



Research Paper

Redox control of yeast Sir2 activity is involved in acetic acid resistance and longevity



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ABSTRACT

Yeast Sir2 is an NAD-dependent histone deacetylase related to oxidative stress and aging. In a previous study, we showed that Sir2 is regulated by S-glutathionylation of key cysteine residues located at the catalytic domain. Mutation of these residues results in strains with increased resistance to disulfide stress. In the present study, these mutant cells were highly resistant to acetic acid and had an increased chronological life span. Mutant cells had increased acetyl-CoA synthetase activity, which converts acetic acid generated by yeast metabolism to acetyl-CoA. This could explain the acetic acid resistance and lower levels of this toxic acid in the extracellular media during aging. Increased acetyl-CoA levels would raise lipid droplets, a source of energy during aging, and fuel glyoxylate-dependent gluconeogenesis. The key enzyme of this pathway, phosphoenolpyruvate carboxykinase (Pck1), showed increased activity in these Sir2 mutant cells during aging. Sir2 activity decreased when cells shifted to the diauxic phase in the mutant strains, compared to the WT strain. Since Pck1 is inactivated through Sir2-dependent deacetylation, the decline in Sir2 activity explained the rise in Pck1 activity. As a consequence, storage of sugars such as trehalose would increase. We conclude that extended longevity observed in the mutants was a combination of increased lipid droplets and trehalose, and decreased acetic acid in the extracellular media. These results offer a deeper understanding of the redox regulation of Sir2 in acetic acid resistance, which is relevant in some food and industrial biotechnology and also in the metabolism associated to calorie restriction, aging and pathologies such as diabetes.

1. Introduction

Under conditions of rich media and high glucose concentration, *S. cerevisiae* ferments glucose to ethanol, also producing acetic acid. The ethanol and the acetic acid excreted to the medium inhibit the growth of other microorganisms. When glucose becomes limiting, yeasts enter the diauxic shift and metabolism changes from glucose fermentation to ethanol respiration, which has been accumulating during the fermentative phase. Finally, when all carbon sources have been exhausted, cells enter the stationary phase (G0). In the course of the diauxic shift, and also at the stationary phase, reactive oxygen species (ROS) generated by aerobic metabolism cause oxidative damage to cell components such as proteins, DNA, and lipids. However, ROS may also act in signaling events and culminate in adaptive responses. In proteins, one of the most reactive groups of amino acid side chains is thiols (-SH) from cysteine residues. Different oxidants can react with thiols to yield a

variety of products [1] that can be detected by proteomic approaches [2]. They include i) sulfenic acid (-SOH), which can react to other thiols to generate mixed disulfides with glutathione (Prot-S-SG), also known as S-glutathionylation, ii) intramolecular disulfides (-S-S-), and iii) intermolecular disulfides between proteins. Sulfenic acid may become further oxidized to sulfinic (-SO₂H) or sulfonic (-SO₃H) acids in the presence of stronger oxidants or prolonged exposure to mild oxidants. When reversible, post-translational modifications of reactive cysteine residues of proteins may act as a regulatory mechanism [3,4]. The thioredoxin and the glutaredoxin systems reduce disulfide bonds, and both systems require NADPH as the final electron donor.

Sirtuins are NAD⁺-dependent histone deacetylases present from bacteria to mammals, and yeast Sir2 was the first sirtuin described [5]. In humans, there are seven mammalian Sir2 orthologues (Sirt1–Sirt7); the biochemical activities of Sirt1 are the most similar to Sir2. Sirt1 participates in a great variety of processes. Directly or indirectly, Sirt1

Abbreviations: Acs, acetyl-CoA synthetase; LD, Lipid droplets; Pck1, phosphoenolpyruvate carboxykinase; ROS, reactive oxygen species; Sod, superoxide dismutase
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regulates insulin secretion, gluconeogenesis, circadian rhythms, adiponectin production, and oxidative stress [6]. Sirt1 has also been involved in different pathologies, including cancer and neurodegenerative diseases [7]. Both selective Sirt1 activators and inhibitors are available, and initial clinical trials have been carried out (reviewed in Ref. [8]). Yeast Sir2 is involved in important functions such as silencing of telomeres, rDNA and the mating type loci, maintaining genome integrity and aging [9,10]. In mammals, the role of Sirt1 in antioxidant defense has been described as dependent on FoxO3a deacetylation [11,12]. Yeast cells have four forkhead transcription factors, three (Hcm1, Fkh1, Fkh2) of which have been related to Sir2 upon oxidative stress [13–15]. Up-regulation of yeast Sir2 has been observed upon exogenous oxidative stress [16,17] or endogenously generated stress [18]. Thus, the relationship of sirtuins with stress resistance is well known. In this context, it has been described that the cellular redox status modulates Sirt1 activity [17,19]. In mammalian [20,21] and zebrafish [22] Sirt1, several cysteines have been identified as S-glutathionylation targets, mostly located in the catalytic domain and near the binding site of the cofactor NAD⁺. Although key cysteine residues involved in these processes have been identified, none of them is conserved in yeast Sir2.

Recently, we showed yeast Sir2 is S-glutathionylated upon strong disulfide stress [23]. Monothiol glutaredoxins Grx3 and Grx4 are able to S-deglutathionylate it, acting as the physiological regulators of Sir2. Three key cysteines (C363, C469 and C513) located at the catalytic domain have been identified as S-glutathionylation targets. Such post-translational modification results in enzyme inactivation, as is described in zebrafish [22], and mammalian Sirt1 [20,21]. Nevertheless, redox modification of key cysteine residues has been described activating many proteins. Among them are transcription factors like oxyR from *E. coli* [24], Yap1 from *S. cerevisiae* [25], Pap1 from *S. pombe* [26], or enzymes such as bacterial Hsp33 [27] and typical 2-Cys peroxiredoxins [28].

Like mammalian Sirt1, yeast Sir2 is described as deacetylating not only histones, but proteins involved in metabolism and related to aging. Sir2 deacetylates, both *in vitro* and *in vivo*, phosphoenolpyruvate carboxykinase (Pck1), a key enzyme in the gluconeogenesis pathway, resulting in the loss of its catalytic activity [29,30]. Acetylation of Lys514 is essential for enzyme activity and the ability of cells to grow in non-fermentable carbon sources [29]. Pck1 inactivation by Sir2 decreases intracellular glucose stores, like trehalose and glycogen, which are necessary for chronological longevity. In humans, activation of Pck1 through acetylation has also been described during fasting and is related to increased gluconeogenesis and longevity [29].

Previously, we showed that Sir2 mutant strains lacking one of three cysteine residues identified as targets of redox regulation are more resistant to strong diamide stress [23]. In the present study, these strains were also more resistant to acetic acid stress and showed increased chronological life span. Our new results may help to elucidate the role of redox-regulated cysteines of Sir2 in the metabolic changes that could explain such phenotypes.

2. Materials and methods

2.1. Yeast strains, genetic methods and culture conditions

Yeast strains used in this study are listed in Table 1. All the mutants are derived from the strain CML128 (*MATa ura3-52 his4 leu2-3,112 trp1*) considered the wild-type (WT) strain in this work [31]. Standard protocols were used for DNA manipulations and transformation of yeast cells. Strains Sir2-C363S, Sir2-C469S and Sir2-C513S, generated by site-directed mutagenesis of Sir2 cysteine residues [23] were also used. To analyze intracellular pH, WT, C363S, C469S and C513S strains were transformed with pYES-P_{ACT1}-pHluorin (*URA3*) plasmid, which contains the pHluorin (a pH-sensitive variant of the green fluorescent protein) open reading frame, under the control of the constitutive *S. cerevisiae* actin (*ACT1*) gene promoter and the *URA3* gene as a

Table 1
Yeast strains used in this work.

Strain	Relevant genotype	Source
CML128	<i>MATa ura3-52 his4 leu2-3,112 trp1</i>	[31]
NVLL4	CML128 <i>sir2::natMX4</i>	[23]
NVLL5	NVLL4 [pNVLL2(<i>SIR2 C363S</i>)]:: <i>LEU2</i>	[23]
NVLL6	NVLL4 [pNVLL3(<i>SIR2 C469S</i>)]:: <i>LEU2</i>	[23]
NVLL7	NVLL4 [pNVLL4(<i>SIR2 C513S</i>)]:: <i>LEU2</i>	[23]
BQS281	CML128 pYES-P _{ACT1} pHluorin:: <i>URA3</i>	This work
BQS283	NVLL5 pYES-P _{ACT1} pHluorin:: <i>URA3</i>	This work
BQS284	NVLL6 pYES-P _{ACT1} pHluorin:: <i>URA3</i>	This work
BQS285	NVLL7 pYES-P _{ACT1} pHluorin:: <i>URA3</i>	This work
BQS304	CML128 <i>RDN1-NTS2::mURA3</i>	This work
BQS305	NVLL5 <i>RDN1-NTS2::mURA3</i>	This work
BQS306	NVLL6 <i>RDN1-NTS2::mURA3</i>	This work
BQS307	NVLL7 <i>RDN1-NTS2::mURA3</i>	This work
BQS308	NVLL4 <i>RDN1-NTS2::mURA3</i>	This work

selectable marker [5,6]. The plasmid pYES-P_{ACT1}-pHluorin (*URA3*) was kindly provided by Dr. Ariño (Universitat Autònoma de Barcelona, Spain). A selection of transformants was performed on solid synthetic medium, and expression of the pHluorin gene in the selected transformants was checked by fluorescence microscopy. Plasmid pDM312 (*RDN1-NTS2::mURA3-LEU2*) was a gift from Dr. Moazed at Harvard Medical School [32]. *mURA3* silencing strains were generated by transformation with pDM312 cut with *Sma*I to integrate at *NTS2*. All transformations were performed with the lithium acetate method [33], and correct integration was confirmed by PCR. Cells were grown at 30 °C by incubation in a rotary shaker using SC medium (0.67% yeast nitrogen base, 2% glucose plus dropout mixture, and auxotrophic requirements). Specific supplements were omitted for selection of the corresponding plasmid-carrying cells. When indicated, YPD medium containing 2% glucose (1% yeast extract, 2% peptone, 2% glucose), YPD medium containing 0.5% glucose (1% yeast extract, 2% peptone, 0.5% glucose) or YPG medium (1% yeast extract, 2% peptone and 3% glycerol) were also used. Diamide, acetic acid, sorbic acid, benzoic acid and sodium chloride were purchased from Sigma. Exponentially growing cells were obtained at OD₆₀₀ = 0.5–1 (day 0); day 1 cells were obtained 24 h after.

2.2. pHluorin calibration and pHluorin measurements

Strains transformed with the plasmid pYES-P_{ACT1}-pHluorin were grown to an OD₆₀₀ of 0.5–0.75 in SC medium, centrifuged and resuspended in PBS containing 0.1 M digitonin (Sigma), which permeabilizes cell membranes. After 10 min, cells were washed with PBS at 4 °C and resuspended in citric acid/Na₂HPO₄ buffer of pH values ranging from 5.0 to 8.0. Cell fluorescence was measured using a fluorimeter (Shimadzu RF-5000). pHluorin fluorescence emission was measured at 508 nm providing excitation bands of 395 nm and 475 nm [34,35]. A calibration curve was generated plotting the ratio of emission intensity resulting from excitation at 395 and 475 nm (395/415 ratio) against the corresponding buffer pH [35]. Background fluorescence for a WT culture not expressing pHluorin was subtracted from the measurements. All pH determination experiments were repeated, at least, three times (biological replicates). To analyze changes in intracellular pH upon acetic acid stress, strains were grown to an OD₆₀₀ of 0.5 in SC medium, centrifuged and resuspended in SC medium plus the indicated amount of acetic acid (pH adjusted to 4.5). After 5 and 10 min, fluorescence emission (395/415 ratio) was measured and converted to intracellular pH according to the calibration curve previously obtained.

2.3. *In vivo* Sir2 silencing activity

In vivo Sir2 telomeric silencing activity was performed by quantifying transcriptional silencing at the naturally occurring telomere

linked gene, *YFR057W* [36]. Sir2-dependent *YFR057W* gene expression was measured by quantitative real-time PCR using the TaqMan System (Applied Biosystems). Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's recommendations. First-strand cDNA synthesis was performed on 1 µg DNase-treated RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen), and 50 ng of cDNA was used for each individual real-time PCR assay in a 48-cycle, three-step PCR reaction using the iCycler (Bio-Rad). Amplification was performed using the TaqMan Universal PCR Master Mix kit (Applied Biosystems). Quantification was completed using iCycler IQ Real-Time detection system software. Actin (*ACT1*) was used as an internal control. Relative expression ratios were calculated based on ΔCq values with efficiency correction based on multiple samples [37]. Data represent three technical repeats of each analysis from, at least, three different experiments.

In vivo Sir2 ribosomal DNA (rDNA) silencing was analyzed using strains transformed with plasmid pDM312 (*RDN1-NTS2::mURA3-LEU2*). Silencing of *NTS2* reporter within rDNA was assessed by monitoring the growth of 5-fold serial dilutions of cells plated on Ura-depleted SC medium (- Ura). Ura-containing SC medium (+ Ura) was used as plating control.

2.4. Enzyme activities

Cells were grown to the desired OD₆₀₀ and centrifuged, and cell extracts were obtained with a Mini-Bead beater cell disrupter (Biospec) using glass beads. All activity assays were measured at 25 °C. Catalase activity was measured by H₂O₂ consumption at 240 nm [38]. Alcohol dehydrogenase was measured spectrophotometrically by NADH formation [39]. Citrate synthase was measured in a coupled assay to reduce 5,5-dithiobis-(2-nitrobenzoic acid) [40]. Glutathione reductase was performed by measuring NADPH consumption at 340 nm [41]. Acetyl CoA synthetase activity was measured using an enzymatic method coupled with malate dehydrogenase and citrate synthase [42]. Phosphoenolpyruvate carboxykinase activity was measured in the OAA formation direction coupled with malate dehydrogenase as described [43]. Superoxide dismutase (SOD) activities were assayed in a native gel by chromogenic staining (zymogram) as described [44], with modifications [13]. In brief, cells were homogenized in 50 mM Tris-HCl buffer, pH 8.5 and after centrifugation, 5% (w/v) saccharose and 0.05% (w/v) Bromophenol blue were added. 25 µg of cell extract were applied directly to a non-denaturing 10% polyacrylamide gel. Following electrophoresis, the gel was stained in a solution containing 50 mM Tris-HCl buffer, pH 7.8, 125 µg/ml thiazolyl blue tetrazolium bromide, 75 µg/ml phenazine methosulfate, and 188 µg/ml MgCl₂. The blue tetrazolium stain for O₂⁻ was developed by exposure to light. Staining was absent at sites of O₂⁻ scavenging.

2.5. Microscopic analysis of lipid droplets

Lipid droplets were visualized by fluorescence microscopy using BODIPY™ 493/503 (Invitrogen, Inc., USA) prepared as a 10 mM stock solution in DMSO and kept at -80 °C [45]. BODIPY 493/503 is a green emitting fluorophore for high-contrast labeling of yeast lipid droplets and shows very high quantum yield and photostability [46]. Aliquots of 5 µl of cellular suspension and 5 µl of 10 mM BODIPY 493/503 were mounted onto a polylysine-treated glass slide. Microscopy was performed on Olympus DP30BW using a 60x oil immersion objective.

2.6. Other analyses

To measure resistance to stress, both cell growth and viability were determined under control conditions or with the stressing agent added to the media at the indicated concentrations. Cell growth was monitored in 0.5 ml cultures in 24-well plates incubated at the indicated temperature and constant agitation in a PowerWave XS microplate

spectrophotometer (Biotek). Plates were sealed with Breathe Easy membranes (Diversified Biotech, USA). Optical density (OD₆₀₀ nm) was recorded every 30 min for 20-25 h and the values were analyzed with Gen 5 1.06 software. Total iron quantitation from yeast cells was performed following the method previously described [47]. Intracellular glutathione (GSH) levels were measured with the ThiolTracker™ Violet fluorescent thiol probe (Invitrogen) following the manufacturer's recommendations. Cell extracts were obtained as previously described [48], separated in SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. Western blot analysis was performed by standard methods and the anti-Sir2 antibody (Santa Cruz Biotechnology) was used. Images were acquired in a ChemiDoc XRS System (Bio-Rad) and analyzed with Quantity One software (Bio-Rad). Ethanol from the extracellular medium was measured spectrophotometrically at 340 nm using the Enzytec™ fluid ethanol kit (Thermo Fisher Scientific). Acetic acid from the extracellular medium was quantified spectrophotometrically at 340 nm using the Acetic Acid kit from Megazyme. Intracellular trehalose was measured according to the Trehalose assay kit from Megazyme.

2.7. Statistical analysis

Data are presented as means ± SD from, at least, three independent experiments. Statistical analysis was performed using two-tailed Student t-test. The *P*-values lower than 0.05(*), 0.01(**) or 0.005 (***) were considered significant.

3. Results

3.1. Lack of redox-regulated Sir2 cysteines increases longevity and acetic acid resistance

In a previous study [23], we have reported that yeast Sir2 activity is post-translationally regulated by reversible S-glutathionylation, with monothiol Grx3 and Grx4 acting as the physiological thiol-reductases. Our study also provides evidence that the redox state of three key cysteine residues of Sir2 (C363, C469 and C513), located at the catalytic domain (Supplementary Figs. 1A–C), influences yeast resistance to disulfide stresses (diamide and diethyl maleate). To more deeply explore the physiological role of such Sir2 cysteine residues, mutant strains in which one of these cysteine residues was mutated to serine (C363S, C469S and C513S) were grown in SC medium and survival along stationary phase (chronological life span) was analyzed and quantified (Fig. 1A). We observed a significantly increased survival in the mutant strains compared with the WT strain. After 24 days, only ~0.01% of cells survived in the WT strain in comparison with ~0.1% in C469S and ~0.5–1% in both C363S and C513S strains. Such increased longevity may be due to a variety of factors, including increased resistance to several stresses. Although mutant cells, expressing physiological levels of Sir2, were more resistant to high disulfide stress [23], no growth differences were detected when H₂O₂ was used as stressing agent, heat shock or osmotic stress (Fig. 1B). In this context, several activities related to resistance to oxidative stress were also measured. As shown, no significant differences in catalase (Fig. 1C), superoxide dismutases (Sod 1 and Sod 2) (Fig. 1D), or intracellular iron were detected (Fig. 1E). Similarly, glutathione reductase activity (Supplementary Fig. 2A) and levels of glutathione (GSH) (Supplementary Fig. 2B), two important parameters under oxidative stress, showed similar levels between mutant and WT cells.

It has also been described that resistance to acetic acid correlates with longevity [49–51] although this is not always fulfilled [52]. Thus, to analyze acetic acid tolerance, WT and mutant strains were grown in SC medium without (control) or with acetic acid addition at the indicated concentration. In all conditions, pH was adjusted to 4.5 (the normal pH of SC medium), and cell growth measured for 20 h. As shown in Fig. 2A, all three mutant (C363S, C469S and C513S) strains showed an improved response upon acetic acid treatment compared

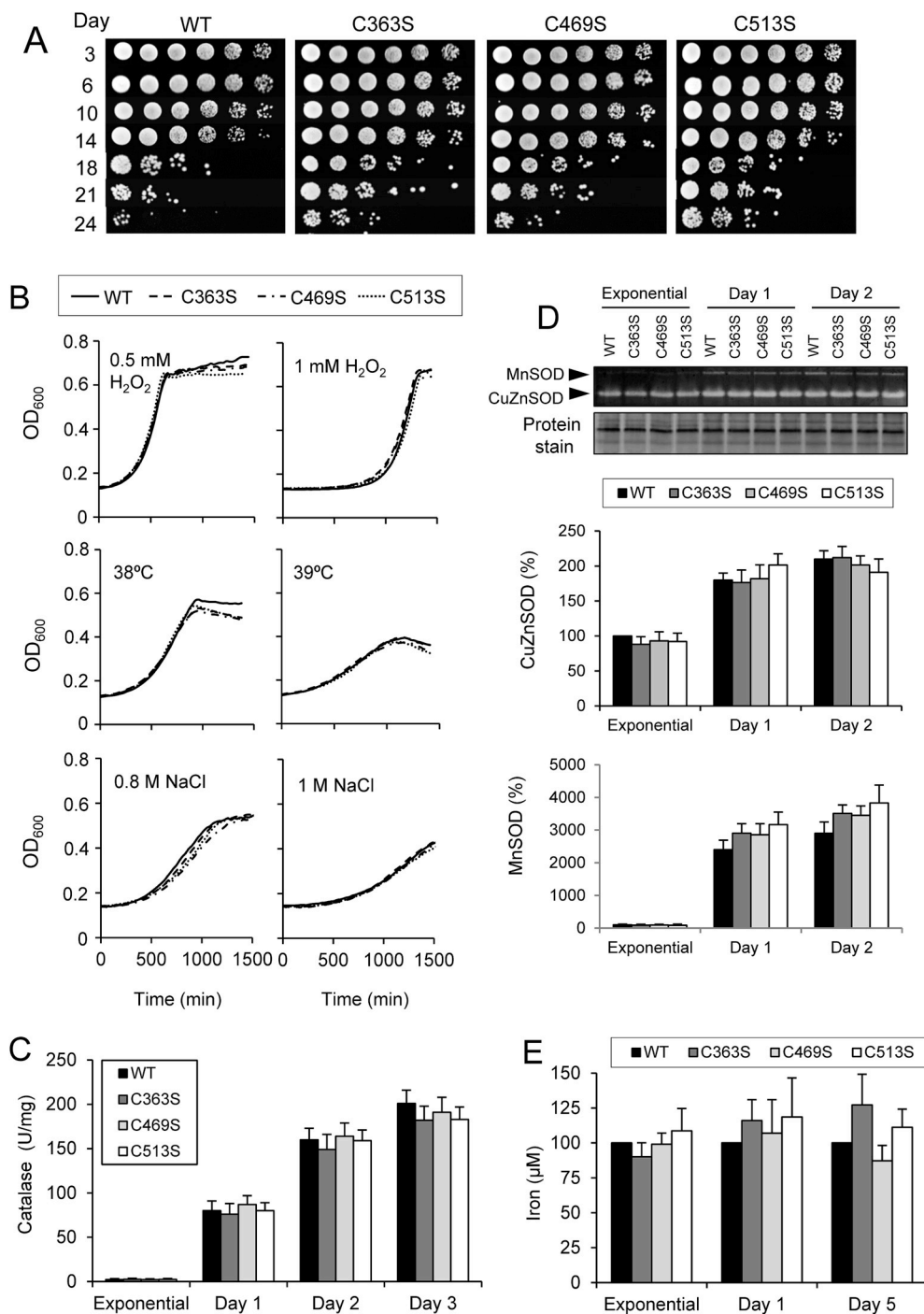


Fig. 1. Increased chronological life span of mutant Sir2 strains is not due to higher antioxidant capacity. Cultures of WT, C363S, C469S and C513S cells were grown in SC medium. (A) Viability was measured during chronological aging by plating serial dilutions (1:5) in YPD plates. (B) Growth curves were measured upon oxidative stress (0.5 mM or 1 mM H₂O₂), heat stress (38 °C or 39 °C) and osmotic stress (0.8 M or 1 M NaCl). The data are the averages of two independent experiments with two replicas each. (C) Catalase activity was analyzed in whole extracts from the exponential phase, days 1, 2 and 3 of culture. (D) Cell lysates from cultures at exponential phase, days 1 and 2 were analyzed by native electrophoresis and SOD staining. Total Coomassie blue protein staining was used as a loading control. Bands corresponding to MnSOD and Cu/ZnSOD activities were measured with a densitometer, and the relative intensity was calculated. (E) Cultures grown at exponential phase, days 1 and 5 were digested with nitric acid and total iron indicated with respect to the exponential WT strain. From C to E, statistical analysis was performed comparing mutant with WT cells. The data are represented as the means ± SD from at least three independent experiments.

with WT, but no significant differences were observed under control conditions. Increased survival of mutant cells, compared with WT, was also observed when cells were plated in media containing acetic acid (Fig. 2B).

We determined viability to other weak organic acids, such as benzoic and sorbic acid, which are used as food preservatives to inhibit the growth of spoilage yeasts, including *S. cerevisiae* [53]. Again, our three mutant strains were more resistant to such acids (Fig. 2C and D).

3.2. Acetic acid tolerance in Sir2 mutant strains is not due to changes in intracellular pH

Although the adaptive response and resistance to these weak organic acids are complex [54,55], it has been published that the capacity

of yeast cells to maintain cytosolic pH (pH_c) plays a key role in acetic acid tolerance [56]. To investigate whether an alteration occurs in the pH_c of mutant strains before and after exposure to the acid, WT and all three mutant strains were transformed with pYES-P_{ACT1}-pHluorin plasmid, carrying a pH-sensitive variant of the green fluorescent protein [34,35]. A calibration curve was performed (Fig. 3A) to analyze the effects of increasing acetic acid concentrations on pH_c. Fig. 3B shows the pH_c values of cells 5 min after exposure to medium containing 0, 30, 60 or 90 mM acetic acid. Non-stressed cells showed no differences among strains (pH_c ~ 7.5). After 30, 60 or 90 mM acetic acid, pH_c decreased to ~6.8, 5.7 and 5.3 respectively, but no significant differences were observed among strains (Fig. 3B). Cells were able to recover rapidly, and after 10 min, cells treated with 30 mM acetic acid showed the same pH_c as untreated cells. Cells treated with 60 and 90 mM

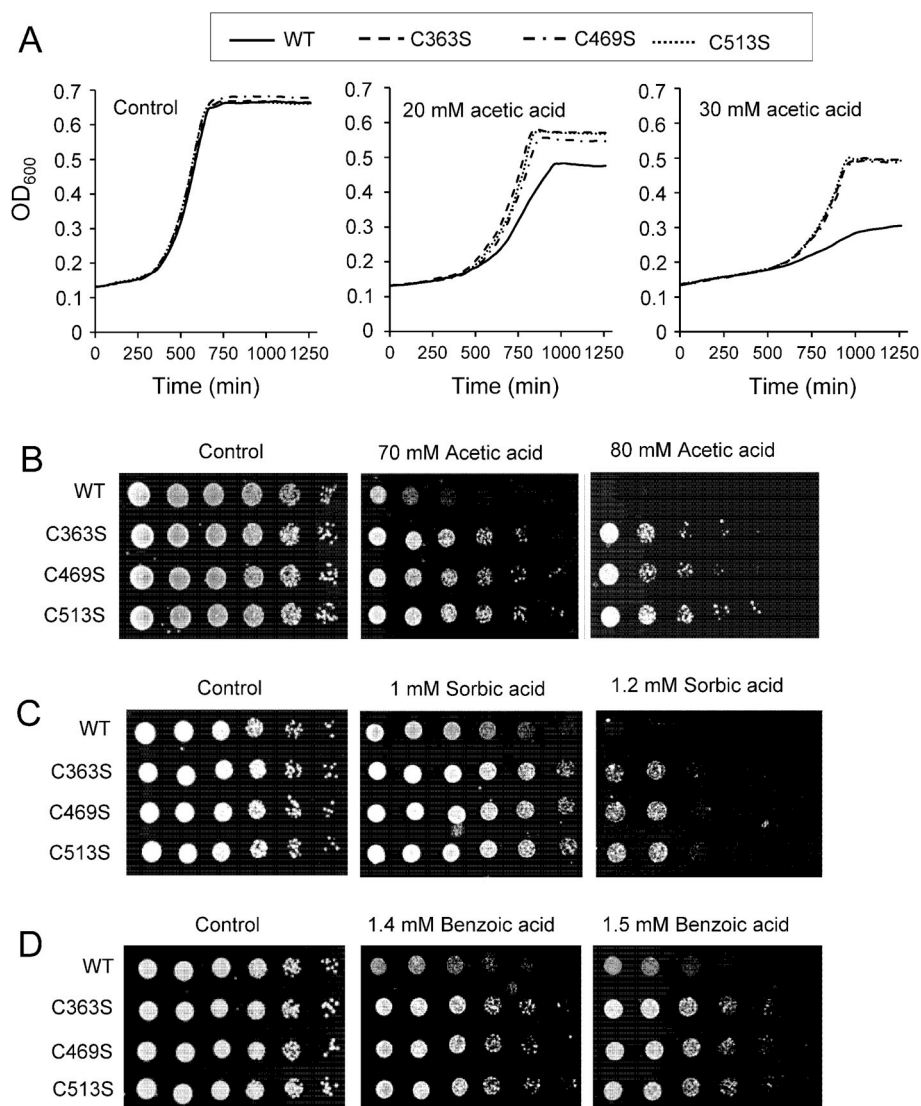


Fig. 2. Mutant Sir2 strains show increased acetic acid tolerance. Cultures of WT, C363S, C469S and C513S cells were grown in SC medium. (A) Growth curves of WT, C363S, C469S, and C513S strains were measured when grown in SC medium without or with 20 or 30 mM acetic acid. The data are the averages of two independent experiments with two replicas each. (C) The viability of WT, C363S, C469S and C513S cells grown in SC medium was measured by plating serial dilutions (1:5) in SC plates containing 70 or 80 mM acetic acid.

showed a pH_c of 6.9 and 6.6, respectively (Fig. 3C). Again, no significant differences between strains were observed during recovery. These results indicated that in exponentially growing cells, acetic acid tolerance is not due to the capacity to maintain and/or recover pH_c upon acute acid stress.

3.3. Sir2 mutants show increased acetyl-CoA synthetase activity and lipid droplets during/along aging

Using cells transformed with pHluorin, we analyzed pH_c throughout chronological aging. As shown in Fig. 3D, pH_c dropped abruptly from 7.5 at the exponential phase to 6.5 at the diauxic shift (day 1). This slightly acidic intracellular pH was maintained for a few days; however, at day 5 and statistically significant at day 7, mutant cells showed an increased pH_c compared with WT cells. We were unable to follow this experiment beyond day 7 because cells started to accumulate lipofuscin, a well-known marker of aged cells with a broad spectrum of autofluorescence [57,58].

To understand such increased pH_c in the mutant cells along aging, we analyzed the metabolic pathways that could be involved. Ethanol

and acetate are two well-known by-products of yeast glucose fermentation that are transiently accumulated in the culture medium. In fact, after the diauxic shift, these C2 compounds are used as substrates of a respiration-based metabolism that, in addition to functional mitochondria, requires the involvement of the glyoxylate/gluconeogenesis pathways. As expected, levels of ethanol in the culture medium were very high on day 1 (diauxic shift) and decreased to undetectable levels after 6 days of culture (Fig. 4A). No significant differences between WT and Sir2 mutant strains were detected. Ethanol levels are the result of the activities of alcohol dehydrogenase (Adh) 1 and 2. At exponential phase, Adh activity is mainly due to the fermentative Adh 1 enzyme, which converts acetaldehyde to ethanol. When glucose decreases, *ADH1* is repressed and *ADH2* induced. Adh2 activity catalyzes the opposite reaction, which is necessary for ethanol respiratory metabolism. Alcohol dehydrogenase activity showed no differences between strains along aging (Fig. 4B), which agrees with the similar levels of ethanol detected.

On the contrary, acetate levels in the culture medium were lower in the Sir2 mutant strains, compared with the WT strain, being statistically significant from day 2 until day 7, when levels were almost

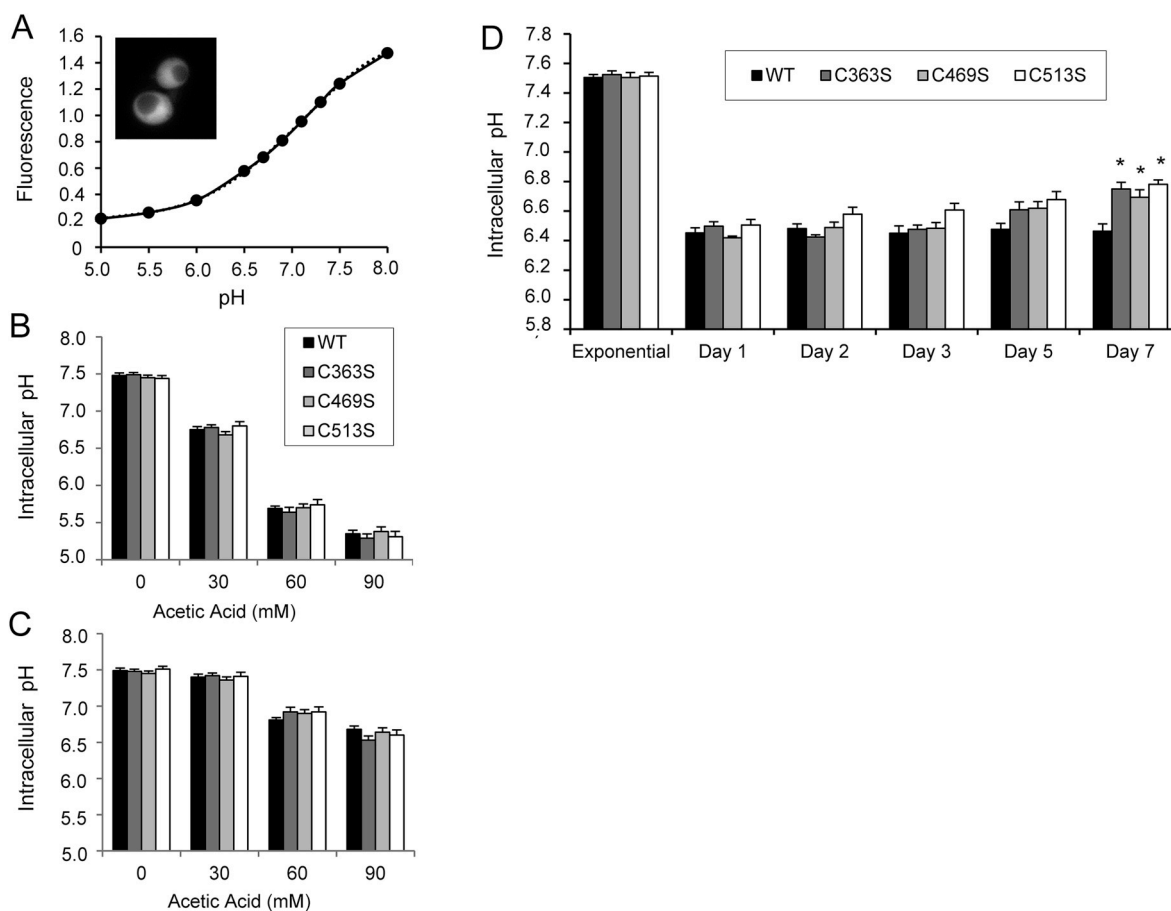


Fig. 3. Intracellular pH shift upon exposure to different concentrations of acetic acid and in chronologically aged cells. WT, C363S, C469S and C513S cells expressing pHluorin were cultivated in SC medium. (A) Calibration curve performed with WT cells expressing pHluorin grown in SC medium to exponential phase. The results are the average of two independent experiments with almost identical results. (B) pHc drop upon exposure to different concentrations of acetic acid for 5 min. (C) Recovery of pHc after 10 min upon exposure to different concentrations of acetic acid. (D) Shift in the pHc in chronologically aged cells at different days of culture. In all cases, statistical analysis was performed comparing mutant with WT cells. From B to D, the data are represented as the means \pm SD from, at least, three independent experiments.

undetectable in the mutant cells (Fig. 4C). To understand why Sir2 mutants displayed this phenotype, we measured acetyl-CoA synthetase (Acs) activity along aging. This enzyme is responsible for generating acetyl-CoA from the acetate previously excreted to the culture medium. As shown in Fig. 4D, Acs activity was higher in Sir2 mutant cells compared with WT cells. In the exponential phase, Acs activity was very low, and no significant differences were observed among strains (not shown).

Lipids droplets (LD), also referred to as lipid bodies, function primarily as organelles for lipid storage, although they have been described as highly dynamic organelles [46]. The neutral lipids (triacylglycerol and cholesteryl esters) stored in LD are used as energy reserve and require acetyl-CoA for their production. We visualized LD using BODIPY 493/503 at days 3 and 5 of cell culture (Fig. 4E). The results showed increased lipid accumulation in the mutant cells, which agrees with their higher Acs activity.

3.4. Chronologically-aged mutant cells have increased phosphoenolpyruvate carboxykinase activity and trehalose stores

At the stationary phase, the storage of sugar as glycogen and trehalose is important for survival. After the diauxic shift, acetyl-CoA obtained from acetate and ethanol is used to fuel the glyoxylate and TCA cycles. During growth on C2 compounds, the glyoxylate cycle plays a key role for anaplerosis of oxaloacetate, which is the substrate of phosphoenolpyruvate carboxykinase (Pck1), the most important

gluconeogenic enzyme. Fig. 5 shows significantly increased Pck1 activity in the mutant strains during chronological aging, compared with the WT strain. In line with an upregulation of gluconeogenesis, increased intracellular trehalose stores were consistently detected in the mutant cells (Fig. 5B).

3.5. Mild oxidative stress increases Sir2 activity through redox regulation

Increased Pck1 activity in Sir2 mutant cells suggested decreased Sir2 activity during chronological aging compared with WT Sir2 [29,30]. *In vivo*, Sir2 activity may be measured by its silencing activity at the telomeric locus. Transcription of a naturally occurring telomere-linked gene, *YFR057W* [59], was assayed by quantitative real-time PCR (Supplementary Fig. 3A). An increase in *YFR057W* mRNA levels in Δ *sir2* cells or in WT cells upon the addition of 5 mM nicotinamide (a Sir2 inhibitor) indicated that the silencing of this gene was strictly Sir2-dependent (Supplementary Fig. 3B) in accordance with previous results [36]. We published that, upon a high disulfide stress (treatment with 5 mM diamide for 1 h), Sir2 is inactivated by glutathionylation of one or more of the key cysteine residues studied in the present work (C363, C469 or C513) [23]. Here, we analyzed the effect of milder stress on WT Sir2 activity. As shown in Fig. 6A, when WT cultures were treated with 2 mM diamide for 1 h, a clear decrease in *YFR057W* expression was detected, resulting in a 5-fold increase in telomeric silencing, compared with untreated cells. As described above, a 5-fold reduction in telomeric silencing occurred upon treatment with 5 mM diamide (Fig. 6A). Such

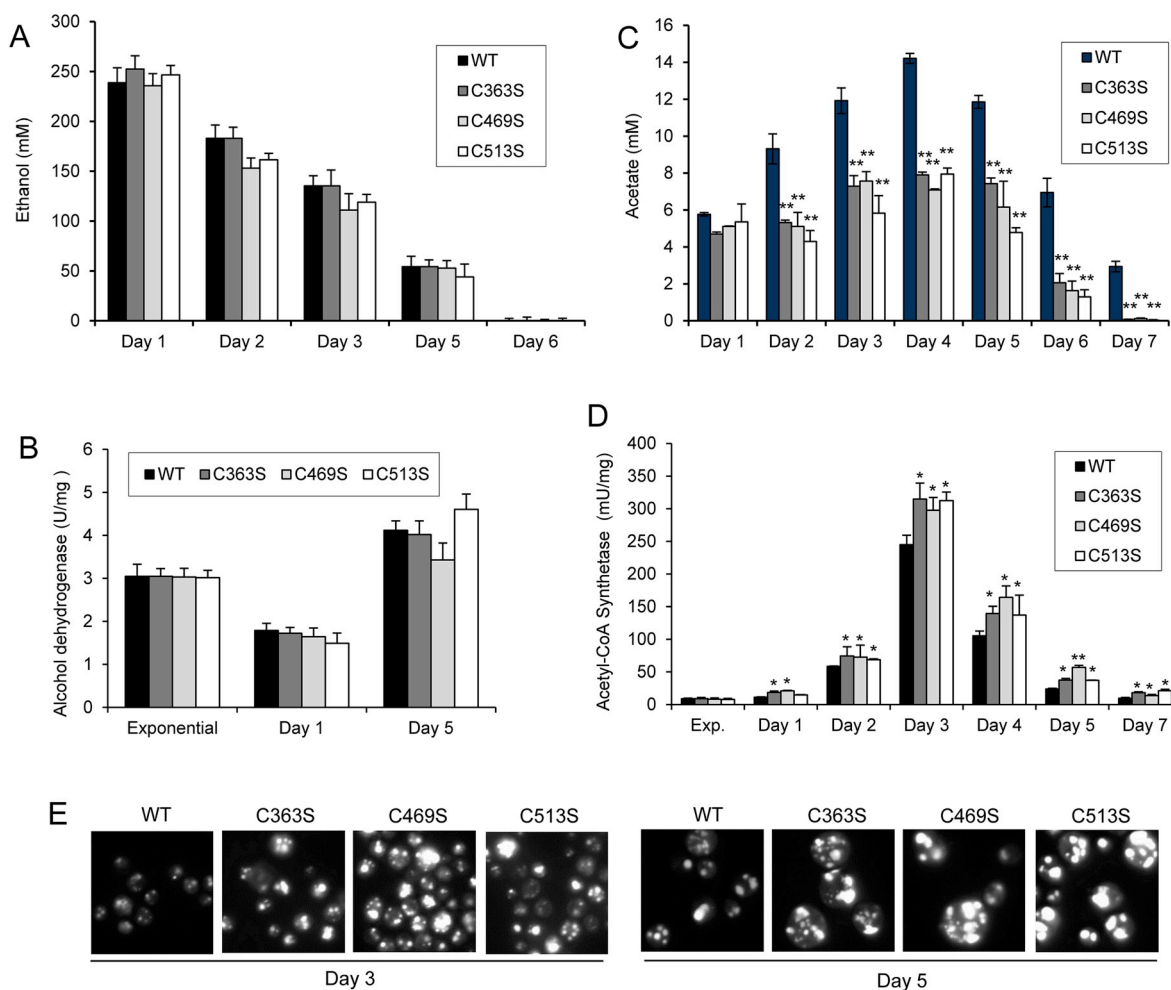


Fig. 4. Ethanol and acetate metabolism in chronologically aged cells. Cultures of WT, C363S, C469S and C513S cells were grown in SC medium and different parameters measured at the indicated days of growth. (A) Ethanol levels in the extracellular media. (B) Alcohol dehydrogenase activity from cell extracts. (C) Acetic acid levels in the extracellular media. (D) Acetyl-CoA synthetase activity from cell extracts. From A to D, statistical analyses were performed comparing Sir2 mutants with WT cells. The data are represented as the means \pm SD from at least, three independent experiments. (E) Representative images of lipid droplets by fluorescence microscopy are shown.

variations in *YFR057W* mRNA levels were not due to changes in the amount of Sir2 protein, which remained unchanged after treatments (Fig. 6A). To investigate whether other kinds of moderate stresses may also increase Sir2 activity, WT cells were grown on a medium containing 0.5% glucose or 3% glycerol as a carbon source and compared with the conventional medium containing 2% glucose. It is known that lower levels of glucose (calorie restriction) increases yeast oxidative metabolism. Glycerol is fully metabolized through mitochondrial oxidation. In both cases, cells are exposed to more oxidative metabolism, which generates mild oxidative stress compared with standard conditions (2% glucose) [58]. As shown in Fig. 6B, when cells were grown in the presence of 0.5% glucose or 3% glycerol, we found a 3- and 10-fold increment in telomeric silencing, respectively. Again, Sir2 levels did not change significantly. Such increase in telomeric silencing after mild stress was not observed when Sir2 mutant cells were treated with 2 mM diamide for 1 h (Fig. 6C).

To analyze whether Sir2 activation after moderate stress can be detected at a target locus other than telomeres, silencing was assessed at rDNA (Supplementary Fig. 4). In budding yeast, 100-200 copies of a 9.1-kb rDNA repeat exist as a tandem array. Each rDNA unit yields a 35S precursor rRNA and a 5S rRNA, separated by two non-transcribed spacers, *NTS1* and *NTS2*. *NTS1* silencing requires Fob1 protein, while *NTS2* silencing is Fob1-independent [60]. WT cells transformed with pDM312 plasmid were untreated or treated with 0.5 or 1 mM diamide

for 1 h, serially diluted and spotted on medium containing uracil as a plating control, and on a medium lacking uracil to monitor the expression of the reporter gene. As shown in Fig. 6D, low levels of diamide decrease viability in uracil-depleted plates, indicating increased Sir2 rDNA silencing activity. As a control, Δ *sir2* cells transformed with pDM312 showed no silencing at this location. To analyze the role of Sir2 cysteines 363, 469 and 513 in silencing, all three mutant strains transformed with pDM312 were submitted to 1 mM diamide stress and rDNA silencing was assayed (Fig. 6E). As shown, compared with WT cells, no increased silencing was detected in the mutant cells.

3.6. Chronologically-aged Sir2 mutant cells have decreased Sir2-dependent silencing activity

As described above, WT Sir2 was activated either by external mild disulfide stress or a physiologically generated stress. This activation did not occur in the Sir2 mutant cells (Fig. 6C and E). To explore if this effect may be physiologically relevant in the context of our research, we measured Sir2 silencing activity along chronological aging. As shown in Fig. 7A, at diauxic shift (day 1), mutant cells showed from 2- to 3-fold decreased telomeric silencing activity compared with WT cells. *ACT1* gene expression was used as an internal control to normalize expression levels. Telomeric silencing could not be measured with confidence along the stationary phase by quantitative real-time PCR due to

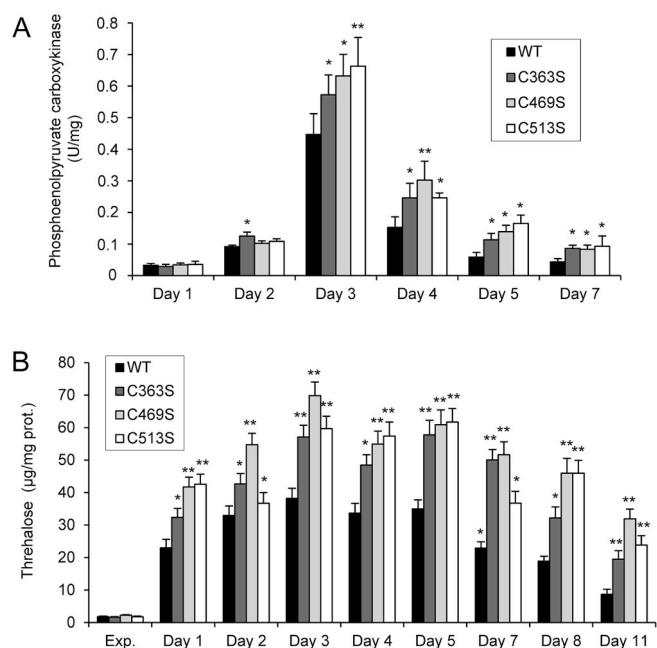


Fig. 5. Gluconeogenesis is increased in chronologically aged Sir2 mutant cells. Cultures of WT, C363S, C469S and C513S cells were grown in SC medium and parameters measured at the indicated days of growth. (A) Phosphoenolpyruvate decarboxylase activity from cell extracts. (B) Trehalose levels from cell extracts. Statistical analyses were performed comparing Sir2 mutants with WT cells. The data are represented as the means \pm SD from, at least, three independent experiments.

expression variability of reference genes. To bypass such a problem, we measured rDNA silencing activity. As shown in Fig. 7B, mutant strains showed decreased rDNA silencing activity (increased growth in plates lacking uracil) along chronological aging, compared with WT strain. Δ sir2 strain was used as a control.

4. Discussion

Yeast Sir2 has been related to aging and oxidative stress. However, despite the huge amount of information available, its regulatory mechanisms are just starting to be elucidated. Recent results from our group have shown that yeast Sir2 is S-glutathionylated upon strong disulfide stress (5 mM diamide), reducing its catalytic activity [23]. We identified three key cysteines (C363, C469 and C513) located at the catalytic domain as the major targets of redox regulation. Cysteine 363 is adjacent to the catalytic histidine 364. Cysteine 469 is located in the catalytic pocket and could be part of a putative nuclear export sequence. Cysteine 513 is one of the 20 amino acids involved in ligand-protein interaction and located in the pocket that contains the active site, but not excessively buried (Supplementary Fig. 1). In the present study we demonstrated that Sir2 mutant strains lacking one of these three cysteine residues were more resistant to acetic acid and showed increased chronological life span. Both phenotypes are complex and different pathways may be involved.

Survival to stationary phase has been extensively associated with resistance to oxidative stress [58]. However, although the role of Sir2 in this context is well-known, no differences in catalase, MnSod, CuZnSod, glutathione reductase, GSH or iron were detected between WT and mutant cells. In agreement, no growth differences were detected when H₂O₂ was used as a stressing agent. At the stationary phase, cells become extremely resistant not only to oxidative stress but other stresses, including heat shock and osmotic stress. Again, no different response in growth upon such treatments was detected among strains. Thus, we hypothesize that the redox regulation of the three cysteines studied in

the present work is not involved in the direct regulation of enzymes and parameters analyzed. It is possible that interaction with the yeast forkhead transcription factors (Hcm1, Fkh1, Fkh2) involved in oxidative stress resistance [13–15] remains unchanged in these mutant strains.

Tolerance to acetic and other weak acids involves genomic and transcriptome responses [61,62]. Understanding acetic acid tolerance of *S. cerevisiae* is important in the field of food and industrial biotechnology. Acetic acid, like other weak acids, such as sorbic acid and benzoic acid, has been traditionally used as a preservative agent in food and beverages by arresting the growth of yeasts and other fungi. In addition, hydrolysates of lignocellulosic biomass are considered renewable feedstock for microbial fermentations [63]. In those hydrolysates, the acetic acid concentrations can be extremely high and become a strong inhibitor of microbial growth and fermentation.

At pH 4.5, the ratio between acetate and acetic acid is close to one [64]. Acetate can be transported through a transporter-mediated mechanism, whereas the undissociated form can diffuse across the plasma membrane by simple diffusion and may trigger regulated cell death [65]. Inside the cytoplasm, acetic acid (pK_a = 4.76) also dissociates into a proton and its counterion, decreasing the intracellular pH and accumulating acetate anions. A variety of mechanisms have evolved to adapt to acetic acid toxicity. The capacity to maintain and recover intracellular pH is a key factor [56,66]. A direct correlation between the initial pH_c and the pH_c drop after exposure to the acid has been described [56]. We demonstrated that, under exponentially growing conditions, all strains have the same intracellular pH (pH_c ~ 7.5). Upon acetic acid stress, pH_c drop was indistinguishable among strains as well as pH_c recovery, which was very fast. This indicates that redox regulation of Sir2 is involved in maintaining neither the pH_c under the control of exponentially growing conditions nor the vacuolar H⁺ - ATPases, which rapidly pump protons out of the cytosol [61,66]. When pH_c was measured along chronological aging, decay in one pH unit was observed after day 1. This was not unexpected since the metabolic change occurring at the diauxic shift generates acetic acid that can be detected extracellularly. In any case, pH_c drop was unchanged between strains, and only after day 5, mutant strains started to recover pH_c better than WT cells.

Why did Sir2 mutant strains show these phenotypes? We demonstrated that chronologically-aged mutant cells presented lower levels of acetate in the extracellular medium because they had increased acetate consumption. Acs activity was higher in Sir2 mutant cells, compared with WT cells, after 1 day of growth with maximum activity on day 3. In yeast, two Acs isoenzymes, Acs1 and Acs2, exist. Although they differ concerning kinetic properties and cellular localization, to date, there is no experimental demonstration of reversible acetylation involved in their enzymatic activity [67]. It has been published that overexpression of ACS2 results in higher resistance to acetic acid as measured by increased growth rate and shorter lag phase relative to the WT strain [68]. Increased Acs activity detected in the Sir2 mutant strains would generate higher levels of acetyl-CoA required for the production of neutral lipids, such as triacylglycerol and cholesteryl esters, which are stored in LD in the cytosol [46]. LD, also known as lipid bodies, lipid particles or oil bodies, are found in most eukaryotic and some prokaryotic cells and serve as an energy reserve, which may contribute to increase longevity in Sir2 mutant cells.

It has been published that a lack of Sir2 increases acetate consumption and decreases extracellular pro-aging factors [51]. In Δ sir2 mutant cells, gluconeogenic pathway is enhanced due to the increase in Pck1 activity, the main flux-controlling enzyme of this route. Lack of Sir2 increases the acetylated active form of Pck1 due to the lack of Sir2-targeted deacetylation [50]. In addition, resveratrol supplementation to WT cells activates Sir2-mediated deacetylation of Pck1, reducing its activity and as a consequence decreasing gluconeogenesis and longevity [69]. Sir2 deacetylates Pck1 both *in vitro* and *in vivo*, and Pck1 acetylation of Lys514, determined by mass spectrometry, was proved to be

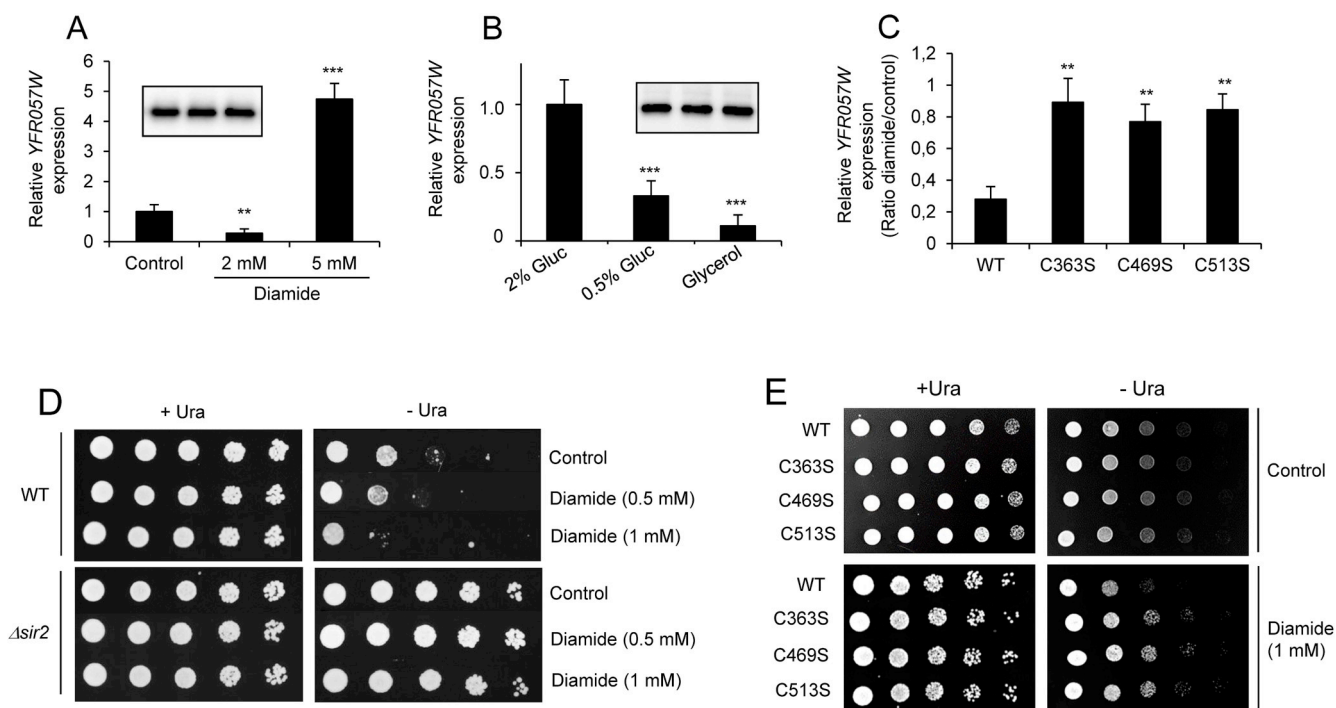


Fig. 6. Wild-type Sir2 but not mutant Sir2 cells increase silencing activities upon mild oxidative stress. (A) Cultures of WT cells grown exponentially in SC medium were treated with 2 or 5 mM diamide for 60 min. Expression of *YFR057W* gene was determined by quantitative real-time PCR. Inset, levels of Sir2 were assessed by Western Blot anti-Sir2. (B) WT cells were grown exponentially in media containing 2% glucose, 0.5% glucose or 3% glycerol as carbon source. Expression of *YFR057W* gene was determined by quantitative real-time PCR. Inset, levels of Sir2 were assessed by Western Blot anti-Sir2. (C) Cultures of WT, C363S, C469S and C513S exponentially grown in SC were treated with 2 mM diamide for 60 min. Expression of *YFR057W* gene was determined by quantitative real-time PCR. Results are expressed as the ratio of values from diamide-treated vs. untreated cells. In all experiments from A to C, *ACT1* gene expression was used as an internal control to normalize expression levels. The data are represented as the means \pm SD from, at least, three independent experiments. (D) Cultures of WT cells grown exponentially in SC medium were treated with 0.5 or 1 mM diamide for 60 min, and silencing of *NTS2* reporter within rDNA was assessed by monitoring the growth of 5-fold serial dilutions of cells plated on Ura-depleted SC medium (- Ura). Ura-containing SC medium (+ Ura) was used as a control. (E) Cultures of WT, C363S, C469S and C513S exponentially grown in SC were treated with 1 mM diamide for 60 min. Silencing of *NTS2* reporter within rDNA was assessed by monitoring the growth of 5-fold serial dilutions of cells plated on Ura-depleted SC medium (- Ura). Ura-containing SC medium (+ Ura) was used as a control.

crucial for enzymatic activity [29]. In agreement, loss of Pck1 activity blocked the extension of yeast chronological life span caused by water starvation. Despite sharing minimal primary structural identity with yeast Pck1, human Pck1 is also activated by acetylation. Although the latter might be deacetylated by multiple deacetylases, yeast Pck1 is exclusively deacetylated by Sir2 [29].

In the present work, we demonstrated that cells lacking one of key redox-regulated cysteines of Sir2 resulted in increased Pck1 activity and, as a consequence, increased trehalose levels. As we proved, WT-Sir2 silencing activity (at both the telomere and the rDNA) was activated under mild disulfide stress. However, this activation did not occur in the Sir2 mutant strains (Fig. 6). Interestingly, these results were obtained after exogenously added diamide and were similar after *in vivo* stress generated during the diauxic and the postdiauxic shifts, pointing out the importance of these cysteine residues on Sir2 regulation. Mutant cells lacking one of the key cysteine residues have decreased Sir2 silencing activity, both telomeric and at rDNA (Fig. 7A and B). Therefore, it is proposed that mutant strains would have increased Pck1 activity due to decreased Sir2-dependent Pck1 deacetylation, compared with WT cells. Thus, in the mutant cells, gluconeogenesis was fueled by i) increased acetyl-CoA produced from acetate metabolism and ii) enhanced Pck1 activity.

As described in Fig. 7C, acetyl-CoA and Pck1 play a crucial role. Acetyl-CoA generated by Acs supplies glyoxylate pathway, which generates oxaloacetate, and is necessary to increase lipid stores. Moreover, acetyl-CoA is an allosteric activator of pyruvate carboxylase (another key enzyme of the gluconeogenesis pathway), which catalyzes the conversion of pyruvate to oxaloacetate [70]. Oxaloacetate is channeled

through the gluconeogenic flux by Pck1, increasing the storage of sugar such as trehalose, needed for cell survival during chronological aging.

In the context of mammalian metabolism, fasting activates the expression of Sir1, promoting the production of acetyl-CoA by deacetylating Acs1 [71]. The increased acetyl-CoA favors the transcription of *PCK1* [72] and activation of Pck1 through acetylation by acetyltransferases like Nut 4 and TIP60 [29]. Like in yeast, increased Pck1 activity ensures a rapid adaptation of the gluconeogenic flux to energy status during fasting and may have a role in longevity. Pck1 activity is also important in the context of pathological status like diabetes [73,74]. To better understand the basis, unraveling the redox regulatory mechanism of key cysteines in human Sirt1 may be important for clarifying how glycemic levels are maintained within a narrow range.

5. Conclusions

Sir2 redox regulation modulates acetic acid metabolism and chronological life span. We have provided evidence that a lack of key redox-regulated cysteines of Sir2 decreased its activity when cells enter to diauxic phase and during the early stationary phase. We propose that the stress generated by the metabolic change occurring at diauxic shift increases Sir2 silencing activity, and such activation occurs to a lesser extent in the mutant Sir2 proteins because they lack one of the redox regulated cysteines. Decreased Sir2 activity, compared with WT cells, inevitably would lead to higher Pck1 acetylation which resulted in increased Pck1 activity. In addition, increased acetyl-CoA synthetase activity observed in the Sir2 mutant cells would generate acetyl-CoA

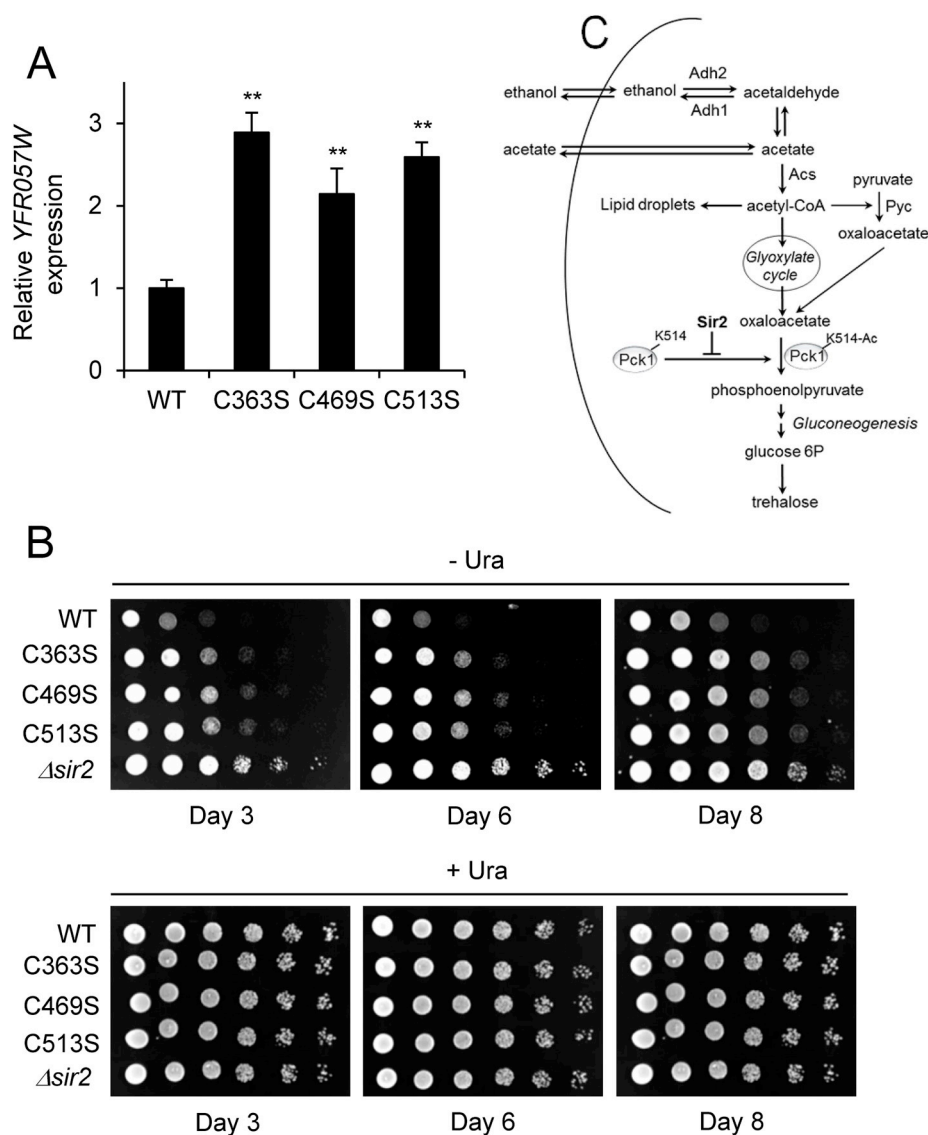


Fig. 7. Mutant Sir2 strains show decreased silencing during chronological aging. (A) Cultures of WT cells were grown in SC medium and at day 1, expression of *YFR057W* gene was determined by quantitative real-time PCR. *ACT1* gene expression was used as an internal control to normalize expression levels. The data are represented as the means \pm SD from, at least, three independent experiments. (B) Cultures of WT, C363S, C469S and C513S were grown in SC and at days 3, 6 and 8, silencing of *NTS2* reporter within rDNA was assessed by monitoring the growth of 5-fold serial dilutions of cells plated on SC -Ura medium. SC medium (complete, +Ura) was used as a control. (C) A simplified scheme of *S. cerevisiae* metabolism showing main pathways affected in the Sir2 mutant strains in chronologically aged cells.

pushing the glyoxylate-requiring gluconeogenesis. In agreement, we reported increased trehalose levels, and decreased acetate in the extracellular medium, both factors known to participate in cell survival during aging. The results presented in this work also open the possibility of exploring the potential effect of sirtuin redox regulation in i) fasting and its role in diabetes and aging, and ii) acetic acid resistance and its role in biotechnology and food processing.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2019.101229>.

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