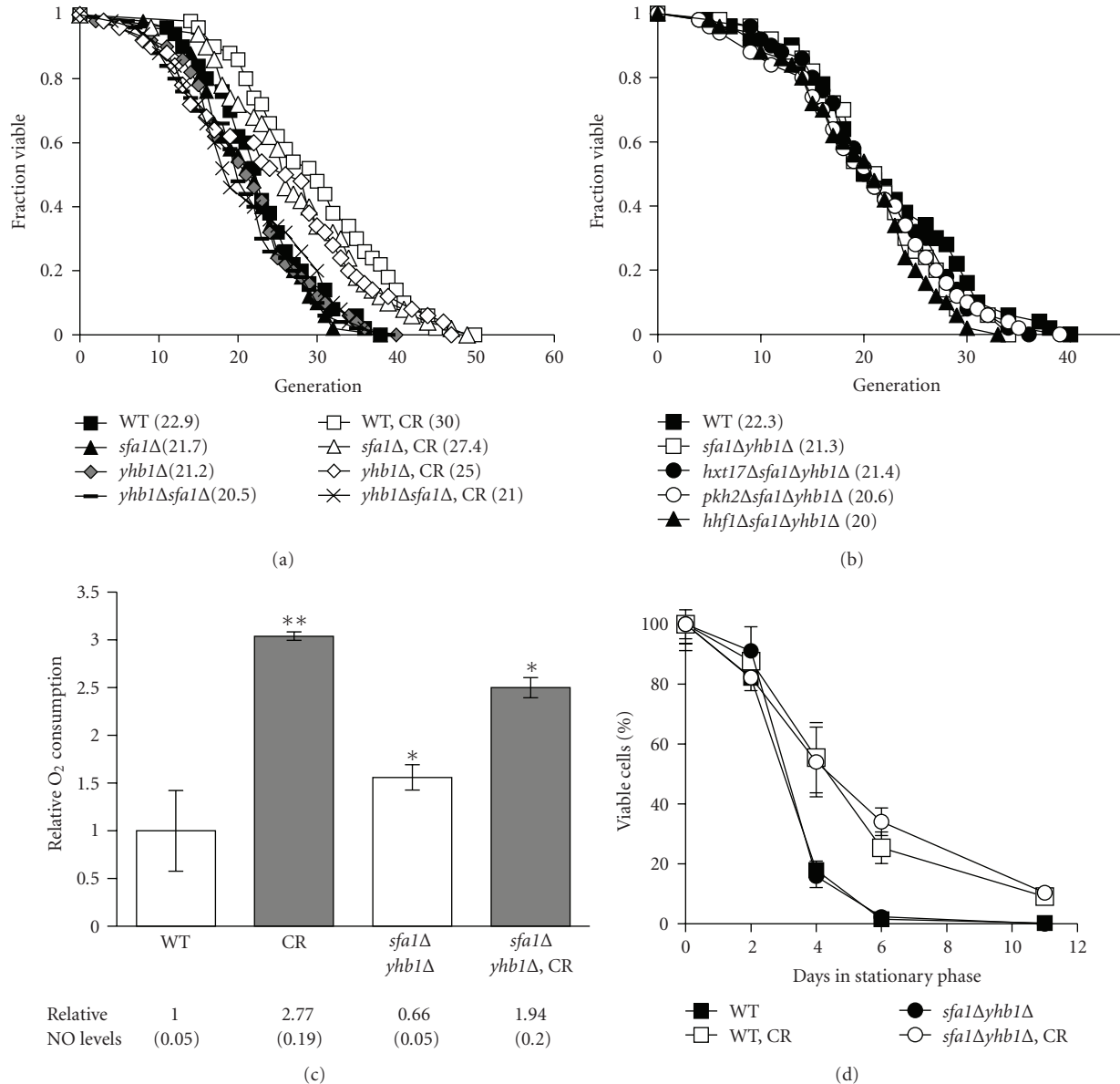
Figure 5: Balanced NO metabolism is required for CR-induced beneficial effects. (a) CR fails to extend RLS in cells lacking Sfa1 and Yhb1. (b) CR genetic mimics fail to extend RLS in cells lacking Sfa1 and Yhb1. (c) Deleting Sfa1 and Yhb1 partially affect CR-induced increase in oxygen consumption and NO levels. (d) Sfa1 and Yhb1 are not required for CR-induced CLS. One representative set of experiments is shown. Error bars denote standard deviations. P values are calculated using Student's t-test ($*P < .05$; $**P < .01$; $***P < .005$).

as well as functional mitochondria are also required for NO-induced lifespan extension. Supporting this model, our results showed that treating the respiratory deficient $cyt1\Delta$ mutant with GSNO only partially restored its CLS (Figure 6(d)). In addition, it has been shown that several stress response factors play important roles in CLS and CR-induced CLS in *S. cerevisiae* [9, 33]. In *Schizosaccharomyces pombe*, the stress-responsive MAP kinase Sty1 was demonstrated to be essential for CR-mediated CLS extension [60].

NO detoxification enzymes Yhb1 and Sfa1 appear to play an important role in CR-induced RLS (Figure 5(a)) but not CLS (Figure 5(d)). One possible explanation is that perhaps actively dividing cells are more susceptible to CR-induced NO stress. In line with this model, CR increases

NO more significantly in early growth stages during which most cells were still actively dividing (Figure 1(a)). Yhb1 and Sfa1 are dispensable for CR-induced CLS, which may be due to the fact that other NO metabolizing factors and/or stress response factors play more important roles in CR-induced CLS. Interestingly, the $sfa1\Delta yhb1\Delta$ double mutant (under non-CR conditions) showed decreased NO levels (Figure 5(c)) suggesting that functional NO detoxification is required to support NO production in yeast cells. These results highlight the differences in sensitivity towards NO production and detoxification activities for CR-induced RLS and CLS. It is possible that yeast RLS and CLS have different sensitivities towards certain NO metabolites such as peroxynitrite. Peroxynitrite is formed by the reaction
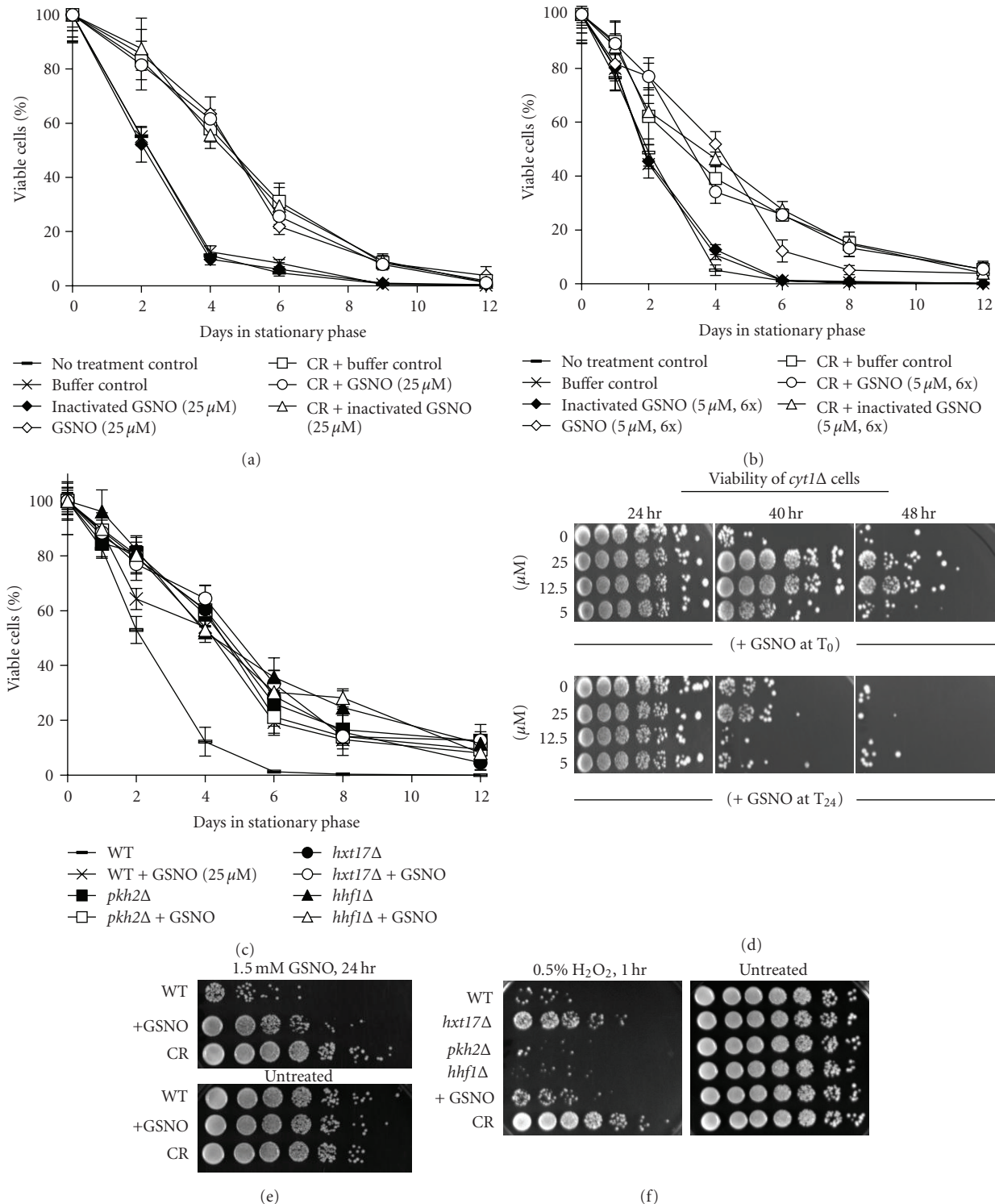
(a)

(b)

(c)

(d)

(e)

(f)

FIGURE 6: GSNO treatments extend chronological lifespan (CLS). (a and b) Treating cells with low doses of GSNO extends CLS. (a) Cells are treated with a single dose of GSNO at a final concentration of 25 μM upon starting the CLS assay (at 0 hr). (b) Cells are treated with GSNO at a final concentration of 5 μM at six different time points (6x): 0, 3, 6, 9, 18, and 24 hr. (c) GSNO treatment does not further increase the long CLS of representative CR mimic mutants. (d) GSNO treatments partially rescue the short CLS of the *cyt1*Δ mutant. Results show relative viability of cells taken from GSNO-treated CLS culture (in 5-fold serial dilutions). T_0: treating cells with GSNO upon starting CLS; T_{24}: treating cells with GSNO 24 hr after starting CLS. (e) Cells pretreated with 25 μM GSNO (+GSNO) show resistance towards GSNO-induced toxicity. (f) Cells pretreated with 25 μM GSNO (+GSNO) show mild resistance towards hydrogen peroxide-induced toxicity. WT: BY4742 wild-type control; CR: 0.5% glucose. One representative set of three independent experiments, each conducted in quadruplicate (for a, b, and c), is shown. Error bars denote standard deviations. For (a) to (c), numbers on *x*-axes denote the number of days after entering stationary phase (nonmitotic state).
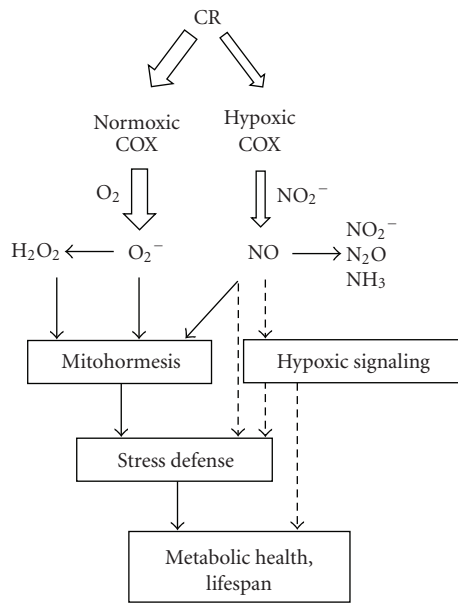
FIGURE 7: A proposed model for the roles of NO and mitochondrial respiration in CR. In yeast, the cytochrome c oxidase (COX) is likely to be responsible for CR-induced NO production. Under normoxic conditions, CR can activate both oxygen-dependent respiration and nitrite-dependent NO production, and both require the cytochrome c oxidase (COX). CR may increase NO production by derepressing expression of the hypoxic isoform subunits of COX and cytochrome c, which are normally repressed by oxygen. ROS detoxification enzymes protect cells from various ROS-associated damages and are required for certain CR-induced beneficial effects. NO and other mitochondrial ROS may induce adaptive gene expression changes as well as a metabolic shift, which optimize metabolism and improve cellular defense system against the oxidative stress that accumulates with age. For clarity and simplicity, many important longevity factors are not shown. Other pathways may work independently or in concert with mitochondrial respiration and stress response to mediate CR.

of NO with superoxide, both of which can be produced by the electron transport chain [32]. It is possible that during replicative aging, superoxide detoxification is not as efficient, making NO scavenging more important. During chronological aging, the high activity of superoxide dismutases can maintain consistently low levels of superoxide, compensating for the lack of NO scavenging. Supporting this possibility, several stress response factors including superoxide dismutase activities have been reported to be upregulated during CLS [9, 33, 58]. In addition, lower levels of superoxide have also been observed in calorie-restricted yeast cells during CLS [58]. Transcription factors associated with respiration (Hap4), stress response (Msn2), and a MAP kinase important in osmoregulation (Hog1) can drive the expression of Yhb1 [61, 62], Sfa1 [63, 64], or both and may regulate increased RLS and resistance to nitrosative stress during CR.

Our results indicate that COX is a major source of NO production under CR (Figure 7) because deleting components of the electron transport chain in the rho^0 (Figure 4(f)), $cyt1\Delta$ (Figures 4(d) and 4(f)), and COX

(B. Li and S.-J. Lin, unpublished) mutants largely abolishes CR-induced NO production and increased longevity (Figures 4(b) and 4(c)). Moreover, GSNO treatments could only partially compensate the loss of respiratory capacity (Figure 6(d)), indicating that both mitochondrial respiration and mitochondrial NO synthesis are required for optimal NO-induced longevity. Interestingly, the hypoxic isoforms of cytochrome c and COX subunit V, which are normally repressed by oxygen, are activated by CR (Figure 4(h)) (P. Castello and R. Poyton, unpublished). It is therefore possible that CR activates/de-represses factors that are normally induced by hypoxia to further augment NO production and respiration capacity. The upregulation of Cox5b in CR cells could explain both the increased rate of NO synthesis and the relaxation of oxygen sensitivity because a COX isozyme containing subunit Vb has been shown to support a higher rate of NO synthesis at high oxygen concentrations [38]. In addition, increased NO has also been suggested to function in the induction of hypoxic genes (hypoxic signaling) [32, 34, 65]. It would be interesting to determine the roles of CR-induced NO production and hypoxic signaling in mitochondrial hormesis (mitohormesis), which also plays an important role increasing stress responses and lifespan [66]. It remains unknown whether COX also plays important roles in CR-induced NO production in mammals, although rat liver mitochondrial COX and human endothelial cell COX also exhibit NO producing activity [34, 65]. In mammals, mitochondrial NO could also come from eNOS, which has been reported to physically interact with the outer mitochondrial membrane [67]. eNOS has been suggested as a mediator of CR in mice, and induction of eNOS caused both mitochondrial biogenesis and enhanced expression of the NAD-dependent protein deacetylase SIRT1 [20]. Together, these studies suggest that NO-mediated signaling and mitochondrial respiration work in concert to adapt cells to metabolic changes induced by CR leading to enhanced stress response and lifespan extension (Figure 7).

5. Conclusions

In summary, we have demonstrated that calorie-restricted yeast cells produce NO under normoxic conditions perhaps by derepressing the hypoxic subunit isoforms of COX and cytochrome c. CR-induced NO production may extend lifespan by increasing the stress response (mitohormesis). Our study may have uncovered potential novel components in the CR pathway and provided tools to analyze the interconnections between NO, mitochondrial respiration, CR, and longevity. Although the CR mimics identified in this study share similar NO levels, lifespan, and oxygen consumption phenotypes with CR, they may activate NO production and regulate mitochondrial respiration and lifespan via different mechanisms. It will be enlightening to examine these differences in future studies. Finally, we propose that CR likely confers its beneficial effects via a mitochondria-NO-mediated adaptive metabolic shift, which optimizes metabolism and at the same time improves cellular defense system against the oxidative stress that accumulates with age.

Acknowledgments

Conflict of Interests

The authors have declared that no conflicts of interest exist.

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