Supporting Information

Mesquita et al. 10.1073/pnas.1004432107

SI Materials and Methods

Chronological Lifespan. Caloric restriction (CR) was accomplished by reducing the glucose concentration from 2 to 0.5% or 0.05% in the initial culture medium. pH values of all of the conditions were monitored during chronological lifespan (CLS) experiments. For growth in buffered medium, a citrate phosphate buffer (64.2 mM Na_2HPO_4 and 17.9 mM citric acid) adjusted to pH 6.0 was added to the medium before inoculation. In a standard experiment, overnight cultures were grown in SC medium containing these different concentrations of glucose and then inoculated into flasks containing medium with the same concentration of glucose at a volume ratio of 1:3. These cultures were then incubated at 26 °C with shaking at 150 rpm. Cultures reached stationary phase 2–3 d later and this was considered day 0 of CLS. Survival was assessed by counting colony-forming units (CFUs) after 2 d of incubation of culture aliquots at 26 °C on YEPD agar plates beginning at day 0 of CLS (when viability was considered to be 100%) and then again every 2–3 d until less than 0.01% of the cells in the culture were viable.

Construction of the *CTA1* Overexpressing and *CTA1CTT1* Double Mutant Strains. To construct the *CTA1* overexpressing strain, *CTA1* was amplified by PCR with the following primers: F (CCGGTCTAGAATGTCGAAATTGGGACAAGA) and R (CCGGAAGCTTGGAGTTACTCGAAAGCTCAG) using genomic DNA isolated from *Saccharomyces cerevisiae* wild-type cells as template. The resulting fragment was cloned into the XbaI–HindlII site of the plasmid pUG35 (EUROSCARF), producing the plasmid pUG 35CTA1. The wild-type *S. cerevisiae* strain BY4742 was transformed by the lithium acetate method with plasmid pUG35CTA1 to produce the strain overexpressing *CTA1* ("*MET-CTA1*") or with the plasmid pUG35 to produce the "empty-vector" control strain.

Double *CTA1CTT1* mutant cells were obtained by *CTT1* disruption in the Δ*cta1* strain. A deletion fragment, containing *URA3* gene and *CTT1* flanking regions, was amplified by PCR using genomic DNA isolated from W303 Δ*ctt1*::*URA3* cells and the following primers: F (ATGGGGATAGAACCTCCGTTAT) and R (GAATTTAAAGTTTTCTCTGCTGG). Cells were transformed by electroporation and Δ*cta1ctt1* mutants were selected in minimal medium lacking uracil. Gene disruption was confirmed by the analysis of catalase activity in a native gel. Double mutants lacking both Cta1p and Ctt1p activity were selected.

Pharmacological Inhibition of Catalases and Glutathione Synthesis. Pharmacological inhibition of catalase was accomplished by treating cells beginning at day 0 of CLS with 10 mM 3-amino-1,2,4-triazole (3AT) (Sigma), which binds covalently to the active center of the active tetrameric heme-containing form of catalases (1, 2). Pharmacological inhibition of glutathione synthesis was accomplished by treating cells at day 0 with 1 mM L-buthionine-sulfoximine (BSO) (Sigma), which indirectly inhibits glutathione synthesis by interacting with γ-glutamylcysteine synthetase (3). The ectopic effects of hydrogen peroxide on CLS were assessed by treating non-CR (2% glucose) wild-type cells with 0, 0.2, 0.3, and 1 mM H_2O_2 (Merck) beginning at day 0 of CLS and survival was measured as described before.

FACS Analysis of Intracellular Reactive Oxygen Species. Levels of intracellular ROS were measured using dihydrorhodamine 123 (DHR) or 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes), compounds that are capable of detecting H_2O_2 (4). Briefly, aliquots were taken at selected time points and DHR was added to a final concentration of 15 μg/mL and cells were incubated for 90 min at 26 °C. For H_2DCF-DA staining cells were incubated with a final concentration of 10 μM for 90 min at 30 °C. Cells were then washed twice in PBS and analyzed by flow cytometry. FACS analysis used an EPICS XL-MCL (Beckman–Coulter) flow cytometer equipped with a 15 mW argon laser emitting at 488 nm. The green fluorescence was collected through a 488-nm blocking filter, a 550-nm long-pass dichroic with a 525 nm band pass. Data acquired from a minimum of 30,000 cells per sample at low flow rate were analyzed with the Multigraph software included in the system II software for the EPICS XL-MCL version 1.0. Intracellular superoxide anions were measured using dihydroethidium (DHE) (Molecular Probes). Aliquots of cells were collected at indicated time points and DHE was added to a final concentration of 5 μM from a 5-mM stock in DMSO. After incubation for 10 min at 30 °C, cells were washed once with 0.5 mL PBS, resuspended in 50 μL PBS, and added to 1 mL PBS. After briefly sonicating the suspension, DHE signals were measured using a FACSCaliber2 flow cytometer (BD-Biosciences) with a 488-nm excitation laser. Signals from 25,000 cells/sample were captured in FL3 (>670 nm) at a flow rate of 5,000 cells/s. FACS measurements of DHR signals presented in Fig. 2 were measured similar to the DHR measurements described for Fig. 1 except that a FACSCaliber flow cytometer was used to capture signals in FL1 (530 nm ± 15 nm) from 25,000 cells/sample at a flow rate of 5,000 cells/s. Data collected with the FACSCaliber2 flow cytometer were processed with Flowjo software (Tree Star) and quantified with WinList software (Verity Software House).

Protein Extract Preparation, Superoxide Dismutase, and Catalase Activity Assays. For determination of catalase and superoxide dismutase activities, yeast extracts were prepared in 25 mM Tris buffer (pH 7.4) containing a mixture of protease inhibitors. Protein content of cellular extracts was estimated by the method of Lowry using BSA as a standard.

Catalase activity was determined as in ref. 5. Briefly, 30 μg of proteins were separated by native PAGE and catalase activity was analyzed in situ in the presence of 3,3′-diaminobenzidine tetrahydrochloride (Sigma), using the H_2O_2/peroxidase system. The gel was incubated in horseradish peroxidase (Sigma) (50 μg/mL) in 50 mM potassium phosphate buffer (pH 6.7) for 45 min. H_2O_2 was then added to a final concentration of 5 mM and incubation was continued for 10 min. The gel was then rapidly rinsed twice with distilled water and incubated in 0.5 mg/mL diaminobenzidine in 50 mM potassium phosphate buffer until staining was complete.

Superoxide dismutase activities were measured on the basis of their ability to inhibit reduction of nitro blue tetrazolium to formazan in nondenaturing polyacrylamide gels (6). Sod2p activity was distinguished from Sod1p activity on the basis of the ability of 2 mM cyanide to inhibit Sod1p, but not Sod2p. Quantification of band intensities was performed by densitometry using Quantity One Basic software from Bio-Rad.

Determination of Oxidative Damage. To measure levels of carbonylated proteins, samples of total cell protein were derivatized by mixing aliquots with one volume of 12% (wt/vol) SDS and two volumes of 20 mM 2,4-dinitrophenylhydrazine in 10% (vol/vol) trifluoroacetic acid (a blank control was treated with two volumes of 10% (vol/vol) trifluoroacetic acid alone) (7). After incubation for 30 min at room temperature in the dark, samples were neutralized and proteins (0.15 μg) were slot blotted onto a poly-

vinylidene fluoride membrane (Hybond-PVDF, GE Healthcare). The PVDF membrane was probed with rabbit IgG anti-DNP (Dako) (1:5,000 dilution) and goat anti-rabbit IgG linked to horseradish peroxidase (Sigma) (1:5,000 dilution) by standard techniques. Detection of derivatized proteins was accomplished by chemiluminescence, using reagents contained in a RPN 2109 kit (GE Healthcare). The membranes were exposed to a Hybond-ECL film (GE Healthcare) for 15 s to 1 min, and the film was developed. Quantitative analysis of carbonyls was performed by densitometry using Quantity One Basic software from Bio-Rad.

Autofluorescence signals indicating oxidative damage to proteins and lipids were collected from 25,000 cells/sample using a FACSCaliber2 flow cytometer as described above for DHE measurements, except that cells were not stained with DHE after the PBS wash step.

Statistical Analysis. Data are reported as mean values of at least three independent assays and presented as mean ± SD or mean ± SEM. The arithmetic means are given with SD with 95% confidence value. Statistical analyses were carried out using Student's *t*-test. $^{*}P < 0.05$ was considered statistically significant.

1. Chang JY, Schroeder WA (1972) Reaction of 3-amino-1:2:4-triazole with bovine liver catalase and human erythrocyte catalase. *Arch Biochem Biophys* 148:505–508.
2. Margoliash E, Novogrodsky A, Schejter A (1960) Irreversible reaction of 3-amino-1:2:4-triazole and related inhibitors with the protein of catalase. *Biochem J* 74:339–348.
3. Griffith OW, Meister A (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J Biol Chem* 254: 7558–7560.
4. Qin Y, Lu M, Gong X (2008) Dihydrorhodamine 123 is superior to 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G in detecting intracellular hydrogen peroxide in tumor cells. *Cell Biol Int* 32:224–228.
5. Aebi H (1984) Catalase in vitro. *Methods Enzymol* 105:121–126.
6. Flohé L, Otting F (1984) Superoxide dismutase assays. *Methods Enzymol* 105:93–104.
7. Levine RL, Williams JA, Stadtman ER, Shacter E (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 233:346–357.

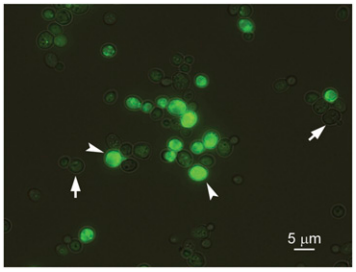

Fig. S1. Determination of dihydrorhodamine 123 (DHR) positive cells containing high levels of ROS. Photomicrograph of DHR-stained *Saccharomyces cerevisiae* wild-type cells (BY4742) from day 3 of stationary phase cultures under caloric restriction (CR) conditions (0.5% glucose). Arrowheads indicate cells displaying bright green fluorescence and correspond to cells with high intracellular ROS levels, designated DHR positive cells. Arrows indicate cells that did not stain with DHR. Cells were visualized by epifluorescence microscopy using an Olympus BX61 microscope equipped with a high-resolution DP70 digital camera and an Olympus PlanApo 60×/oil objective, with a numerical aperture of 1.42. Total magnification 600×. (Bar, 5 μm.)

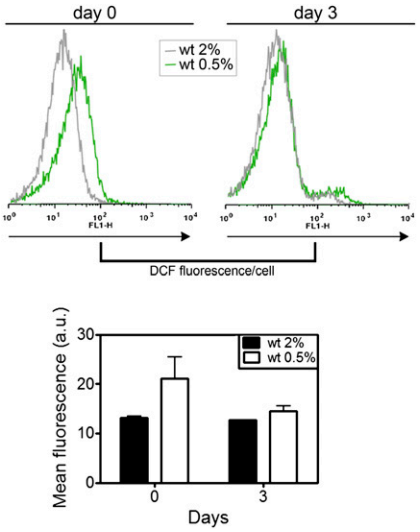

Fig. S2. Increased levels of ROS in caloric-restricted *Saccharomyces cerevisiae* wild-type cells. FACS measurements of intracellular ROS using the probe H_2DCF-DA at day 0 and day 3 of stationary phase (histograms). Bar graphs indicate mean ± SD fluorescence/cell (arbitrary units) measured in 30,000 cells/sample in three independent experiments.

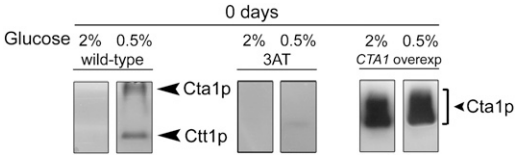

Fig. S3. Caloric restriction of stationary phase cells induces the activity of the peroxisomal catalase *CTA1*, as well as the cytosolic catalase *CTT1*. Catalase activities of wild-type *Saccharomyces cerevisiae* (BY4742) cells were increased by CR conditions beginning at day 0 of stationary phase. The pharmacological inhibition of catalase with 10 mM 3-amino-1,2,4-triazole (3AT) resulted in complete inhibition of CR-induced Cta1p and Ctt1p activity. Overexpression of *CTA1* resulted in a substantial increase in Cta1p activity.

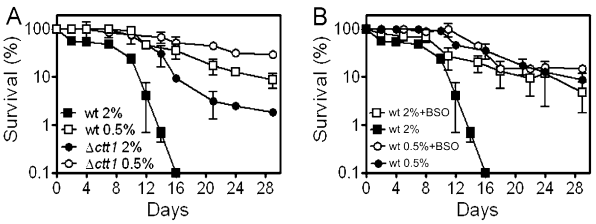

Fig. S4. CLS was also extended by mutational inactivation of *CTT1* or upon inhibition of glutathione synthesis, and the longer CLS detected under these conditions was extended further by CR. Survival of (*A*) wild-type (BY4742) and Δ*ctt1* cells and (*B*) wild-type cells in the absence or presence of 1 mM L-buthionine-sulfoximine (BSO), which indirectly inhibits glutathione synthesis. Cell viability was measured every 2–3 d starting at day 0 when cultures reached the stationary phase. Three to five biological replicas of each experiment were performed and measurements were made in three samples per experiment. Survival values indicate mean ± SD of all experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t* test.

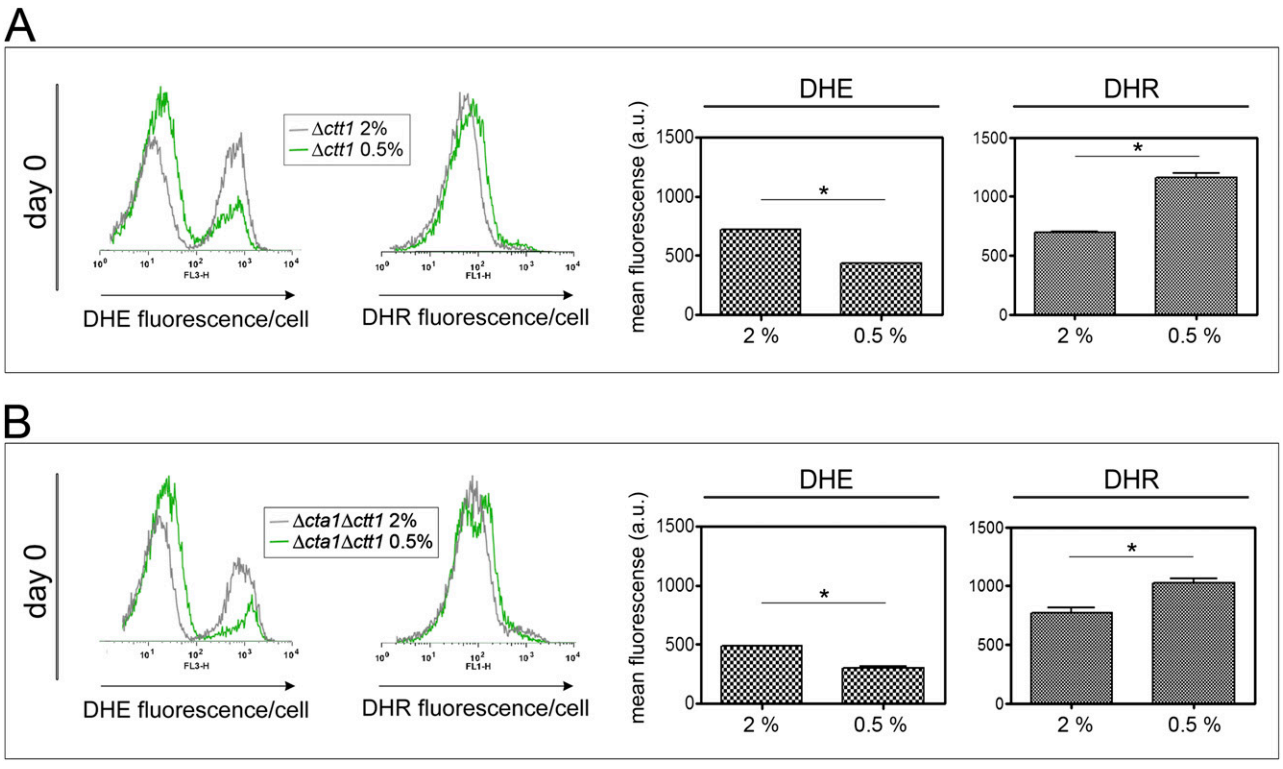

Fig. S5. Abrogation of *CTT1* or of both catalases (*CTA1CTT1*) mimics the CR longevity-promoting effects associated with high intracellular H_2O_2 levels and reduction in the chronological age-dependent accumulation of superoxide anions. FACS measurements of superoxide anions using dihydroethidium (DHE) in parallel with measurements of H_2O_2 using dihydrorhodamine 123 (DHR) at day 0 of CLS in Δ*ctt1* (*A*) and Δ*cta1ctt1* (*B*) cells. Bar graphs indicate mean ± SD fluorescence/cell (arbitrary units) measured in 30,000 cells/sample in three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t* test.

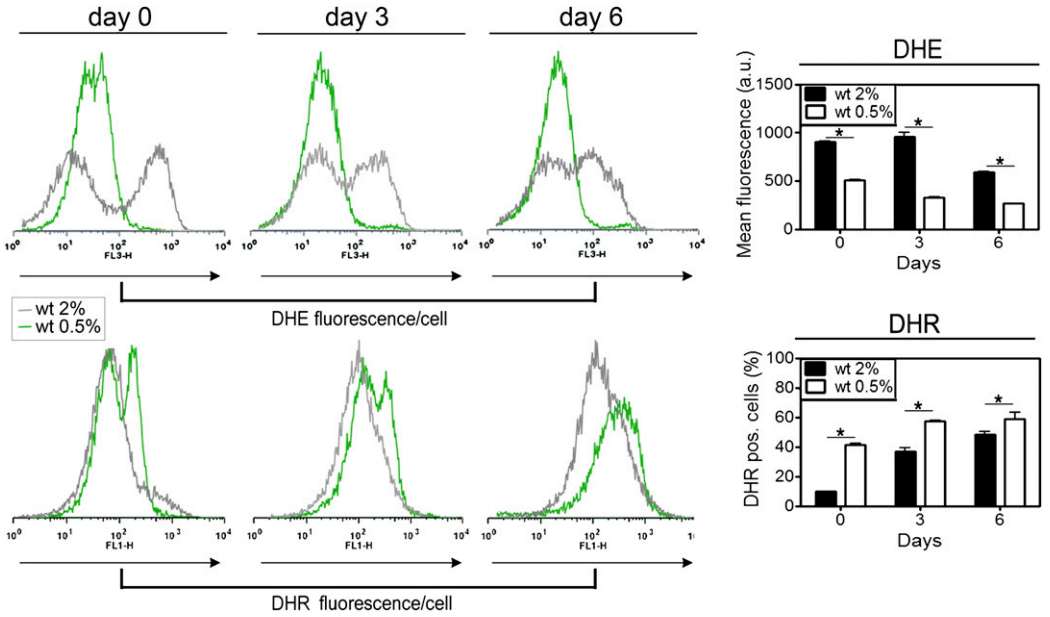

Fig. S6. CR-induced increases in intracellular H_2O_2 accompanied by a reduction in the accumulation of superoxide anions occurs independently of changes in levels of acetic acid as demonstrated in cells cultured in buffered medium. FACS measurements of superoxide anions using dihydroethidium (DHE) in parallel with measurements of H_2O_2 using dihydrorhodamine 123 (DHR) in wild-type cells cultured in buffered medium (citrate phosphate buffer, pH 6.0) at days 0, 3, and 6 of stationary phase. Bar graphs indicate mean ± SD fluorescence/cell (arbitrary units) measured in 30,000 cells/sample in three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t* test.

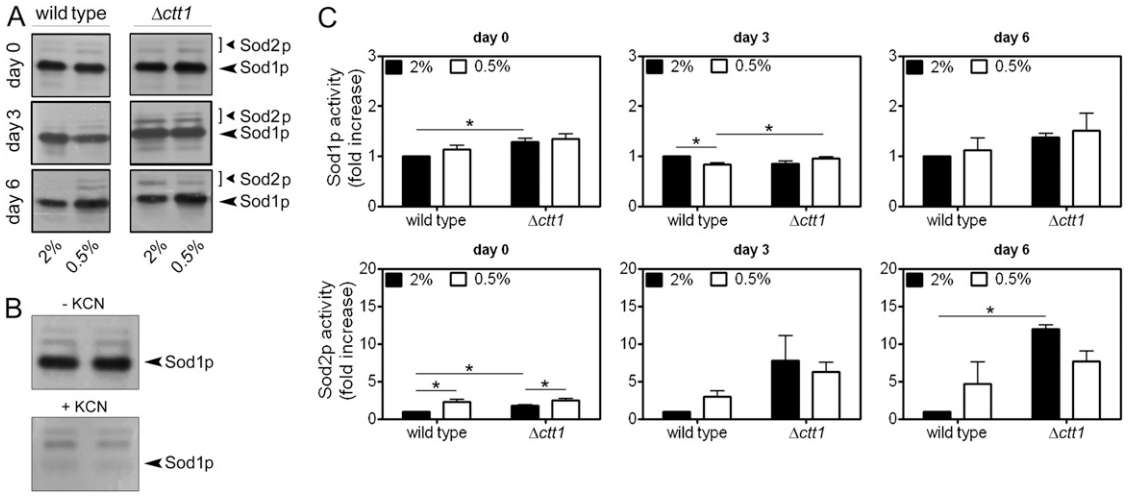

Fig. S7. Induction of superoxide dismutase activity in Δ*ctt1* cells. (*A*) In situ determination of superoxide dismutase activities, as previously described (6), in stationary phase wild-type and Δ*ctt1* cells. (*B*) MnSOD (Sod2p) activity detected in the presence of 2 mM potassium cyanide, which inhibits Sod1p activity. (*C*) Quantification of fold increase in Sod1p and Sod2p activity in wild-type and Δ*ctt1* cells. Sod1p and Sod2p activities measured at each time point were normalized to the activity of wild-type cells under non-CR conditions (2% glucose). Values indicate mean ± SEM from three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t* test.

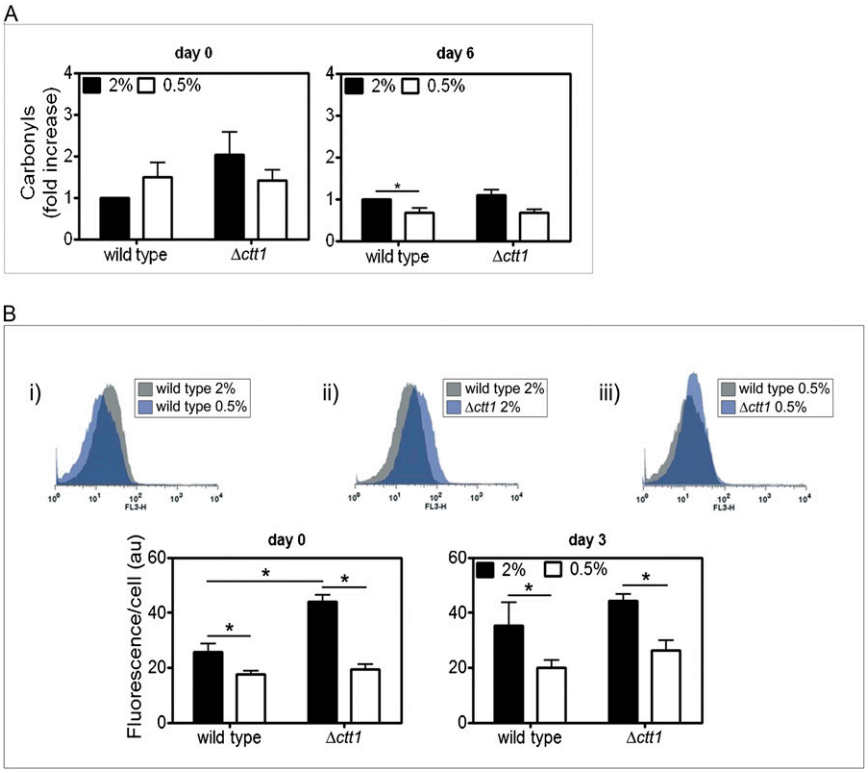

Fig. S8. Increased H_2O_2 in Δ*ctt1* cells is associated with higher levels of oxidative damage despite the increased longevity of these cells. (*A*) Oxidative damage was measured as the fold increase in levels of oxidized proteins (carbonyls) (7) in stationary phase wild-type and Δ*ctt1* cells under non-CR and CR conditions. The levels of carbonyls measured at each time point were normalized to those of wild-type cells under non-CR conditions (2% glucose). (*B*) Determination of oxidative damage to proteins and lipids indicated by autofluorescence of stationary phase wild-type and Δ*ctt1* cells under non-CR and CR conditions. Histograms are representative of data collected at day 3. Values indicate mean ± SEM from three independent experiments. Statistical significance (*$P < 0.05$) was determined by Student's *t* test.

Table S1. pH of aging cultures

	SC 2%	SC 0.5%
BY4742	2.95 (± 0.02)	3.16 (± 0.02)
BY4742 Δ*cta1*	2.93 (± 0.01)	3.10 (± 0.03)
BY4742 Δ*ctt1*	2.93 (± 0.00)	3.26 (± 0.06)
BY4742 Δ*cta1ctt1*	2.96 (± 0.00)	3.16 (± 0.01)

Cells were inoculated into the indicated medium and pH was measured at 72 h of aging. Data are presented as mean pH of three biological replicates with SD in parentheses. SC, synthetic complete medium.