

Loss of mitochondrial DNA accelerates the early-age decline in stress resistance during yeast replicative aging

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Abstract

Baker's yeast is a well-established model for studying fundamental mechanisms of aging. Due to asymmetric division, yeast mother and daughter cells are distinguishable, and mother cells typically cease division after 15–25 budding events. Meanwhile, yeast suspensions consist predominantly of young cells. Here we studied whether, under harsh environmental conditions, replicative aging — defined by the number of buds produced — manifests at early replicative ages. To test this, we adopted a flow-cytometry-based pulse-chase labeling method to trace survival of yeast age cohorts from daughter cells up to mothers that had produced several — on average up to eight — buds. We found that stress-induced mortality increased with replicative age across all nine stress conditions tested. Mitochondrial dysfunction, caused by depletion of mitochondrial DNA, increased the regression coefficient of replicative age against mortality induced by acetic acid stress, oxidative stress induced by menadione, and sugar-induced cell death. Deletion of SIR2 — a genetic perturbation known to extend replicative lifespan under optimal conditions — also steepened the age–mortality slope under acetic acid stress. Together, our data indicate that aging manifests early in life under extreme environments and that genetic factors influencing this process partially overlap with those limiting lifespan in optimal conditions.

Highlights

- Stress resistance declines during early replicative aging in yeast, generating heterogeneity in stress resistance within clonal populations.
- The rate of this decline for acetic acid, menadione, and sugar stresses is markedly accelerated in cells lacking mitochondrial DNA.
- Deletion of the histone deacetylase gene accelerates the early decline in acetic acid stress resistance with age.

1. Introduction

Baker's yeast, *Saccharomyces cerevisiae*, serves as a simple model for studying aging. Yeast cells exhibit a finite replicative lifespan (RLS), where a mother cell can produce a limited number of daughter cells before it stops dividing and dies, typically after 15–25 buddings (Ashrafi et al. 1999). Studies in yeast have been instrumental in identifying genes and signaling pathways that also play a role in aging of multicellular eukaryotes (Medvedik et al. 2007; Johnson et al. 2013; Beupere et al. 2017; Mittal et al. 2017). For instance, genetic screens in yeast have identified a set of genes whose orthologs, when mutated, modulate lifespan of the nematode *Caenorhabditis elegans* (McCormick et al. 2015). Moreover, the yeast replicative aging model helped to unveil calorie restriction mimetics, such as resveratrol (Howitz et al. 2003), whose efficacy was later validated in animal systems (Okamoto et al. 2022). This makes yeast an accessible model for investigating aging and implies some level of conservation of the underlying molecular processes between unicellular and multicellular organisms.

Yeast aging proceeds via one of two chain-of-event pathways. One pathway is linked to the loss of chromatin silencing and the accumulation of extrachromosomal rDNA circles (ERCs), while the other is linked to mitochondrial dysfunction (Jin et al. 2019; Li et al. 2020). Within the rDNA/ERC pathway, the *SIR2* gene limits ERC formation and extends lifespan, whereas shorter rDNA arrays reduce Sir2 activity, increasing ERCs and shortening lifespan (Sinclair and Guarente 1997; Kim et al. 1999; Hotz et al. 2022). Deleting *FOB1*, which encodes a nucleolar protein that binds the rDNA replication fork, suppresses ERC formation and abolishes the link between rDNA copy number and RLS (Defossez et al. 1999). Mitochondria play a central role in the second trajectory, where age-dependent loss of membrane potential impairs protein import, leading to mtDNA loss and the cytoplasmic accumulation of toxic mitochondrial precursors (Veatch et al. 2009; Poveda-Huertes et al. 2021).

While these two terminal pathways define the limits of lifespan, much less is known about the earliest events that take place during the first several buddings of a mother cell. However, some manifestations of yeast aging start early in life — just after a mother cell has produced a few buds. For example, reactive oxygen species (ROS) production increases during the first several divisions (Lam et al. 2011), a phenotype likely associated with early manifestation of mitochondrial dysfunction (Hughes and Gottschling 2012; Azbarova et al. 2017), see for review (Knorre et al. 2018). Moreover, there are pronounced changes in metabolic fluxes during the first quarter of yeast replicative lifespan, possibly aimed to compensate for the ROS production or damage (Leupold et al. 2019).

These early-life changes may have a disproportionate impact on population fitness because exponentially growing cultures consist predominantly of young cells: each round of division dilutes the mother cell population by half, such that the vast majority of cells are either daughters or mother cells that have undergone only a few buddings (Knorre et al. 2018). We have previously shown that young mother cells tend to have higher resistance to certain stresses than older mother cells. However, in those studies, the replicative age of mother cells was determined in exponentially growing cultures by manually counting chitin-rich bud scars. While informative, this approach is labor-intensive and has limited statistical power for older age cohorts due to their rarity.

Here, we employed a flow-cytometry-based method to assess yeast stress resistance as a function of replicative age during early life. This approach circumvents the statistical limitations of bud-scar counting by enabling the analysis of large, defined cohorts of mother cells after a specified number of divisions. Using this method, we studied the kinetics of the age-dependent decrease in stress resistance during the first few buddings. We assessed the rates of aging for several environmental stresses (heat shock, hyperosmotic conditions, acidification of cytoplasm by acetic acid) as well as the resistances to some xenobiotics, such as surfactants (benzalkonium chloride, SDS), prooxidants menadione and hydrogen peroxide.

Because declining stress resistance is an early hallmark of cellular aging, we asked whether this decline is linked to the two established lifespan-determining pathways. To test this, we compared the kinetics of stress resistance loss in mutants known to alter maximum RLS: $\Delta sir2$ (short-lived, rDNA/ERC pathway)

and $\Delta fob1$ (long-lived, rDNA/ERC pathway), as well as a strain lacking mitochondrial DNA (ρ^0), representing the mitochondrial dysfunction pathway described above. By resolving the temporal onset of stress vulnerability and its dependence on canonical aging pathways, our study aims to clarify whether the known determinants of lifespan also moderate the earliest declines in cellular fitness.

2. Material and methods

2.1 Strains and media

In this study, we utilized the standard laboratory strain *W303 1-A* (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) along with its derivatives, *W303 ρ^0* (strain without mitochondrial DNA) (Zyrina et al. 2017), $\Delta sir2$ (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, KanMX::sir2*) and $\Delta fob1$ (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, KanMX::fob1*). The $\Delta sir2$ strain was previously generated in our laboratory (Knorre et al. 2010). The $\Delta fob1$ strain was created in this study by amplifying a PCR fragment from the *pFA6a-3HA-kanMX6* plasmid (Bähler et al. 1998) as a template using primers listed in Table S1. The resulting DNA fragment, which comprises the KanMX cassette conferring resistance to G418 and is flanked by sequences from the 5' and 3' ends of the *FOB1* gene, was subsequently integrated into the *W303 1-A* strain genome. The gene deletion in clones was validated by using test PCR product primers listed in Table S1.

We used YPD (Yeast Peptone Dextrose) medium for routine yeast cell growth and stress survival experiments (acetic stress, heat shock, menadione, osmotic stress). Additionally, synthetic complete medium (YNB Base (Sigma-Aldrich Y0626) with addition of amino acids) was utilized for some stress survival tests (tBOOH, benzalkonium chloride, SDS). All media were prepared according to the methods outlined in Sherman (Sherman 2002).

2.2 Pulse-chase labeling of mother cells with AF488 NHS ester

To mark mother cells, we adapted our previously described pulse-chase approach (Azbarova et al. 2017) with a different fluorescent dye and several modifications. The original method used TRITC-ConA, which provided insufficient contrast for flow cytometry after the pulse-chase procedure; we therefore substituted it with AF488 NHS ester (Lumiprobe, Russia; cat. no. 11820), an amine-reactive activated ester of a sulfonated rhodamine dye (a sulfonated derivative of rhodamine 110; excitation/emission maxima 495/519 nm), which covalently labels primary amines on cell-surface proteins.

Yeast cells were inoculated into two 50 ml tubes with 5 mL of liquid YPD medium each and cultured overnight at 30°C until reaching an optical density of OD₅₅₀ in the range of 0.1–0.3 (exponential growth phase; the exact value within this range was not critical). A culture volume containing a number of cells equivalent to 6 mL of culture at OD₅₅₀ 0.1 (approximately 12×10^6 cells) was then centrifuged (2,500 rpm, 2 min) and washed twice with 500 μ L of MQ. The cells were then stained with 0.6 μ g/mL AF488 NHS ester in PBS (pH 8.0) for 10 minutes after which they were washed twice with MQ to remove unbound

dye. Cells were then resuspended in YPD medium and divided into three groups (three 50 mL tubes with 5 mL YPD each) according to their final incubation time after staining: 2 h, 4 h, and 6 h.

To obtain cell populations with varying incubation times following staining — and consequently different average replicative ages, since labeled mother cells give rise to unlabeled progeny — we conducted a 2-day experiment. As the labeled mother cells divide during the incubation period, each division produces an unlabeled daughter cell while the labeled mother cell is retained within the population; consequently, longer incubation times after staining allow more divisions to accumulate, yielding populations enriched for progressively older (i.e., more replicatively aged) mother cells alongside a growing proportion of unlabeled daughter cells. The 2 h group was immediately placed at 4°C, while the 4 h and 6 h groups were cultured for an additional 2 and 4 hours, respectively, before being placed at 4°C. All populations were kept overnight at 4°C and then cultured for an additional 2 hours at 30°C the following morning. For subsequent stress survival experiments, a volume containing a number of cells equivalent to 1 mL of culture at OD₅₅₀ 0.1 was taken from each population.

2.3 Stress survival experiments

The three cell populations (2 h, 4 h, and 6 h of enrichment) were subjected to the stress treatments described in previous studies (Sorokin et al. 2014; Zyrina et al. 2017; Valiakhmetov et al. 2019; Kireeva et al. 2021), the exact incubation conditions are listed in Table S2. For each stress condition, a matched control sample was processed identically but without stress exposure (Table S2). For all stress treatments except heat shock, cells were incubated on a shaking incubator at 30°C and 250 rpm during stress exposure.

Following stress exposure, cells were centrifuged (2,500 rpm, 2 min), the supernatant was discarded, and the pellet was resuspended in 500 µL PBS. Propidium iodide (PI, 0.1 µg/mL; Sigma-Aldrich) was then added to the suspension to identify dead cells. After incubation at 30°C for 10 min, cells were centrifuged again (2,500 rpm, 2 min), the supernatant was discarded, and cells were resuspended in fresh 500 µL PBS to remove unbound PI. Immediately after this washing step, samples were analyzed on a CytoFLEX flow cytometer (Beckman Coulter), and all data were processed using CytExpert software (Beckman Coulter). A minimum of 10,000 cells was recorded per sample in each experiment.

For each sample, three plots were generated: a histogram of PI fluorescence (PC-7 channel; PC7-A on the x-axis, cell count on the y-axis), a histogram of the customized parameter AF488 (AF488 on the x-axis, cell count on the y-axis), and a contour plot of AF488 (y-axis) versus PC7-A (x-axis). The customized parameter AF488 — distinct from the raw FITC channel — was defined using the Set Customized Parameter function in CytExpert: $AF488 = (FITC-H / FSC-H) \times 100$, where FSC-H is the forward scatter height reflecting cell size. Normalizing FITC-H to cell size improved subpopulation resolution and, therefore, were used for manual gate selection instead of the raw FITC channel.

For each sample, subpopulations were gated on the AF488-versus-PC7-A contour plot using the Four Quadrant tool, with the vertical and horizontal quadrant boundaries positioned at the valley between the

two peaks corresponding to labeled and unlabeled cells on the AF488 and PC-7 histograms, respectively. This gating strategy yielded four subpopulations for each sample: double-negative (Q1-LL; PI-/AF488⁻), PI-single-positive (Q1-LR; PI+/AF488⁻), AF488-single-positive (Q1-UL; PI-/AF488⁺), and double-positive (Q1-UR; PI+/AF488⁺) cells. Since only mother cells retained the AF488 NHS ester label while their progeny remained entirely unlabeled, AF488⁺ cells were classified as mother cells, whereas AF488⁻ cells represented daughter cells formed after the initial staining; PI positivity was used to identify dead cells within each subpopulation, allowing survival to be assessed separately for mother and daughter cells following stress exposure. For each of the four gated subpopulations the percentage of the subpopulation relative to the total number of events (% Total) were exported from the Statistics table in CytExpert.

2.4 Fluorescent Microscopy

To estimate the average replicative age of stained and unstained AF488 cells with different incubation times after staining, we stained AF488 cells as described in section 2.2 and then placed them in rich growth medium and grew them for 2, 4, or 6 hours. The cells were then stained with 5 μ M Calcofluor White (CW, Sigma-Aldrich F3542) to visualize bud scars, which were counted in different focal planes under a microscope to determine cell age. This age was compared with the number of stained and unstained cells from the same samples analyzed on a cytometer.

Fluorescence microscopy was performed using an Olympus BX51 upright microscope equipped with a UPLANFLN 100x lens (N.A. 1.3) and the filter set specified in Table S3. Images were captured with an Olympus DP30BW CCD camera utilizing Olympus CellF software. To create color images of the cells, pseudocoloring was applied: the black and white image from the AF488 channel was transferred to the green channel of the RGB file, while the black and white image from the PI channel was moved to the red channel. GIMP and Inkscape software were employed to crop and arrange the figure panels.

2.5 Data Analysis and Visualization

Data analysis and visualisation was performed using R (version 4.4.1) and tidyverse libraries (Wickham et al. 2019), as well as CytExpert software (Beckman Coulter). All individual data points are shown where feasible

To compare the correlations between yeast age and death rate under stress conditions for different mutants, linear regression slopes were estimated for each condition using the `lm()` command with default parameters. We then used a permutation test to determine whether the difference in slopes between conditions was statistically significant. Condition labels were randomly shuffled 10,000 times, and the slope difference was recalculated for each permutation to construct a null distribution. The two-sided p-value was computed as the frequency of permuted differences with an absolute value greater than or equal to the observed absolute difference.

3. Results

3.1 Pulse-chase labeling of yeast mother cells with AF488

To distinguish yeast cell populations of different age cohorts, we selected AF488, an NHS-activated ester of sulfonated Rhodamine 110. AF488 binds to the yeast cell surface, enabling clear discrimination between stained and unstained cells by flow cytometry (Fig. S1). To test whether AF488 allows tracing of mother cells, we performed a pulse-chase labeling experiment on a yeast suspension. After washing out unbound dye, we incubated the cells for 6 hours in YPD. Figure 1 shows that after this chase period, mother cells retained AF488 staining, whereas daughter cells did not. To distinguish mother cells from newborn daughter cells, we used Calcofluor White staining, which reveals bud scars present exclusively on the cell wall of mother cells (Fig. 1A). Expectedly, longer periods of chase decrease the proportion of AF488-negative cells within the overall population (see Fig. 1B). Importantly, we showed that AF488 doesn't affect cell growth in a wide range of concentrations, up to 10 mg/L (Fig. S2), while we used an order of magnitude lower concentrations (0.6 mg/L) to visualise the cells.

We reasoned that because dividing mother cells produce unstained daughters while retaining their own label, the stained population becomes progressively diluted over time. Therefore, a smaller AF488-positive subpopulation corresponds to an older average age of those stained cells. To test this hypothesis, we incubated cells for 1–6 hours after AF488 staining and then measured (1) the proportion of stained cells by flow cytometry and (2) the amount of scars in AF488-positive and AF488-negative cells. It should be noted that we did not distinguish bud and birth scars in our analysis, thus the cells with one scar are daughter cells. The longer the incubation time after washing off the AF488, the more scars there were on the cells on average (Fig. S3). Figure 2 shows that the log₂-transformed proportion of AF488-positive cells in the population negatively correlated with the average number of bud scars in this subpopulation (Kendall's tau = -0.82, p-value = 3.6×10^{-4}). A simple linear regression analysis was performed to examine the relationship between the average number of bud scars and the log₂-transformed size of the AF488-positive subpopulations. The regression yielded the following equation: Scars = $-1.26 \times \log_2(\text{subpopulation size}) + 2.34$ ($R^2 = 0.88$). These results suggested that the average number of scars in yeast exponentially growth subpopulation is 2.34 (when the AF488-positive subpopulation size is equal to 1), which suggests a small enrichment in mother cells. The regression provided us with the conversion factors between population size and average cell age of the AF488-positive subpopulation. We used these coefficients to transform the size of the subpopulation to the average age of AF-488 positive cells in the subsequent experiments.

Correlation between the proportion of log₂-transformed proportion of AF488-positive cells in the population and the average number of bud scars per cell. Thin lines connect subpopulations from the same flow cytometry run.

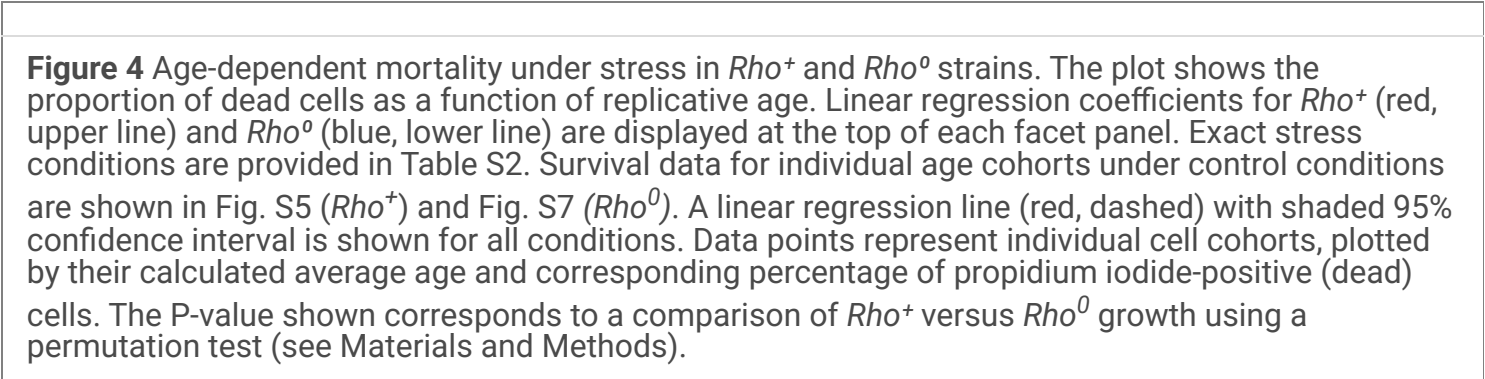
3.2 Stress Resistances of yeast cells as a function of replicative age

To quantify the rate at which stress resistance declines with each cell division, we selected a panel of environmental and chemical stresses that induce a loss of cell viability detectable by propidium iodide (PI) staining. These included environmental stresses: heat shock (HS), hyperosmotic stress (NaCl), cytoplasmic acidification (acetic acid), oxidative stress (Menadione or H₂O₂). We also assessed resistance to high concentrations of ethanol and glucose (SICD), as well as to two surfactants—sodium dodecyl sulfate (SDS) and benzalkonium chloride (BAC). A complete list of stress conditions and corresponding treatment protocols is provided in Table S2.

To distinguish stress resistance of yeast populations of different replicative ages, we employed a pulse-chase staining approach and then subjected yeast cells to the stresses mentioned above. As a result, we obtained yeast suspensions with four subpopulations based on their AF488 and PI fluorescence profiles (Fig. 3). Fluorescence microscopy confirmed the presence of all four cell types in our experiments (Fig. S4), and these populations were clearly clustered into three subpopulations in flow cytometry data (Fig. 3). For each chase incubation time, we were therefore able to calculate the average age of the suspension, as well as the percentages of PI-positive (dead) and PI-negative (alive) cells.

Following stress exposure, we measured the percentage of dead cells within each age-defined population and performed linear regression analysis for every condition tested. Across all examined stresses, the regression coefficient for replicative age was consistently positive, indicating that increasing replicative age correlates with higher death rates Fig. 4. The regression coefficient for different stresses fell in range 2.5–8.5 suggesting that each round of budding renders several percent of the yeast mother cell cohort incapable of withstanding the stress. Interestingly, the age-dependent susceptibility was also observed under non-stress conditions, where mother-cell-enriched populations contained significantly more dead cells than daughter-cell-enriched populations Fig. S5. Importantly, we verified that the AF488 staining used for age discrimination did not sensitize cells to the applied stresses. Control experiments showed that AF488-labeled cells exhibited stress resistance comparable to unstained cells Fig. S6.

Collectively, these findings demonstrate that each successive mother-cell division leads to a progressive decline in resistance to multiple types of stress, thereby reducing the likelihood of maintaining viability upon environmental challenge. However, the rate of this decline depends on the type of stress we studied.



3.3 Mitochondrial dysfunction changes the rate of yeast deterioration of stress resistance in early life

Mitochondria play a central role in aging and stress resistance. To determine whether mitochondrial dysfunction alters the relationship between replicative age and stress-induced mortality, we employed the pulse-chase labeling approach in a *rho*⁰ strain, which lacks mitochondrial DNA and is incapable of oxidative phosphorylation. We then assessed the rate of stress resistance decline in these cells across the same panel of stresses. As observed for the control *rho*⁺ strain, regression analysis yielded consistently positive coefficients between replicative age and cell death in *rho*⁰ cells across all conditions tested (Fig. 4, see Fig. S7 for the comparison of stress with control conditions), indicating that the age-dependent decline in stress resistance is recapitulated even in the absence of functional mitochondria. Interestingly, average stress tolerance differed markedly between strains for some stresses. *Rho*⁰ cells exhibited increased sensitivity to ethanol and hydrogen peroxide, yet showed higher resistance to SDS at all tested age intervals (Fig. 4).

For some stresses, the rate of the decrease in stress resistance per division was faster in *rho*⁰ than in *rho*⁺ cells (Fig. 4). With the exception of hydrogen peroxide, the regression slopes for *rho*⁰ were steeper across all stresses. Permutation testing (see Materials and Methods) revealed that these slope differences were statistically significant for SICD (*P* = 0.03), oxidative stress induced by menadione (*P* = 0.03) and acetic acid stress (*P* = 0.0005). Collectively, these results demonstrate that mitochondrial DNA depletion not only alters basal stress resistance for some stresses but also modulates the age-dependent loss of viability, in other conditions.

3.4 Age-dependent profile of stress resistance during early replicative age in mutant strains *sir2Δ* and *fob1Δ* known to have altered maximum replicative lifespan

To investigate whether genetic modifications that alter replicative lifespan also modulate the age-dependent decline in stress resistance during early divisions, we examined two deletion strains: *sir2Δ*, which exhibits shortened replicative lifespan, and *fob1Δ*, which exhibits extended replicative lifespan. Using our pulse-chase mother-cell staining approach, we measured the rate of stress resistance decline under heat shock and acetic acid stress for each strain (Fig. 5).

For *fob1Δ* cells, the regression coefficient between replicative age and mortality showed no pronounced or statistically significant difference from that of wild-type (WT) *rho*⁺ cells under both tested stress condition. Similarly, *sir2Δ* cells showed no significant difference from WT under heat shock. However, under acetic acid stress, *sir2Δ* cells exhibited a significantly steeper age-dependent increase in mortality compared to WT (*P* = 0.0024, permutation test; see Materials and Methods).

These results indicate that *SIR2* deletion accelerates the age-related decline in resistance to acetic acid during early replicative life, whereas *FOB1* deletion does not affect this parameter. Notably, neither

genetic manipulation influenced the age-dependent response to heat shock, suggesting that the effect of *SIR2* on stress resistance decline is stressor-specific.

Figure 5 Age-dependent mortality under stress in wild type, *sir2Δ* and *fob1Δ* strains. Deletion of *SIR2* increases the age-dependent component of acetic acid sensitivity with age (* P = 0.0024). Linear regression line (red, dashed) with shaded 95% confidence interval is shown for all conditions. Data points represent individual cell cohorts with calculated average age and percentage of propidium positive cells. Note, that WT control data are the same as in Fig. 4.

4. Discussion

4.1 Main findings

In this study, we used a flow-cytometry–based approach to compare the survival of yeast cells under stress conditions as a function of replicative age, focusing on the young age cohorts. Analysed age range accounts for the vast majority of cells in a yeast suspension culture (for a detailed demographic analysis of yeast suspension see (Knorre et al. 2018)). Consistent with our previous work (Knorre et al. 2010; Sorokin et al. 2014; Azbarova et al. 2017), we found that even a few rounds of division reduce the ability of yeast cells to withstand cytotoxic stresses. Importantly, in all cases the regression of mortality on replicative age showed (1) a positive slope and (2) a steeper slope under stress than under control conditions (Fig. 4, S5, S7). These results indicate that (1) younger cells are more resilient under both normal and stressful conditions, and (2) stress amplifies this age-dependent difference. Together, these findings reveal a pronounced age-structured pattern of stress resistance even among age cohorts which are the most represented in proliferating populations.

Depletion of mtDNA can influence stress survival and aging through two opposing mechanisms. On the one hand, it can promote reactive oxygen species production and increase cellular damage; on the other hand (in multicellular organisms), mitochondrial dysfunction can inhibit cancer cell proliferation and thereby reduce cancer-associated mortality (Knorre et al. 2016). In yeast, mitochondrial dysfunction has been reported to either increase (Jiang et al. 2016) or decrease (Heeren et al. 2004) maximum replicative lifespan. These discrepancies likely reflect the net balance between the deleterious effects of mitochondrial dysfunction and the activation of compensatory pathways (Azbarova and Knorre 2023). Here we show that in early life, the *rho⁰* mutation – with hydrogen peroxide as the exception – accelerates aging, manifested as a more rapid decline in stress resistance with replicative age (Fig. 4). This contrasts with studies of maximum replicative lifespan conducted under optimal growth conditions, where mitochondrial dysfunction can sometimes be beneficial (Jiang et al. 2016). We suggest that under the harsh stress conditions tested here, the inability to perform oxidative phosphorylation decisively outweighs any potential benefits of compensatory mechanisms activation.

Of the two stresses tested, only under acetic acid stress did deletion of *SIR2* increase the slope of mortality with replicative age (Fig. 5). Notably, acetic acid supplementation induces cytoplasmic

acidification (Chaves et al. 2021), a phenotype that also precedes age-associated death under normal conditions (Knieß and Mayer 2016). This raises the possibility that acetic acid stress partially mimics a state of advanced replicative age, and that the contribution of *SIR2* to the age-dependent survival of young cell cohorts resembles its role in old cells dying under normal aging. Taken together, our experiments with the short-lived *sir2Δ* and long-lived *fob1Δ* mutants, like those with the *rho^o* mutant, only partially align with established mechanisms of cell death during normal replicative aging.

4.2 Evolutionary aging theories perspective

Accumulation of mutations that are neutral or beneficial in early life but have deleterious effects during post-reproductive stages limits the lifespan and drives evolution of aging (Medawar 1952; Williams 1957; Hamilton 1966). This mutation accumulation process is enabled by weakening of selection pressure after onset of reproduction. Indeed, alleles whose adverse effects manifest only in late life are already transmitted to offspring before they can be purified. As a result weakened selection allows deleterious-in-late-life alleles to accumulate in the populations (see for review (Flatt and Partridge 2018; Turan et al. 2019; Wensink and Cohen 2021)). These explanations are referred to as the mutation accumulation (Medawar 1952) and antagonistic pleiotropy (Williams 1957) evolutionary theories of aging, which are not mutually exclusive.

This evolutionary theory of aging can be extended to asymmetrically divided unicellular organisms, such as *S.cerevisiae*, where parental and progeny cells can be readily distinguished. Indeed, increase of mutation burden in the mutator yeast strains decrease their replicative lifespan (Lee et al. 2019)). However, it should be mentioned that mutation accumulation in the asymmetrically dividing bacteria experimental evolution with *Caulobacter crescentus* provided a decrease of the slope of age associated mortality in some of the studied populations (Ackermann et al. 2007).

Although the trajectories of aging significantly vary between species (Jones et al. 2014), under normal conditions, the age of death from intrinsic factors usually occurs considerably later than the onset of reproduction (Hamilton 1966). This delay is consistent with evolutionary aging theories as the selection pressure on deleterious-in-late-life alleles weakens gradually, as the parent individuals are diluted in the offspring (Charlesworth 2000). However, the question remains whether this relationship (increasing mortality probability with age) holds true under extreme conditions, when the mortality is extremely high. Indeed, on the one hand, the evolutionary logic that selection weakens with age might still apply. Selection is expected to act more strongly on young organisms before they reproduce, whereas a mother cell that has already produced many offspring has already passed on her genes, so selection to keep her alive weakens. Therefore, in evolutionary history, selection for stress resistance likely weakened after the first round of budding. Gene variants that disable stress resistance after several rounds of division would have had a higher chance of persisting than alleles that abolish this defence entirely from the start. Accordingly, it is known that human peak performance in sports declines early in life, at age of 20–40 years depending on specific challenges (Huey et al. 2007; Allen and Hopkins 2015). On the other hand, with age, cells accumulate exposure to stress factors and may therefore become increasingly tolerant or acclimated to stress.

Our data showed that in all studied stress conditions there is an increase in stress-dependent mortality with age among young cohorts (Fig. 4, Fig. S5). Therefore, the first explanation overweight in these two mutually non-exclusive factors that shape the age-dependency of stress resistance in yeast population. Meanwhile, we suggest that different weights of these two opposing factors: preadaptation on one hand and accumulated mutations, on the other hand, determine the rate of aging — the slope of mortality over the age — for each specific stress. It should be noted, that although in our study the stresses were not equalised by intensity, there was no apparent dependency between mortality slope and stress intensity: compare for instance BAC and HS conditions (Fig. 4, Fig. S5). Moreover, we suggest that the steeper age-dependent mortality under stressful conditions (Fig. 4) we observed for *rho*⁰ cells is explained by the fact that the combination of mitochondrial dysfunction and stress conditions is an extremely rare event that yeast cells have encountered in their evolutionary history. Therefore, their genomes contain an even greater number of late-life deleterious alleles that reduce stress resistance under these combined conditions.

4.3 Limitations of the study

In our study, we observed age-dependent mortality of yeast cells under various stressful conditions. However, our approach only allows analysis of stresses that cause rapid membrane permeabilisation, detectable by propidium iodide (PI) staining. Many stresses, however — for example, the toxic effects of antifungal drugs like Amphotericin B and azoles — primarily inhibit growth rather than kill cells (Hill et al. 2013; Vincent et al. 2013; Galkina et al. 2020). To the best of our knowledge, these drugs do not induce membrane permeabilisation or PI staining at standard working concentrations. Consequently, our study was limited to cytotoxic stresses, and we could not assess cytostatic agents; whether age-dependent resistance also applies to growth-inhibitory stresses remains an open question. Moreover, PI staining has its own limitations: under certain conditions, it can transiently stain live cells, yielding false positives (Davey and Hexley 2011). Nonetheless, given that we analysed multiple stresses and that PI-positive and PI-negative populations were clearly distinguishable (Fig. 3, Fig. S5), we believe that — even if exact values can be biased — the general conclusion that stress resistance declines with age in early age cohorts still holds.

Another limitation of our approach is that it enables us to analyse only relatively young age cohorts. Longer incubation times lead to decreased AF488 fluorescence intensity and further dilution of the AF488-labelled cohort. These two factors together prevented us from extending the analysis to older ages. We suggest that the use of more stable fluorophores in the future would help extend this approach to a broader age range.

Finally, potential source of error arises from the age heterogeneity within each measured population (see Fig. S3). Although our method labels mother cells and longer incubation with AF488 increases the average age of the cells in the sample, each data point nevertheless represents a mixture of cells of different ages. This age spread may blur the effects we observe, because the true age range within each AF488 + and AF488 – sample is wider than the nominal age values assigned in the figures and used in the regression analysis. Because the age distributions show a pronounced mode, this limitation prevents

us from linking stress resistance to a precise age, but still permits us to extract general patterns — specifically, comparing the slopes that relate age to survival under stress.

Concluding remarks

In this study, we demonstrated a relationship between replicative age in young yeast cell cohorts and their stress resistance. This relationship, for certain stresses, changes when cells lose mtDNA. The slope relating mean population age to the proportion of dead cells in these experiments reaches 8.5 (for hyperosmotic stress in ρ^+ cells), indicating that even in clonal, genetically identical populations, substantial heterogeneity in stress resistance exists — and that this heterogeneity is determined by the age structure of the population.

Our study demonstrates that deletion of the *SIR2* gene, which reduces maximum replicative lifespan (Kaeberlein et al. 2005), accelerates the early-life decline in acetic acid resistance (Fig. 5). However, this effect was not observed for heat shock stress, nor in another mutant strain, *fob1 Δ* which is known for its extended replicative lifespan under optimal conditions (Kaeberlein et al. 2005). This suggests that the mechanisms governing early replicative senescence overlap only partially with those limiting maximum replicative lifespan.

We believe our work expands the understanding of yeast aging by extending its implications to younger cells, which constitute the vast majority of cells in proliferating populations. Our data are consistent with predictions from evolutionary theories of aging. To our knowledge, this is one of the few studies to examine age-dependent mortality under extreme conditions specifically in young cohorts, where age-structured heterogeneity has been largely overlooked.

Declarations

Author contributions

D.K. designed the study and obtained funding. A.A. generated the *fob1 Δ* strain. A.A. and K.G. conducted the experiments and analyzed the data. A.A. and D.K. prepared the illustrations. D.K. supervised the study and drafted the manuscript. All authors contributed to text editing and approved the final version of the manuscript.

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Data availability

The data presented in this study are available within this paper and Supplementary Materials

Conflict of interest

The authors declare no competing interests.

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Figures

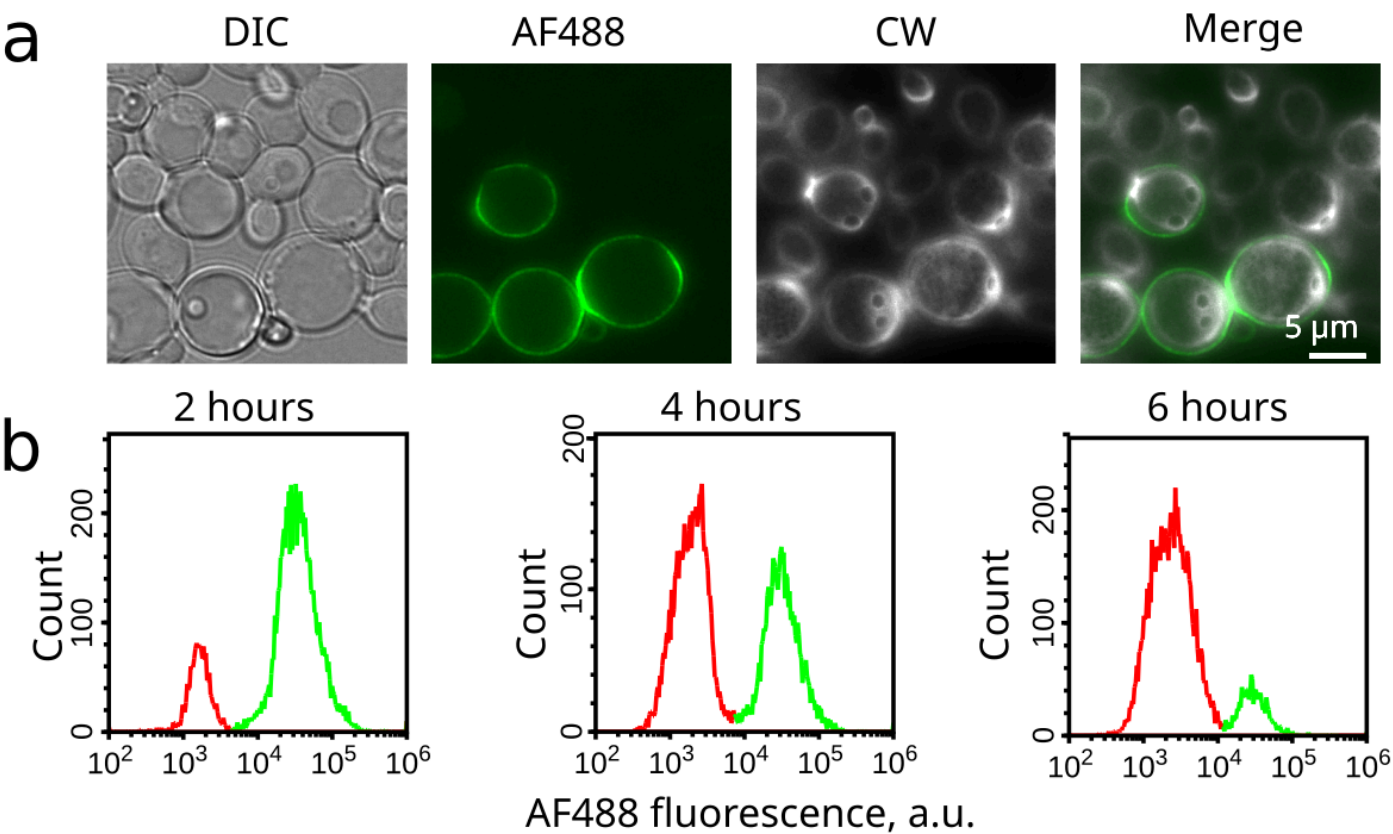


Figure 1

Pulse-chase labeling with AF488 distinguishes old and newly formed yeast cells. **a** Fluorescence microscopy of yeast cells following pulse-chase labeling with AF488 ($0.6\ \mu\text{g/mL}$; see Materials and Methods for details). After washing to remove unbound dye, cells were incubated for 4 h and then stained with Calcofluor White (CW) to visualize bud scars. DIC, differential interference contrast. **b** AF488-positive cells are diluted by newly formed AF488-negative cells as proliferation proceeds. Flow cytometry reveals a bimodal fluorescence distribution: stained (older, labeled in green) and unstained (newly formed, labeled in red) populations. Incubation time after labeling is indicated above each histogram. For clarity, the two distribution modes were manually annotated.

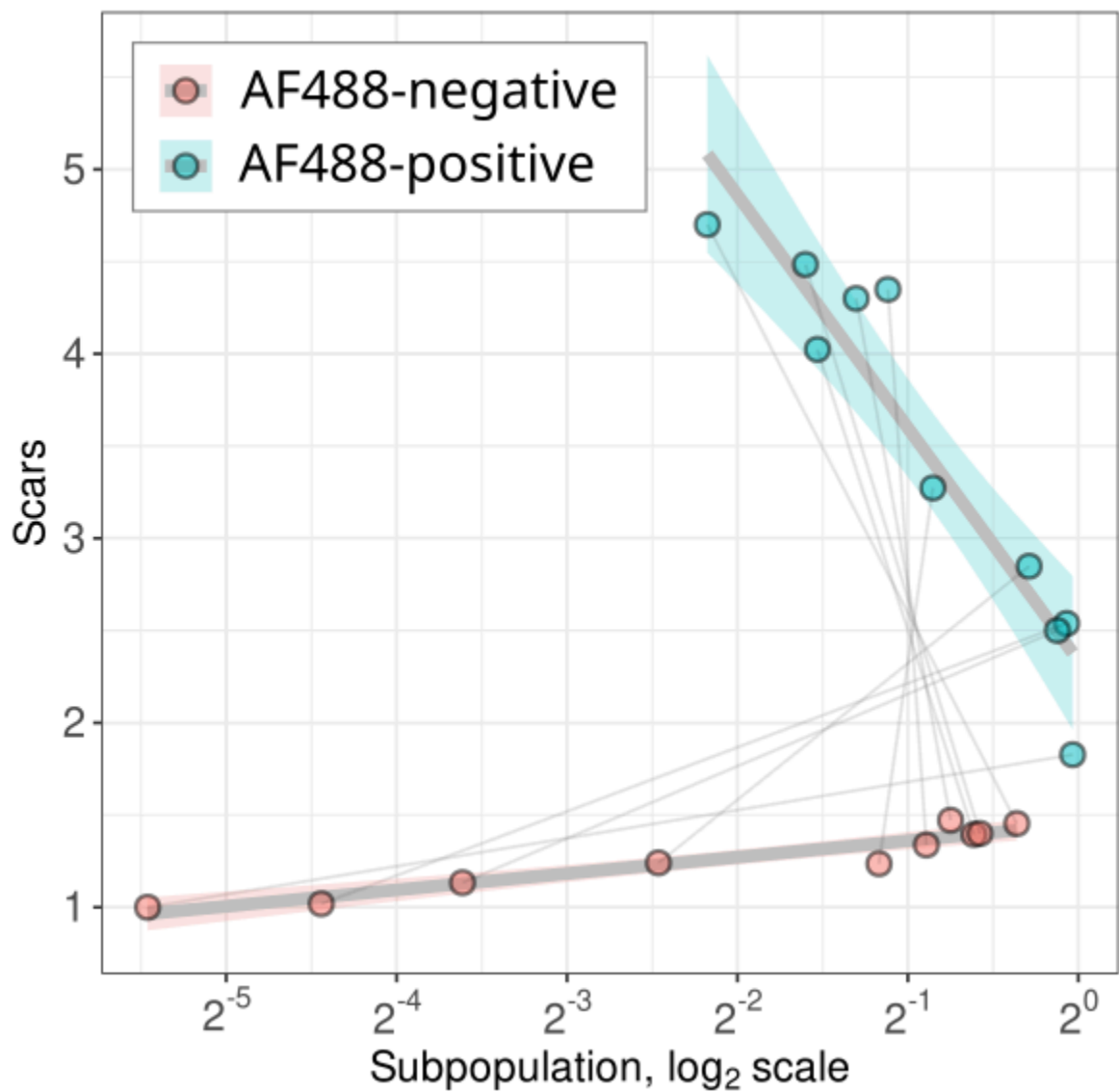


Figure 2

Proportion of AF488-stained cells correlates with average cell age

Correlation between the proportion of log₂-transformed proportion of AF488-positive cells in the population and the average number of bud scars per cell. Thin lines connect subpopulations from the same flow cytometry run.

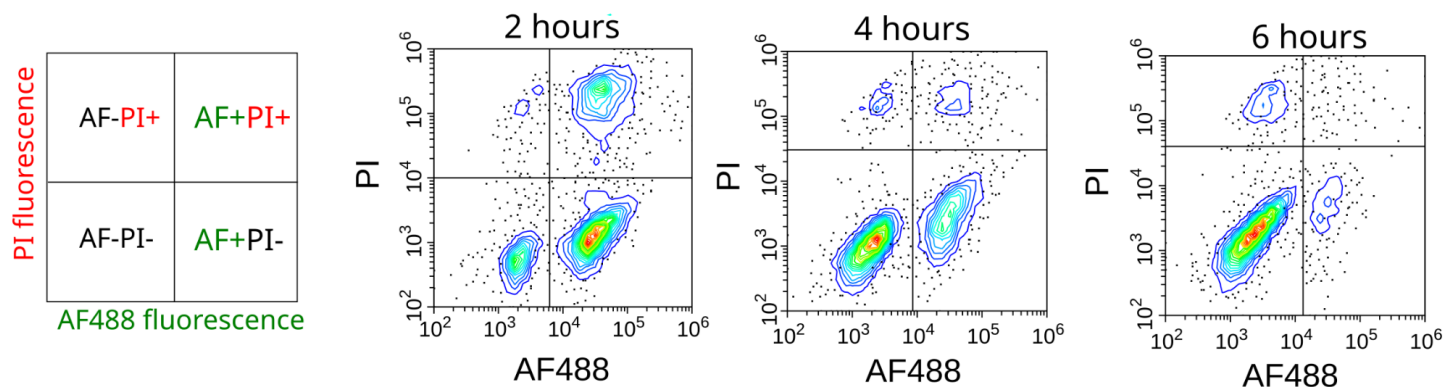


Figure 3

AF488 enables differential assessment of cell death in newborn and old cells under stress conditions. Cells were pulse-labeled with AF488, exposed to stress (SCID), and subsequently stained with propidium iodide (PI). Flow cytometry dot plots show four distinct subpopulations based on AF488 and PI fluorescence: AF488-/PI- (young live), AF488+/PI- (old live), AF488-/PI+ (young dead), and AF488+/PI+ (old dead).

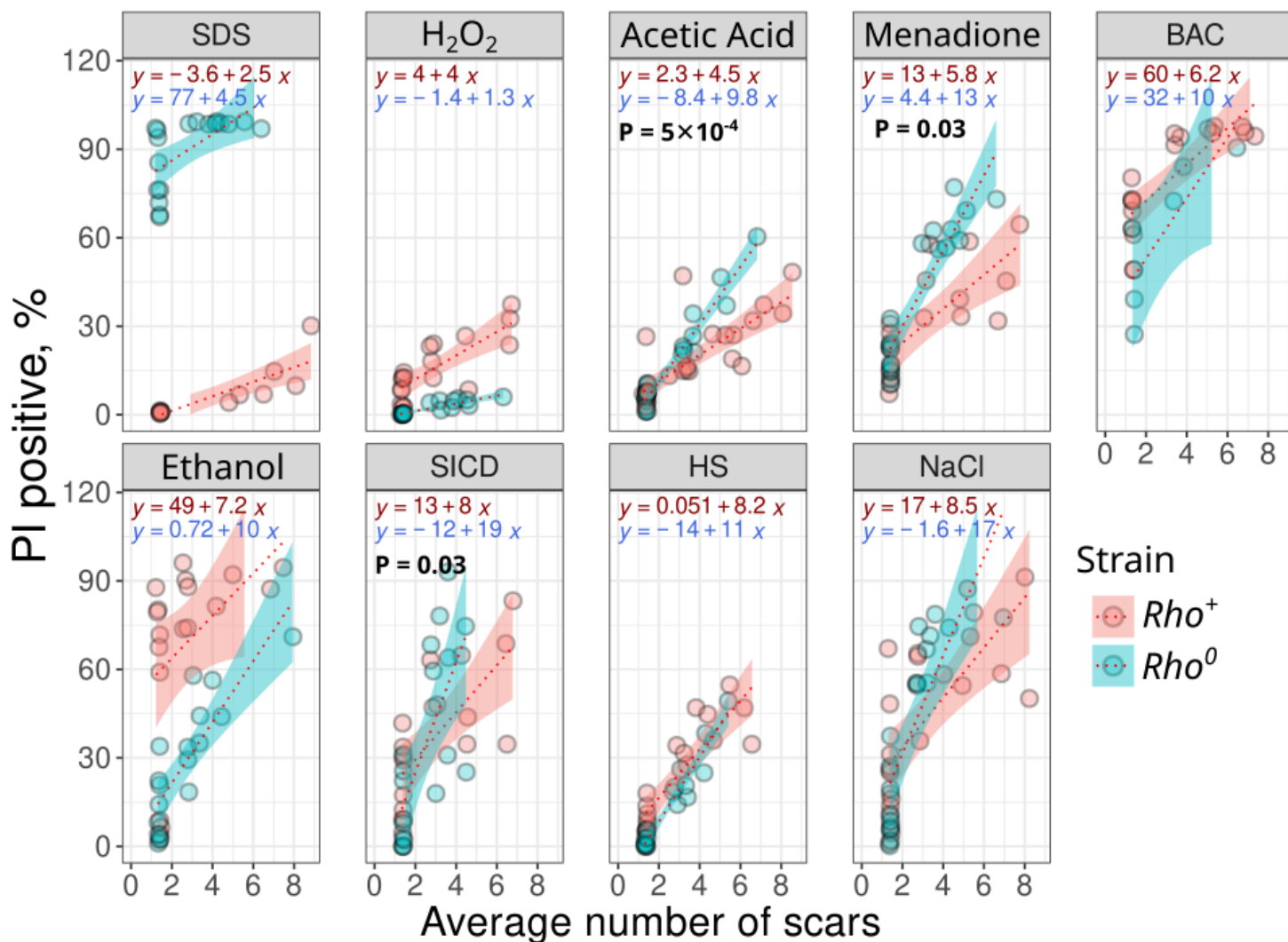


Figure 4

Age-dependent mortality under stress in Rho^+ and Rho^0 strains. The plot shows the proportion of dead cells as a function of replicative age. Linear regression coefficients for Rho^+ (red, upper line) and Rho^0 (blue, lower line) are displayed at the top of each facet panel. Exact stress conditions are provided in Table S2. Survival data for individual age cohorts under control conditions are shown in Fig. S5 (Rho^+) and Fig. S7 (Rho^0). A linear regression line (red, dashed) with shaded 95% confidence interval is shown for all conditions. Data points represent individual cell cohorts, plotted by their calculated average age and corresponding percentage of propidium iodide-positive (dead) cells. The P-value shown corresponds to a comparison of Rho^+ versus Rho^0 growth using a permutation test (see Materials and Methods).

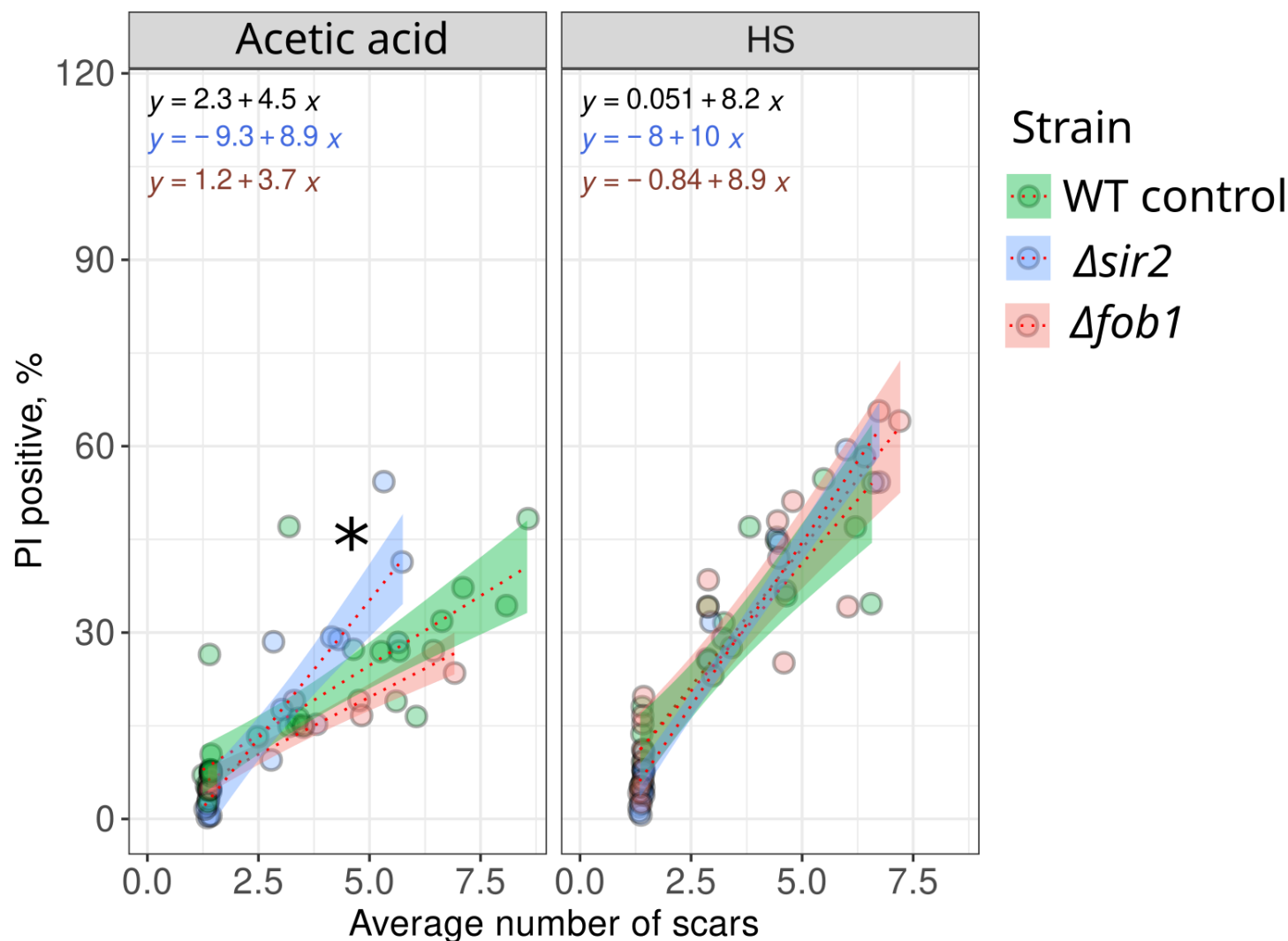


Figure 5

Age-dependent mortality under stress in wild type, $sir2\Delta$ and $fob1\Delta$ strains. Deletion of $SIR2$ increases the age-dependent component of acetic acid sensitivity with age (* $P = 0.0024$). Linear regression line (red, dashed) with shaded 95% confidence interval is shown for all conditions. Data points represent

individual cell cohorts with calculated average age and percentage of propidium positive cells. Note, that WT control data are the same as in Fig. 4.

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