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## Low doses of DNA damaging agents extend *Saccharomyces cerevisiae* chronological lifespan by promoting entry into quiescence

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### Abstract

A variety of mild stresses have been shown to extend lifespan in diverse species through hormesis, which is a beneficial response to a stress or toxin that would cause a negative response at a higher exposure. Whether particular stresses induce hormesis can vary with genotype for a given species, and the underlying mechanisms of lifespan extension are only partly understood in most cases. We show that low doses of the DNA damaging or replication stress agents hydroxyurea, methyl methanesulfonate, 4-nitroquinoline 1-oxide, or Zeocin (a phleomycin derivative) lengthened chronological lifespan in *Saccharomyces cerevisiae* if cells were exposed during growth, but not if they were exposed during stationary phase. Treatment with these agents did not change mitochondrial activity, increase resistance to acetic acid, ethanol, or heat stress, and three of four treatments did not increase resistance to hydrogen peroxide. Stationary phase yeast populations consist of both quiescent and nonquiescent cells, and all four treatments increased the proportion of quiescent cells. Several mutant strains with deletions in genes that influence quiescence prevented Zeocin treatment from extending lifespan and from increasing the proportion of quiescent stationary phase cells. These data indicate that mild DNA damage stress can extend lifespan by promoting quiescence in the absence of mitohormesis or improved general stress responses that have been frequently associated with improved longevity in other cases of hormesis. Further study of the underlying mechanism may yield new insights into quiescence regulation that will be relevant to healthy aging.

### Keywords

Aging; Chronological lifespan; DNA damage; Hormesis; Quiescence; *Saccharomyces cerevisiae*

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### Conflict of interest

The authors declare no conflict of interest.

## 1. Introduction

Hormesis is a beneficial response in cells or organisms triggered by a low dose of a toxin or a mild stress that would otherwise have a negative effect at a higher dose/exposure (Calabrese et al., 2015). Extension of lifespan due to hormesis has been observed in many organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammalian cells (Cypser and Johnson, 2002; Khazaeli et al., 1997; Le Bourg et al., 2000; Lithgow et al., 1995; Mesquita et al., 2010; Moskalev, 2007; Pan et al., 2011; Perez et al., 2008; Rattan, 1998). A variety of stressors have been used to induce hormetic responses, including toxic chemicals, natural compounds in plants, radiation, heat or cold stress, oxidative stress, and hypergravity (Cypser and Johnson, 2002; Fang et al., 2017; Hunt et al., 2011; Khazaeli et al., 1997; Le Bourg et al., 2000; Moskalev, 2007; Pan et al., 2011; Schmeisser et al., 2013). However, genetic background and gender can influence whether lifespan effects are observed (Defays et al., 2011; Henten et al., 2016; Le Bourg et al., 2000; Perez-Benito, 2006; Rodriguez et al., 2012), and lifespan extension is not always consistently observed across multiple studies for a given stressor (Lagisz et al., 2013). Further studies of conditions that induce hormesis, the influences of genotypic variation, and the underlying mechanisms are necessary to better understand the potential for it to be used as a strategy to promote healthy aging.

Hormesis induced by one stressor often leads to improved resistance to other stresses, which may at least partly account for some hormetic effects (Anderson et al., 2016; Cañuelo et al., 2012; Le Bourg, 2011; Rattan, 2004; Schmeisser et al., 2013). There are examples of mild stresses inducing expression of heat shock proteins, oxidative stress response pathways, and autophagy to increase stress resistance and lifespan (Cañuelo et al., 2012; Fang et al., 2017; Hercus et al., 2003; Hunt et al., 2011; Kumsta and Hansen, 2017; Zuo et al., 2013). Calorie restriction and physical exercise, considered as mild stresses, stimulate mitochondrial metabolism, production of reactive oxygen species (ROS), and ROS defense mechanisms to promote longevity or characteristics of healthy aging (Ristow and Zarse, 2010). This has led to the development of the mitohormesis concept to explain some forms of hormesis. This theory states that increased mitochondrial respiration and ROS production protect against stresses to extend lifespan (Ristow and Zarse, 2010; Yun and Finkel, 2014). While some studies of hormesis-induced lifespan extension have noted increases in mitochondrial activity, transient increases in ROS, and increased activity of oxidative stress response pathways (De Haes et al., 2014; Fang et al., 2017; Pan et al., 2011; Schmeisser et al., 2013), decreasing mitochondrial function can also increase lifespan in certain contexts (Baumgart et al., 2016; Lee et al., 2003). Mitohormesis or increased overall stress resistance may account for certain examples of hormesis, but there is a lack of evidence that any one particular mechanism is universally required for various stressors to extend lifespan through hormesis. Autophagy, heat-shock proteins, and mitochondrial function are relevant to aging and aging-related diseases independent of hormesis (Goloubinoff, 2016; López-Otín et al., 2013; Schiavi and Ventura, 2014), and further studies of hormesis will provide additional understanding of how stress responses and mitochondrial function are relevant to aging.

*Saccharomyces cerevisiae* has been a useful model organism for characterizing conserved factors relevant to aging (Longo et al., 2012; Ruetenik and Barrientos, 2015). Yeast

chronological lifespan (CLS) is a measure of how long cells remain viable during stationary phase in nutrient-depleted medium (Longo et al., 2012). Yeast stationary phase populations consist of a nonquiescent cell subpopulation that has not appropriately adapted for quiescence and a quiescent cell subpopulation that has arrested in G1/G0 and appropriately adapted for quiescence (Allen et al., 2006; De Virgilio, 2012). Quiescent cells have improved stress-resistance, long-term viability, and re-entry into the cell cycle in the presence of fresh nutrients compared to nonquiescent cells (Allen et al., 2006; De Virgilio, 2012). There are some examples of conditions that extend yeast CLS by triggering hormesis. Calorie restriction or a low dose of hydrogen peroxide extended yeast CLS through hormesis, and both of these effects were associated with higher hydrogen peroxide levels, higher superoxide dismutase activity, and reduced superoxide levels (Mesquita et al., 2010). In another example, extension of CLS by treatment of growing yeast cells with menadione resulted from the generation of mitochondrial ROS that signaled through nuclear DNA damage response kinases to repress subtelomeric DNA transcription (Pan et al., 2011; Schroeder et al., 2013).

Here we report that low doses of three DNA damaging agents and an inhibitor of DNA replication extend yeast CLS when cells are exposed during growth. Three of these four agents decreased ROS levels at late exponential phase, but none of them decreased ROS at the start of stationary phase, and none of the agents changed mitochondrial membrane potential, indicating that mitohormesis may not account for their effect. The treatments did not improve resistance to multiple stresses, but did consistently promote appropriate entry into quiescence during stationary phase. Several mutants that prevented one of these treatments from increasing the proportion of quiescent stationary phase cells also prevented that treatment from extending lifespan. These data indicate that DNA damaging agents can produce a hormetic extension of yeast lifespan that depends on regulation of quiescence, rather than general stress responses or changes in mitochondrial activity.

## 2. Methods

### 2.1. Yeast strains and media

Yeast strains were grown in standard rich (YPD) or synthetic complete (SC) medium with 2% (w/v) glucose (Amberg et al., 2005). For some experiments SC medium contained a final concentration of 1× phosphate-buffered saline (PBS). Treatments with DNA damaging or replication stress agents included chronic exposure beginning on day zero to 200 μM (0.0022%, v/v) methyl methanesulfonate (MMS), 30 mM hydroxyurea (HU), 0.5 μg/ml Zeocin, or 0.2 μg/ml 4-nitroquinoline 1-oxide (4NQO), except where noted otherwise in the results text and figures. Day zero refers to the day that cells were first inoculated into media. All experiments were performed using *S. cerevisiae* strain JC5516, which is a derivative of the BY4741 background (*MATa his3 1 leu2 0 met15 0 ura3 0*) that has a partial deletion allele of *kanMX* in place of the *TRP1* open reading frame (*trp1::kanMX NsiI*), a partial deletion of *CAN1* at its normal location (*can1 1*, lacking positions 84-1427 of the open reading frame), an insertion of *TRP1* on the left arm of chromosome VIII, and insertions of *CAN1*, *HIS3*, and *URA3* on the right arm of chromosome VIII, as previously described (Maxwell et al., 2011). Mutant derivatives of JC5516 with single gene deletions

of *ATG1*, *ATP10*, *DCC1*, *ECM27*, *EDC3*, *LSM1*, *MSS51*, *MTL1*, *RAS2*, *SIR2*, *SSD1*, or *TOR1* were generated by one-step gene replacement of the wild type allele with a *kanMX* cassette amplified from the corresponding *S. cerevisiae* *MATa* deletion collection strain (Thermo Scientific Open Biosystems) using lithium acetate transformation. Presence of the *kanMX* cassette and absence of the wild type allele was verified by PCR in each case, and experiments were conducted with two or three independent transformants for each genotype.

## 2.2. Chronological aging experiments

Triplicate cultures of each strain were inoculated at 5000 cells/ml in YPD, SC, or buffered SC medium with or without the treatments described in Section 2.1. Cells were grown at 30 °C either in 5 ml of medium in glass culture tubes on rotators or in 50 ml of medium in 250 ml flasks in shaking incubators. Day zero was the day that cells were inoculated into media. Cultures were sampled initially at day two or three, and then every three to seven days thereafter until the viability of the cell populations was < 10%. Viability was determined using trypan blue dye exclusion and examination of cells on hemacytometers by microscopy, as described previously (Maxwell et al., 2011). Trend lines were fit to graphs of viability to determine the days to 50% and 10% viability for each set of triplicate cultures as representations of median and maximum lifespan, respectively.

## 2.3. Cell doubling times and percentages of budding cells

For measuring doubling times, 5000 cells/ml were inoculated into YPD medium in duplicate with or without DNA damaging agents (Section 2.1) and grown at 30 °C. Cell densities were determined by counting cells on hemacytometers periodically over the course of 20–24 h. Exponential trend lines were fit to plots of cell densities versus the number of hours, and doubling times in hours were calculated as the natural log of two divided by the coefficient for the exponent from the trend line equation. Cells grown in duplicate cultures for three days using the same conditions described in Section 2.2 were diluted into 20 mM EDTA pH 8 and vortexed for 5 min to separate clumped cells prior to determining the percentage of budded cells by microscopy. Approximately 200 cells were examined per sample and categorized as having no, small (< 50% the size of the mother cell), or large (> 50% the size of the mother cell) buds.

## 2.4. *CAN1* and *URA3* mutation frequencies

*CAN1* and *URA3* mutation frequencies were assayed for cells grown for three days in YPD broth from an initial density of 5000 cells/ml with or without the DNA damaging agents described in Section 2.1. Cells were diluted in water and spread onto YPD medium to determine colony forming units per ml (cfu/ml), and appropriate volumes of cells were pelleted and suspended in water to spread onto SC medium with 1 mg/ml 5-fluoroorotic acid (FOA) or SC medium lacking arginine with 60 µg/ml canavanine to select for cells with loss of function mutations in *URA3* or *CAN1*, respectively (Boeke et al., 1984; Whelan et al., 1979). Plates were incubated at 30 °C for 2–3 days before counting colonies, and frequencies were determined by dividing the number of colonies formed on selective medium by the number of cfu spread onto the medium. The mean of seven replicate cultures was determined for each trial.

## 2.5. Examination of ROS levels and mitochondrial membrane potential

Cells grown in duplicate in YPD medium with or without DNA damaging agents as described in Sections 2.1 and 2.2 were sampled on days one and three. Samples of  $10^7$  cells were washed twice with  $1\times$  PBS, suspended in PBS containing  $50\ \mu\text{M}$  of either dihydroethidium (DHE) or dihydrorhodamine (DHR) and incubated in the dark on a shaker for 1 h at room temperature to measure ROS, or suspended in PBS containing  $200\ \text{nM}$  3,3'-dihexyloxycarbocyanine iodide ( $\text{DiOC}_6$ ) and incubated in the dark for 30 min at  $30\ ^\circ\text{C}$  to measure mitochondrial membrane potential. After incubation, cells were washed two times with  $1\times$  PBS, suspended in PBS, and analyzed on a BD LSRII flow cytometer using excitation at 488 nm and a 530/30 nm (long pass 505) emission filter for DHR or  $\text{DiOC}_6$ , or excitation at 515 nm and a 610/20 nm (long pass 600) emission filter for DHE. The mean fluorescence intensity for each sample was determined using FlowJo version 10.2 software (TreeStar) and normalized to the mean fluorescence intensity of the corresponding unstained sample.

## 2.6. Stress resistance assays

Cells were grown in YPD medium with or without DNA damaging agents as described in Section 2.2. To test resistance to moderate stresses, triplicate cultures were grown at  $30\ ^\circ\text{C}$  to mid-to-late exponential phase (20–24 h) for each treatment and cells were exposed to  $50\ ^\circ\text{C}$  for 7 min, 15% (v/v) ethanol for 20 min at room temperature, 3 mM hydrogen peroxide at  $30\ ^\circ\text{C}$  with shaking for 1 h, or 100 mM acetic acid at  $30\ ^\circ\text{C}$  with shaking for 1.5 h. Mock-treated and treated cells were diluted and spread onto YPD agar to determine the number of cfu/ml. Relative cfu/ml values were obtained by dividing values for the treated samples by values for the mock samples. To test resistance to severe stresses, cells were grown at  $30\ ^\circ\text{C}$  in duplicate to late exponential phase (24 h) and exposed to 20% (v/v) ethanol at room temperature with shaking for 1 h or incubation at  $52\ ^\circ\text{C}$  for 45 min. For these severe stresses, cells were diluted in water and incubated with trypan blue to directly determine viability, as described in Section 2.2.

## 2.7. Fractionation of quiescent cells and plating efficiency

Duplicate cultures grown for four days in YPD with or without DNA damaging agents as described in Sections 2.1 and 2.2 were separated into quiescent and nonquiescent cell populations using a previously reported Percoll density gradient fractionation method (Allen et al., 2006). The quiescent and nonquiescent fractions were separately collected by pipetting from the top of the gradient and washed 2–3 times in water before counting cells on hemacytometers by microscopy to determine cell densities and calculate the percentage of quiescent cells for each population. Cells from each fraction were diluted in water and spread onto YPD agar in duplicate. Plates were incubated at  $30\ ^\circ\text{C}$  for 2–3 days before counting colonies. Plating efficiency was determined by dividing the number of colonies formed on each plate by the number of cells spread on the plate.

## 2.8. Statistical analysis

Significant differences in mean values were determined using unpaired two-tailed *t*-tests assuming either equal or unequal variance, as appropriate for the data sets being compared.

The levels of significance are indicated in figure legends. All experiments were repeated at least three times, and the number of trials is indicated in figure legends.

### 3. Results

#### 3.1. Exposure of dividing cells to low doses of DNA damaging agents in rich medium extends chronological lifespan

During initial experiments, we observed that treatment of *S. cerevisiae* cell populations with low concentrations of DNA damaging agents appeared to extend chronological lifespan (CLS). To further investigate this effect, we grew a wild type strain from the BY4741 background (JC5516) with chronic exposure to 200  $\mu$ M (0.0022% v/v) methyl methanesulfonate (MMS, an alkylating agent), 0.5  $\mu$ g/ml Zeocin (a phleomycin derivative, induces DNA breaks), 30 mM hydroxyurea (HU, inhibits ribonucleotide reductase), or 0.2  $\mu$ g/ml 4-nitroquinoline 1-oxide (4NQO, a UV mimetic). Day zero for these experiments refers to the day when cells were inoculated into media at low densities (Section 2.2), on day one cells were in late exponential phase, and on day three cell densities plateaued. CLS was significantly increased when cells were inoculated into rich medium (YPD) with any of the four treatments (day zero exposure) and grown to stationary phase, as measured by trypan blue dye exclusion (Fig. 1A). Days to reach 50% and 10% viability in these populations were calculated as representations of median and maximum CLS, respectively. The four treatments resulted in 63–83% increases in median lifespan and 36–52% increases in maximum lifespan (Fig. 1B).

We further examined this effect by testing the timing of the treatments and whether the effect would be observed in synthetic complete medium (SC). Cells were exposed from the time of inoculation throughout their aging for the initial experiments, so CLS extension could have resulted from effects during the growth phase, stationary phase, or both phases. We repeated the CLS experiments but did not expose cells to treatments until day three, at which point they had reached stationary phase and many cells were in G1/G0 phase of the cell cycle. No increase in CLS was observed when cells were treated beginning on day three after their inoculation into YPD (Fig. 1C). Growth in SC medium substantially reduces CLS due to the accumulation of acetic acid in the culture medium (Burtner et al., 2009). CLS was not increased when cells were treated on day zero in SC medium, and lifespans were overall much shorter than those observed in YPD, as expected (Fig. 1D, note change in y-axis scale for SC experiments). To test whether this was due to reduced medium pH as a result of accumulation of acetic acid, the experiments were repeated with the addition of phosphate-buffered saline (PBS) to the SC medium. In this case, MMS treatment significantly extended median and maximum lifespan by 50% and 35%, respectively, and HU extended maximum lifespan by 30% (Fig. 1E). No changes were observed with Zeocin or 4NQO (Fig. 1E), indicating that low pH due to acetic acid only partially accounts for the different results between YPD and SC media.

### 3.2. Treatments with DNA damaging agents slow growth, increase the proportion of budded cells, and increase mutation frequencies

We characterized the effects of the treatments on growth and mutation accumulation to begin to explore the factors responsible for the increased CLS when cells were exposed on day zero in YPD. The doubling times of cell populations exposed to the treatments in YPD medium moderately increased, except for the 4NQO treatment, in which case doubling time increased just over two-fold (Fig. 2A). However, the much greater effect of 4NQO on doubling time was not correlated with a greater change in CLS (Fig. 1B). All four treatments led to similar increases in the percentage of cells with small buds at the start of stationary phase in YPD, but there was no change in the percentage of cells with large buds (Fig. 2B). In contrast, when cells were treated in SC medium, there was no difference in the percentage of cells with small buds for most treatments, but there was a moderate increase in cells with large buds (Fig. 2C). These results are consistent with more cells being arrested in or delayed in progression through S phase in YPD or G2/M phase in SC. The frequencies of loss of function mutations in the *CAN1* and *URA3* genes, each of which is present on the right arm of chromosome VIII in strain JC5516 (Maxwell et al., 2011), were measured in cells grown to early stationary phase with or without the DNA damage/replication stress agents. *CAN1* mutations in this strain were previously shown to result primarily from non-allelic recombination events (Maxwell, 2016), and were elevated 1.9–6.2 fold by all treatments except MMS (Fig. 2D). *URA3* mutations in this strain were previously shown to result primarily from point mutations (Maxwell, 2016), and were elevated 3.9–37 fold by the treatments (Fig. 2E). These data indicate that the doses of these agents were high enough to influence cell cycle progression and mutation accumulation, but there was no correlation between the magnitude of the increase in mutation accumulation and the amount of lifespan extension.

### 3.3. Stress resistance is not improved and ROS levels are not decreased at stationary phase by exposure to the DNA damaging agents

Treatments that extend lifespan through hormesis are often associated with increased stress resistance (Anderson et al., 2016; Le Bourg, 2011; Rattan, 2004), so we tested whether treated cells exhibited a general increase in stress resistance. We tested a range of moderate to severe stresses to be sure that effects would not be missed because of testing stresses that were too mild or severe. Cells were grown to mid-to-late exponential phase (20–24 h) and then exposed to one of four moderate stresses: 50 °C for 7 min, 15% ethanol for 20 min, 3 mM hydrogen peroxide for 1 h, or 100 mM acetic acid for 1.5 h. Acetic acid stress was included since acetic acid accumulates during chronological aging, dependent on the media conditions (Burtner et al., 2009). Treated and mock-treated cells were spread onto YPD medium to determine colony-forming units (cfu)/ml as a measure of cell reproductive potential, and values are reported as relative to mock-treated samples. Incubation at 50 °C and with 3 mM hydrogen peroxide strongly reduced relative cfu/ml to 1.0% and 12%, respectively, for the control cultures (Fig. 3A, note log scale for y-axis). None of the treatments with DNA damaging or replication stress agents increased resistance to the heat stress and only HU treatment moderately increased resistance to hydrogen peroxide (Fig. 3A). The incubations with ethanol and acetic acid moderately decreased relative cfu/ml to

85% and 57%, respectively, for the control cultures (Fig. 3B). None of the four treatments affected resistance to these two stresses (Fig. 3B).

Cells were also grown to late exponential phase (24 h) and exposed to severe stresses: 20% ethanol for 1 h or 52 °C for 45 min. Cell viability was directly assayed by trypan blue dye exclusion, and mean viability of the untreated cells was 79% or 47% for the ethanol or heat stress, respectively. None of the treatments improved resistance to either stress (Fig. 3C). In contrast, 4NQO reduced resistance to ethanol, and all four treatments reduced resistance to the heat stress. Based on the results for all the moderate and severe stresses tested, increased general stress resistance does not account for the effect of these treatments on CLS.

We tested whether ROS levels and mitochondrial membrane potential were altered by the four treatments, since changes in ROS and mitochondrial function contribute to hormesis in other contexts (Pan et al., 2011; Schmeisser et al., 2013; Yun and Finkel, 2014). Superoxide levels measured by DHE fluorescence were significantly reduced by MMS and HU at late exponential phase (Day 1) and significantly increased by HU at stationary phase (Day 3, Fig. 4A). All treatments except Zeocin significantly reduced DHR fluorescence, a measure of peroxide levels, at late exponential phase, while HU modestly increased DHR fluorescence at stationary phase (Fig. 4B). Since the four treatments did not consistently alter ROS and none of them decreased ROS at the start of stationary phase, either ROS changes are not crucial to the lifespan extension, or the different agents are working through different mechanisms that do not all depend on ROS. We observed no significant changes in mitochondrial membrane potential measured by DiOC<sub>6</sub> fluorescence at either late exponential or stationary phase for any of the treatments (Fig. 4C).

#### 3.4. Exposure to the DNA damaging agents promotes entry into quiescence

Stationary phase yeast cell populations consist of cells that have correctly adapted to the depletion of nutrients (quiescent) and cells that have not correctly adapted (nonquiescent), and quiescent (Q) cells live longer than nonquiescent (NQ) cells (Allen et al., 2006; Li et al., 2009). We tested whether the four treatments increased the proportion of Q cells in stationary phase cultures by separating NQ and Q cell sub-populations using a previously reported Percoll density gradient fractionation method (Allen et al., 2006). Cells are usually grown for seven days prior to fractionation (Allen et al., 2006; Davidson et al., 2011; Li et al., 2013), but the original report of this method showed that distinct fractions could be observed after 24–36 h of growth (Allen et al., 2006). Our cultures were initiated at low densities, and we observed consistent fractionation of Q and NQ cells after four days of growth at 30 °C. We used cells grown for four days for all of our fractionations so that they would be closer to the point at which we measured budding, mutation frequencies, ROS, and mitochondrial membrane potential.

All four treatments increased the proportion of quiescent (Q) cells when cells were exposed beginning on day zero in YPD medium (Fig. 5A). The plating efficiency (ability to form colonies on fresh medium) after growth to stationary phase in YPD medium was much higher for Q cells compared to NQ cells, as expected (Fig. 5B) (Allen et al., 2006). The four treatments tended to decrease the plating efficiency of Q cells, though this was only significant for Zeocin and HU (Fig. 5B). No consistent effect was observed on NQ cells,

since HU strongly reduced NQ cell plating efficiency while 4NQO led to a nearly significant increase in NQ cell plating efficiency (Fig. 5B). In contrast to the results for growth in YPD, the proportion of Q cells was not increased when cells were exposed beginning on day zero in SC medium, and treatment with HU moderately decreased the proportion of Q cells (Fig. 5C). These data are consistent with the inability of the treatments to extend CLS in SC medium (Fig. 1D). Administering Zeocin treatment on day three also did not increase the proportion of Q cells (Fig. 5D), consistent with the absence of CLS extension for cells treated beginning on day three (Fig. 1C).

We tested other media conditions known to extend yeast CLS to determine whether they would lead to similar increases in the proportion of Q cells. Growth in media containing carbon sources that require aerobic respiration for utilization or containing low concentrations of glucose extend yeast CLS (Barros et al., 2004; Smith et al., 2007). Buffering SC medium to raise the pH and counteract the negative influence of acetic acid accumulation during stationary phase also extends CLS (Burtner et al., 2009). We found that 97% of stationary phase cells fractionated as Q cells in rich medium containing 2% ethanol as a carbon source (YPE), as compared to 60% Q cells in YPD medium containing 2% glucose (Fig. 5E). Virtually no cells fractionated as Q cells when cells were grown in rich medium containing 0.2% glucose (YPlowG). This was not due to growing the cells for only four days prior to fractionation, since cell populations grown for seven days also yielded almost no Q cells (Fig. 5E). This result is consistent with a prior report that cells suddenly transferred to medium lacking glucose do not correctly transition to a quiescent state (Li et al., 2013). Only 37% of cells grown in SC medium fractionated as Q cells, consistent with the shorter lifespan of cells in SC compared to YPD (Fig. 1), but addition of PBS to buffer the medium increased the proportion of Q cells to 53% (Fig. 5E). The relative increases in Q cells for the cultures treated with DNA damaging agents compared to untreated cultures ranged from 18 to 31%, while the relative increases observed for YPE and SC + PBS were 62% and 40%, respectively. Therefore, other factors that extend yeast CLS also increase the proportion of Q cells, but the low glucose experiments demonstrate that an increased proportion of Q cells is not a requirement for increased CLS.

We tested a set of strains with single deletions of genes known to influence quiescence entry/exit or that function in pathways relevant to quiescence and CLS to determine whether these genes were required for the treatments to increase the proportion of Q cells. These genes included *ECM27*, *LSM1*, and *SSD1* that contribute to calcium uptake during stationary phase, mRNA decay/P-body functions, and trafficking/translation of many mRNAs, respectively, and which have all been shown to influence quiescence entry/exit (Klukovich and Courchesne, 2016; Li et al., 2009; Li et al., 2013). We also tested strains with a deletion of *EDC3*, which encodes a P-body scaffold protein (Parker and Sheth, 2007), or the cell wall integrity pathway gene *MTL1* (Rajavel et al., 1999), since *SSD1* contributes to proper quiescence entry at least in part by promoting cell wall changes (Li et al., 2013). Zeocin was chosen as a treatment for these experiments because it resulted in the greatest increase in Q cells. In the absence of Zeocin treatment, *ssd1* mutants failed to form Q cells (Fig. 6A). Zeocin treatment did not increase the proportion of Q cells for *ecm27*, *lsm1*, and *ssd1* mutants, but was still able to increase the proportion of Q cells for *edc3* and

*mtl1* mutants (Fig. 6A). These data indicate that *ECM27*, *LSM1*, and *SSD1* are required for the increase in Q cells.

We next tested strains with deletions of genes that contribute to mitochondrial function and growth signaling, since mitochondrial function and defects in growth-signaling pathways can lengthen CLS (Longo et al., 2012). Mitochondrial function is also important for Q cell function, and Q cells have abundant mRNAs for genes in the Ras-PKA and TOR1 growth-signaling pathways (Aragon et al., 2008; Davidson et al., 2011). In the absence of Zeocin, Q cell formation was strongly inhibited in *atp10* mutants that are defective for assembly of the mitochondrial F1-F0 ATP synthase for respiratory growth (Ackerman and Tzagoloff, 1990), and Q cells were not observed in *mss51* mutants that are defective for translation of a subunit of cytochrome *c* oxidase (Perez-Martinez et al., 2003) (Fig. 6B). In contrast, higher proportions of Q cells were observed for *ras2* mutants defective for Ras-cAMP signaling (Broek et al., 1985) and *tor1* mutants defective for activity of the TOR growth-signaling pathway (Evans et al., 2011) (Fig. 6B). Zeocin treatment was unable to increase the proportion of Q cells for any of these mutants, and decreased the proportion of Q cells in *atp10* mutants (Fig. 6B). These data indicate that mitochondrial function may be required for the increased proportion of Q cells, and that defects in growth signaling may mask the ability of Zeocin to promote Q cell formation.

A final set of mutants was used to test the possible contributions of autophagy, which involves the breakdown and recycling of macromolecules and organelles (Feng et al., 2014), and genomic instability to the increase in the proportion of Q cells. Autophagy is induced during stationary phase (Alvers et al., 2009), and a novel form of autophagy is triggered by DNA damage in yeast (Eapen et al., 2017). The proportion of Q cells was significantly increased without Zeocin treatment in *atg1* mutants defective for the initiation of autophagy (Feng et al., 2014) and in *sir2* mutants defective for sirtuin function and regulation of autophagy during stationary phase (Sampaio-Marques et al., 2012) (Fig. 6C). No further increase in Q cells was observed when these mutants were treated with Zeocin (Fig. 6C). *DCC1* encodes a component of an alternative replication factor C complex that promotes sister chromatid cohesion and genomic stability (Mayer et al., 2001). *DCC1* also exhibits genetic interactions with *LSM1*, *PKA* pathway genes, *TOR1*, *ATG1*, and *SIR2*, all of which we found to influence the ability of Zeocin to promote Q cell formation (Bandyopadhyay et al., 2010; Costanzo et al., 2010; Dixon et al., 2008; Pan et al., 2006). However, there was no change in the proportion of Q cells in *dcc1* mutants without or with Zeocin treatment (Fig. 6C). These results indicate that defects in autophagy and sirtuin function can also mask the ability of Zeocin to increase the proportion of Q cells.

We then determined whether the mutations that prevented Zeocin from increasing the proportion of Q cells would also prevent Zeocin from extending lifespan. Without Zeocin treatment, *tor1* mutants had increased CLS, as expected (Bonawitz et al., 2007), while the *mss51* and *ssd1* mutants had decreased CLS (Fig. 7A–B), consistent with the absence of Q cell fractions for the *mss51* and *ssd1* mutants (Fig. 6A–B). Seven of the nine sets of mutant strains that prevented Zeocin from increasing the proportion of Q cells (*ecm27*, *lsm1*, *ssd1*, *atp10*, *mss51*, *ras2*, *tor1*, *atg1*, and *sir2*) also prevented Zeocin treatment from extending median and maximum CLS (Fig. 7A–B). The two exceptions that

still exhibited extension of maximum CLS were the *atp10* and *ras2* mutants. Maximum CLS was extended by Zeocin treatment for all three sets of mutant strains for which Zeocin treatment still increased the proportion of Q cells (*edc3*, *mtl1*, and *dcc1*), though median CLS was only extended in *mtl1* mutants (Fig. 7C–D). These results indicate that the increase in Q cells is likely to contribute to lifespan extension by treatment with Zeocin.

#### 4. Discussion

We have found that four distinct chemical agents that cause DNA damage or replication stress extend yeast CLS when administered in low doses. Since these agents generate different types of chemical damage to DNA, and HU does not directly damage DNA, these results establish a general connection between mild DNA damage/replication stress and longevity. Overall, the treatments moderately reduced growth rate, increased the proportion of small-budded cells (characteristic of cells in S phase), and increased mutation frequencies. All treatments significantly increased the proportion of cells that appropriately entered quiescence during stationary phase. Since these Q cells maintain their viability much longer than NQ cells that fail to become quiescent (Li et al., 2009), this difference could account for the increased CLS of the cell populations. The increase in Q cells was not observed when cells were treated in SC medium, and lifespan also was not increased when cells were treated in SC medium. The absence of an effect in SC might result from increased DNA replication stress during aging in SC medium (Weinberger et al., 2007), which could result in the doses of chemicals we used moving out of a hormetic range and into a neutral or slightly toxic range, or possibly from depletion for different nutrients at stationary phase due to the different concentrations of nutrients in SC and YPD. Most of the mutant strains that prevented treatment with Zeocin from increasing the proportion of Q cells also prevented Zeocin from extending CLS, further supporting a connection between quiescence entry and lifespan extension.

Lifespan extension was only observed if cells were exposed while they were growing in rich medium, not after the start of stationary phase when cells are typically no longer dividing. Changes in gene expression and cell physiology that occur or are initiated during the growth phase are therefore likely to be required for lifespan extension. Our data are consistent with prior studies in *C. elegans* and yeast that have also demonstrated that exposure to stress needed to occur during growth in order to produce a hormetic response (Burstein et al., 2012; Dillin et al., 2002; Pan et al., 2011). Cold stress in *D. melanogaster* only produced a hormetic response when it occurred early during adult lifespan (Le Bourg, 2011). Together, these studies indicate that for at least some stresses the adaptations necessary to promote longevity can only occur at certain points during development or growth. For our study, the need for treatment to occur during growth to extend lifespan could be explained by our observation that all of the treatments increased the proportion of stationary phase cells that appropriately entered a quiescent state. It could be that treatment after the beginning of stationary phase can no longer produce the cellular changes that promote quiescence entry, and for Zeocin treatment we confirmed that treating cells at the beginning of stationary phase did not increase the proportion of Q cells.

In contrast to a number of studies on hormesis, we did not observe a general increase in stress resistance of treated cells or any evidence for mitohormesis. For instance, repeated mild heat stress in human fibroblasts increased their resistance to ethanol, UV, and hydrogen peroxide (Rattan, 2004), exposure of *C. elegans* to a phenol from extra virgin olive oil increased thermotolerance and oxidative stress resistance (Cañuelo et al., 2012), and cold stress in *D. melanogaster* improved resistance to heat stress and fungal infection (Le Bourg, 2011). We tested resistance to several stresses for cells treated with each of the four agents that increased CLS, and the only increase in stress resistance observed was a moderate increase in resistance to hydrogen peroxide for cells treated with HU. We tested a range of mild to severe stresses, so it is unlikely that we failed to observe increased stress resistance because of testing too mild or harsh of a stress. Mitohormesis is associated with increased mitochondrial activity, transient increases in ROS that trigger adaptive responses, and often reduced ROS later in lifespan (Ristow and Zarse, 2010). Extension of lifespan by ROS in yeast (Pan et al., 2011), arsenite in *C. elegans* (Schmeisser et al., 2013), a natural product from tomatoes in *C. elegans* (Fang et al., 2017), and transient repression of a subunit of mitochondrial complex I in *D. melanogaster* (Owusu-Ansah et al., 2013) have all been proposed to promote longevity through mitohormesis. We observed no evidence for a transient increase in ROS or an increase in mitochondrial activity in response to DNA damaging agents. The mechanism of the hormetic effect we observed is therefore likely to be distinct from these other examples of hormesis. However, it remains possible that there is an underlying factor or process not yet identified that is common to all of these examples.

It is formally possible that each of the four treatments that we used to extend lifespan works through a distinct mechanism, despite leading to similar increases in lifespan and proportion of Q cells. For instance, 4NQO treatment increased the doubling time of cells much more than the other treatments. This could be an indication that there is selection for a subset of cells able to grow in the presence of 4NQO, and this subset of cells could have characteristics leading to longer lifespan. HU treatment moderately increased resistance to hydrogen peroxide, and increased resistance to ROS could potentially increase lifespan. MMS, HU, and 4NQO treatments reduced ROS levels during exponential phase, which could possibly contribute to longer CLS. However, we favor the simpler interpretation that the agents all work through a common mechanism that involves regulation of quiescence to promote similar changes in CLS.

The mechanism(s) through which the four chemical agents promote entry into quiescence remains to be determined. We found that five genes with diverse functions were required for Zeocin to increase the proportion of Q cells: *ATP10*, *ECM27*, *LSM1*, *MSS51*, and *SSDI*. These genes contribute to Q cell formation and function through mitochondrial function (*ATP10* and *MSS51*), increasing intracellular calcium (*ECM27*), promoting cell wall modifications (*LSM1* and *SSDI*), and carbohydrate accumulation (*ECM27* and *LSM1*) (Aragon et al., 2008; Davidson et al., 2011; Klukovich and Courchesne, 2016; Li et al., 2013). The requirement of genes with these various functions complicates the identification of what specific pathways are responsible for the increased proportion of Q cells due to mild DNA damage stress. The requirement for these genes may instead reflect their importance for quiescence entry, rather than a specific interaction with mild DNA damage stress. Deletion of *ATG1*, *RAS2*, *SIR2*, or *TOR1* increased the proportion of Q cells without

treatment and prevented Zeocin treatment from further increasing Q cells. One commonality between these four genes is that each regulates autophagy (Budovskaya et al., 2004; Feng et al., 2014; Kamada et al., 2000; Sampaio-Marques et al., 2012). Defective autophagy inhibits DNA repair, and DNA damage has been shown to induce autophagy (Eapen et al., 2017; Lin et al., 2015; Liu et al., 2015; Park et al., 2015). Whether changes in autophagy could mask the effect of mild DNA damage stress on quiescence entry will require further investigation.

DNA damage and DNA repair genes regulate cell cycle progression so that DNA damage can be repaired prior to continuation of the cell cycle, and cell cycle genes can also regulate DNA repair activities (Langerak and Russell, 2011). Our treatments significantly increased mutation frequencies and the proportion of budded cells, indicating that they caused DNA damage and influenced cell cycle progression. We speculate that mild DNA damage/replication stress increases the proportion of cells transiently arresting the cell cycle during the growth phase, which increases the likelihood of cells arresting growth and entering quiescence in subsequent cell cycles during the transition to stationary phase. This speculation is supported by recent work in mammalian cells showing that the likelihood of cells to proliferate or enter quiescence is regulated by competition between growth signaling and DNA damage signaling, can be affected by the transmission of protein or mRNA for DNA repair and cell cycle genes from mother to daughter cells, and that DNA replication stress in one cell cycle can promote entry into quiescence during the next cell cycle (Arora et al., 2017; Heldt et al., 2018; Yang et al., 2017).

Overall, we have identified a novel connection between mild DNA damage stress and improved quiescence entry that lengthens yeast CLS. Our data indicate that hormesis-induced lifespan extension can occur independently of a general increase in stress resistance and mitohormesis. The trigger for quiescence in yeast is nutrient depletion, whereas quiescence in mammalian cells is regulated by the presence/ absence of growth factors, cytokines, signaling between cells, and signaling between cells and their microenvironment (Rumman et al., 2015; Valcourt et al., 2012). However, commonalities have been observed between certain aspects of quiescence in yeast and some types of adult mammalian stem cells. These include the existence of distinct quiescent states due partly to different means of inducing quiescence, distinct subpopulations of cells with differing responsiveness to proliferative signals, as well as the influence of DNA replication stress, autophagy, and TOR signaling on quiescence (Dhawan and Laxman, 2015; Flach et al., 2014; Ho et al., 2017; Rumman et al., 2015; Weinberger et al., 2007). Further study of the ability of mild DNA damage stress to promote quiescence may therefore provide insights relevant to quiescence regulation in some types of mammalian stem cells.

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## Abbreviations

<b>CFU</b>	colony-forming units
<b>CLS</b>	chronological lifespan
<b>DHE</b>	dihydroethidium
<b>DHR</b>	dihydrorhodamine
<b>DiOC<sub>6</sub></b>	3,3'-dihexyloxycarbocyanine iodide
<b>FOA</b>	5-fluoroorotic acid
<b>HU</b>	hydroxyurea
<b>MMS</b>	methyl methanesulfonate
<b>NQ</b>	non-quiescent
<b>4NQO</b>	4-nitroquinoline 1-oxide
<b>PBS</b>	phosphate buffered saline
<b>Q</b>	quiescent
<b>ROS</b>	reactive oxygen species
<b>SC</b>	synthetic complete medium
<b>YPD</b>	yeast extract-peptone-dextrose (rich) medium

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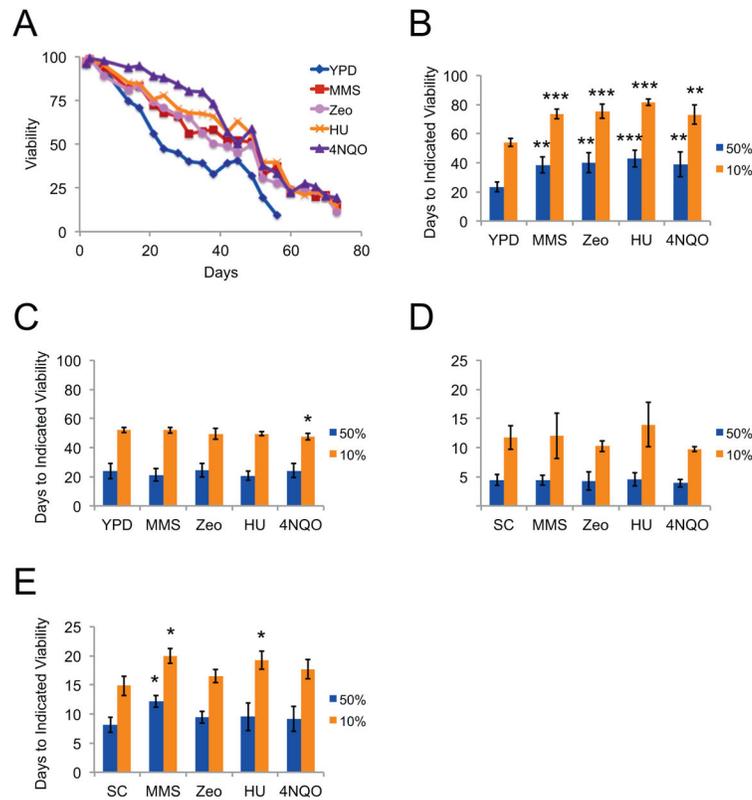
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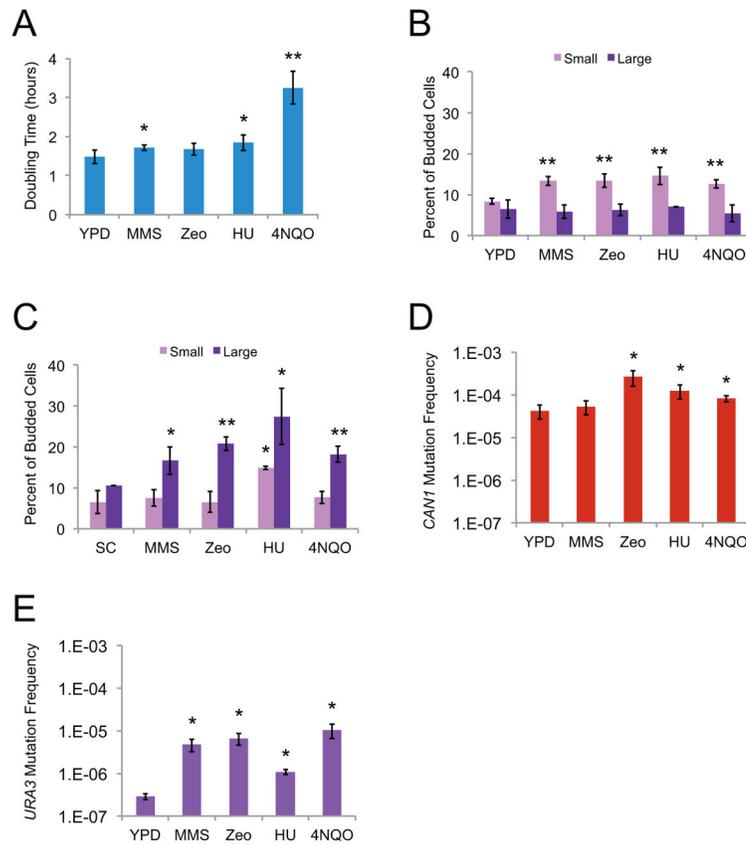
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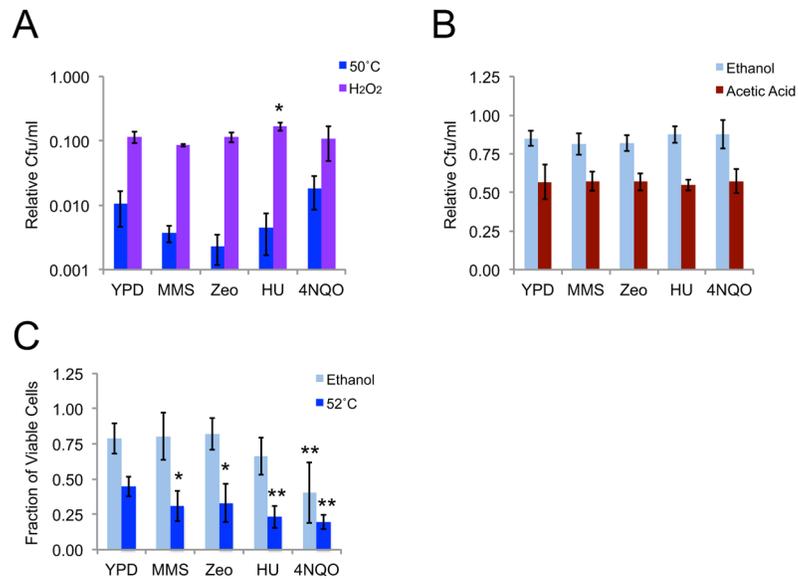
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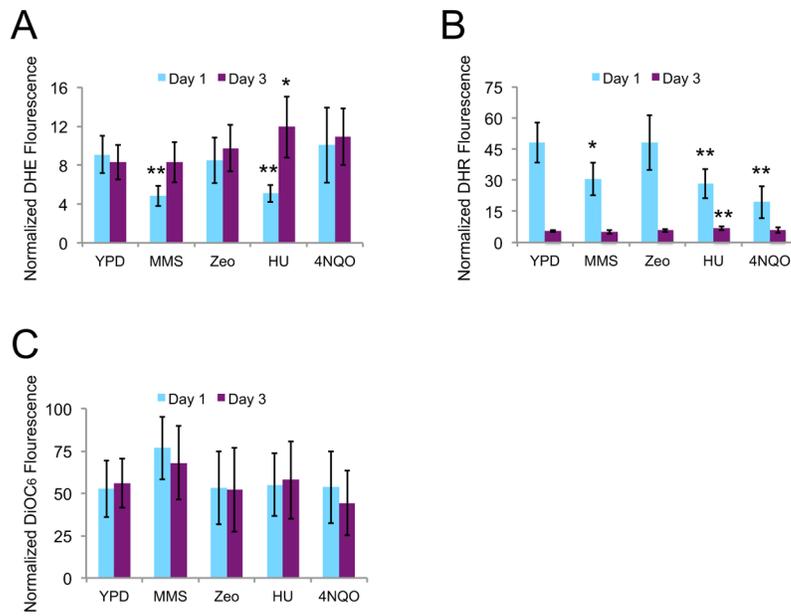
**Fig. 1.** Exposure to low doses of DNA damaging agents during growth in rich medium extends yeast CLS. (A) A representative chronological aging experiment for cells grown in control YPD medium or YPD with the indicated chemical agents, using trypan blue dye exclusion to determine viability. Mean and standard deviation for median (days to 50% viability) or maximum CLS (days to 10% viability) for three trials with treatment beginning on day zero in YPD medium (B), on day three in YPD medium (C), on day zero in SC medium (D), and on day zero in SC medium with PBS (E). Asterisks indicate significant differences: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .



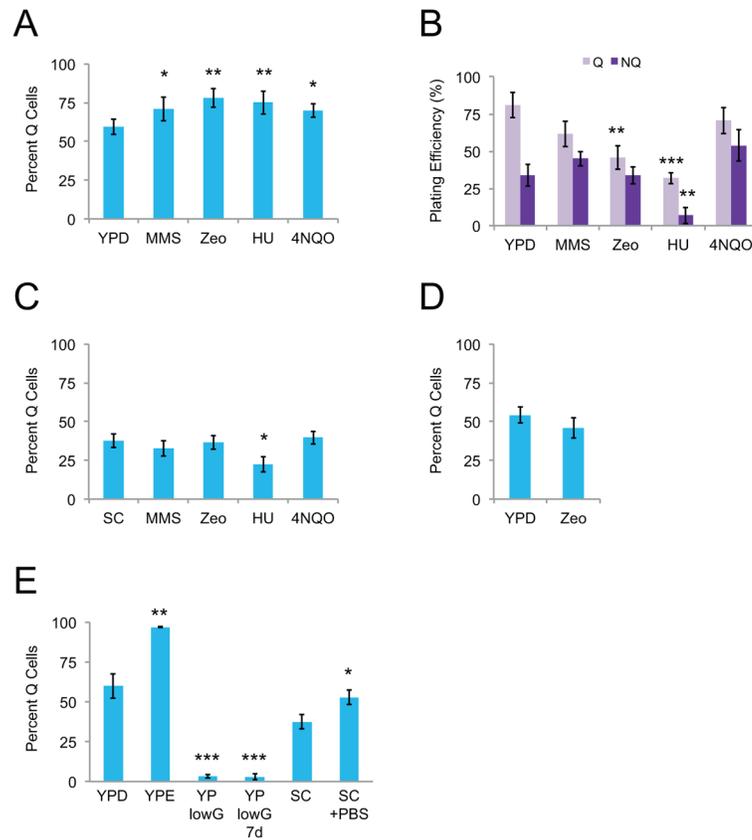
**Fig. 2.** Exposure to DNA damaging agents slows growth, increases the proportion of budded cells, and increases mutation frequencies. (A) Doubling time in hours during exponential phase for cells grown in control YPD medium or YPD with the indicated chemical agents determined by counting cells by microscopy. (B) Percentage of cells in early stationary phase (day three) with small or large buds in YPD medium without or with the indicated chemical agents added at day zero. (C) Percentage of cells at day three with small or large buds in SC medium without or with the indicated chemical agents added at day zero. Frequency of canavanine-resistant (D) or FOA-resistant (E) cells at early stationary phase for cells grown in YPD medium without or with chronic exposure to the indicated chemical agents. Mean and standard deviation are shown for three trials in each case. Asterisks indicate significant differences: \* =  $p < 0.05$  and \*\* =  $p < 0.01$ .



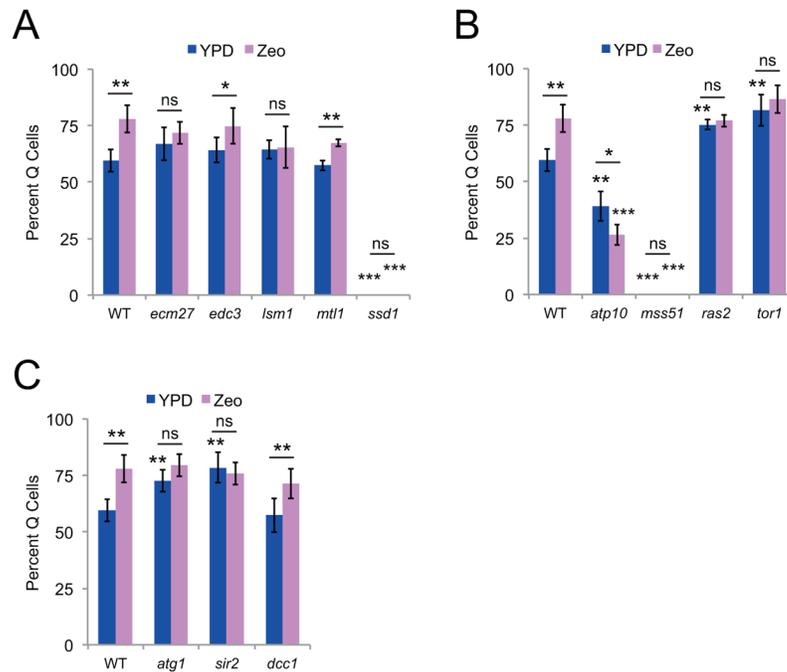
**Fig. 3.** Increased stress resistance does not account for lifespan extension induced by DNA damaging agents. Colony-forming units (cfu) per ml relative to mock treatment for cells grown in YPD without or with the indicated chemical treatments for 20–24 h and exposed to 50 °C for 7 min or 3 mM hydrogen peroxide for 1 h (A) or 15% ethanol for 20 min or 100 mM acetic acid for 1.5 h (B). (C) Viability determined by trypan blue dye exclusion for cells grown for 24 h in YPD without or with the indicated chemical agents and then exposed to 20% ethanol for 1 h or 52 °C for 45 min. Mean and standard deviation are shown for three (A and B) or five trials (C). Asterisks indicate significant differences compared to YPD with no chemical treatment: \* =  $p < 0.05$  and \*\* =  $p < 0.01$ .



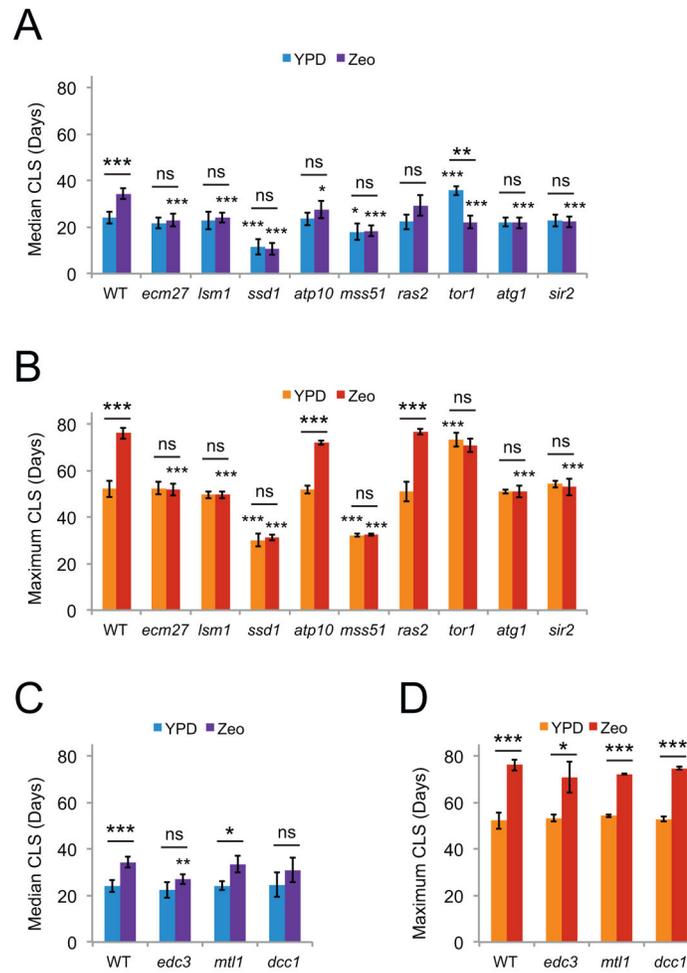
**Fig. 4.** Mitohormesis does not account for lifespan extension induced by DNA damaging agents. Fluorescence signal normalized to unstained controls to measure superoxide levels by DHE fluorescence (A), peroxide levels by DHR fluorescence (B), or mitochondrial membrane potential by DiOC<sub>6</sub> fluorescence (C) for five to six trials of cells grown in YPD medium without or with chronic exposure to the DNA damaging agents and sampled on day one or three. In all cases, mean and standard deviation values are shown, and asterisks indicate significant differences: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ .



**Fig. 5.** Exposure to DNA damaging agents promotes entry into quiescence. (A) Percentage of quiescent cells obtained by Percoll density gradient fractionation of cells on day four following growth in YPD without or with the indicated DNA damaging agents. Data for four to five trials are shown for each treatment. (B) Plating efficiency of fractionated Q and NQ cells from experiments shown in A. (C) Percentage of Q cells obtained by Percoll density gradient fractionation of cells on day four after growth in SC without or with chronic exposure to the indicated DNA damaging agents. Data for three trials are shown. (D) Percentage of Q cells obtained after treating cells in YPD medium with Zeocin on day three and fractionating cells on day four. Data for five trials are shown. (E) Percentage of Q cells obtained after growing cells in YPD, YP + 2% ethanol (YPE), YP + 0.2% glucose (YPlowG), SC, or SC + PBS for four days, or seven days (7d) for YPlowG. Data for three trials are shown. Mean and standard deviation are shown in all cases, and asterisks indicate significant differences: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .



**Fig. 6.** Deletion of genes that contribute to Q cell formation and function prevent Zeocin treatment from increasing the proportion of Q cells. Each panel shows the percentage of Q cells obtained by Percoll density gradient fractionation of wild type (WT) or mutant strains on day four after growth in YPD without or with chronic Zeocin treatment for a different set of mutant strains. Mean and standard deviation for three to seven trials per strain are shown. Horizontal lines indicate comparisons between untreated and treated cells for each strain, and asterisks over individual columns indicate differences compared to WT. Asterisks indicate significant differences, as in Fig. 5, and “ns” indicates not significant.



**Fig. 7.** Many mutants that prevent Zeocin from increasing the proportion of Q cells also prevent lifespan extension. Median (days to 50% viability, panels A and C) or maximum CLS (days to 10% viability, panels B and D) for wild type (WT) or the indicated mutant strains grown in YPD without or with chronic exposure to Zeocin beginning on day zero. Mutants in panels A and B prevented Zeocin from increasing the proportion of Q cells, and mutants in panels C and D did not. Mean and standard deviation for three trials are shown. Horizontal lines, asterisks, and “ns” are used to indicate the presence or absence of significant differences as for Fig. 6.