Chronological and replicative life-span extension in *Saccharomyces cerevisiae* by increased dosage of alcohol dehydrogenase 1

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Alcohol dehydrogenase 1 (Adh1)p catalyses the conversion of acetaldehyde to ethanol, regenerating NAD⁺. In *Saccharomyces cerevisiae*, Adh1p is oxidatively modified during ageing and, consequently, its activity becomes reduced. To analyse whether maintaining this activity is advantageous for the cell, a yeast strain with an extra copy of the *ADH1* gene (2×*ADH1*) was constructed, and the effects on chronological and replicative ageing were analysed. The strain showed increased survival in stationary phase (chronological ageing) due to induction of antioxidant enzymes such as catalase and superoxide dismutases. In addition, 2×*ADH1* cells displayed an increased activity of silent information regulator 2 (Sir2)p, an NAD⁺-dependent histone deacetylase, due to a higher NAD⁺/NADH ratio. As a consequence, a 30% extension in replicative life span was observed. Taken together, these results suggest that the maintenance of enzymes that participate in NAD⁺/NADH balancing is important to chronological and replicative life-span parameters.

INTRODUCTION

One of the features of ageing in *Saccharomyces cerevisiae*, as well as in other organisms, is the oxidative modification of specific protein targets, whose functions are impaired by such modification. A mild oxidation contributes to marking the protein for degradation (Stadtman & Oliver, 1991), while strong oxidation can promote protein aggregation, making proteins unavailable for proteasome degradation (Grune et al., 2004). The accumulation of these modified proteins in a cell contributes to the ageing phenotype (Harman, 1981; Stadtman, 1992; Stadtman & Levine, 2000; Levine, 2002; Nyström, 2005). In yeasts, replicative ageing refers to the number of cell divisions occurring in a mother yeast cell (Sinclair, 2002). Chronological ageing refers to the ability of cells to maintain viability in stationary phase (Herman, 2002).

During the last decade investigations of ageing have uncovered physiological and molecular mechanisms involved in this process, as well as providing some clues towards understanding life-span lengthening (Bordone & Guarente, 2005). Calorie restriction is one of the mechanisms that has been consistently proven to extend life span in organisms ranging from yeasts to mammals (Sohal & Weindruch, 1996). In yeast, the replicative life span can be increased by shifting the cells from 2 to 0.5% glucose (calorie restriction) (Lin et al., 2002, 2004). One of the key molecules is the silent information regulator 2 (Sir2)p, a class III NAD⁺-dependent histone deacetylase, which is involved in several physiologically important functions such as silencing telomeres and rDNA, maintaining genome integrity, and ageing (Bitterman et al., 2003). Strains that have an extra copy of the *SIR2* gene have a life span extended by 40%, while deletion of *SIR2* shortens life span by 50% (Kaeberlein et al., 1999). Under calorie restriction, the oxygen consumption increases, thus raising the NAD⁺/NADH ratio by lowering the concentration of NADH. Since NADH has been described as a Sir2p inhibitor, calorie restriction results in higher Sir2p activity (Lin et al., 2002; Lin & Guarente, 2003), contributing to life-span extension. Nevertheless, the importance of this ratio to the activation of Sir2p has been questioned (Anderson et al., 2003; Kaeberlein et al., 2004; Grubisha et al., 2005).

Chronological ageing in yeast has been used as a valuable model to study oxidative damage and ageing of the post-mitotic tissues of higher organisms (Sinclair, 2002; MacLean et al., 2001). In post-diauxic cultures, respiration is the main source of energy for the yeast cell. It is obtained from previously stored nutrients such as glycogen and trehalose. The cell wall thickens and cells become more resistant to heat and oxidative stresses. Chronological

Abbreviations: Adh1, alcohol dehydrogenase 1; FOXO, forkhead transcription factor; DHE, dihydroethidium; NAM, nicotinamide; ROS, reactive oxygen species; Sir2, silent information regulator 2; Sod, superoxide dismutase; WT, wild-type.
life-span extension is highly dependent on stress resistance (Fabrizio et al., 2001) and has been observed under calorie restriction (Fabrizio et al., 2004; Reverter-Branchat et al., 2004) and in cells overexpressing superoxide dismutase (Sod)2p (Harris et al., 2003).

Replicative and chronological ageing show markers of oxidative stress and selective oxidative modification of proteins. These major protein targets have been identified as enzymes of glucose and energy metabolism, as well as stress resistance and cytoskeletal proteins (Reverter-Branchat et al., 2004). Such oxidative damage to proteins and other stress markers are reduced in cells cultured under calorie restriction.

In this context, one could ask whether the impairment of a given protein function caused by such modifications (i) actively contributes to the ageing phenotype, (ii) is merely a consequence of the ageing process or (iii) has been evolutionarily favoured (e.g. impairment of glycolytic flux to favour the pentose phosphate pathway under stress conditions). Whichever is the case, one might think that the preservation of cell viability would be the main goal. With the aim of getting an insight into question (i) above, we prepared strains that overproduced one of these target proteins and analysed the impact of this extra dose on life span. In other words, is the additional activity of a particular protein advantageous or detrimental to cell life span?

We have previously reported that one of the oxidatively modified targets is alcohol dehydrogenase 1 (Adh1)p (Reverter-Branchat et al., 2004). This enzyme is involved in glucose metabolism in S. cerevisiae, and catalyses the conversion of acetaldehyde to ethanol by a concomitant conversion of NADH into NAD+, thus contributing to balancing the levels of the oxidised cofactor. As a consequence of decreased enzymic activity, the steady state of NAD+/NADH levels would vary. Such variation would affect the activity of Sir2p, thus affecting life span. For these reasons we focused our investigation on cells possessing an extra copy of the dehydrogenase gene. The results presented in this paper provide evidence that this strain shows extended replicative and chronological life span. Responses to mild endogenous oxidative stress in combination with an increased activity of Sir2p are the main traits responsible for the life-span lengthening observed.

### METHODS

**Organisms and culture conditions.** Strains used in this work are listed in Table 1. S. cerevisiae CML128 (MATa ura3-52 his4 leu2-3,112 trpl) was used as a parental wild-type (WT) strain (Gallego et al., 1997). The strain GRB2402 carried a second copy of the ADH1 gene in the genome. This strain was constructed by integrating plasmid pTB106 in the chromosome at the LEU2 locus after Clal-mediated linearization of the plasmid. Plasmid pTB106 was constructed as follows. The ADH1 gene product was obtained using the oligonucleotides ADHI1 (GACACCGGGTTCATTGTTGGTGTC) and ADH1R (GACAGAATTCTAGGCTAGGCTGTGCA) and was subsequently cloned into the EcoRI and SmaI sites of the Yiplac128 vector. The strain GRB2401 was obtained by integrating the empty Yiplac128 vector in CML128. Deletion of the SIR2 gene was obtained by replacing the ORF with a kanMX cassette system using p43965, a pUG26-derived vector (from the Euroscarf collection), digested with NotI. Strains GRB2401UT and GRB2402UT were constructed by transformation with plasmid pADH4UCA-IV (a kind gift of D. Gottschling, Fred Hutchinson Cancer Research Center) in GRB2401 and GRB2402 backgrounds, respectively. Plasmid pADH4UCA-IV, containing the URA3 reporter gene, was digested with SalI and EcoRI prior to transformation, and was integrated into the subtelomeric region of chromosome VII as described elsewhere (Gottschling et al., 1990).

Yeast cells were grown aerobically at 30 °C in rich YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose) or in YPG medium (1 % yeast extract, 2 % peptone, 3 %, v/v, glycerol) by incubation in a rotary shaker at 30 °C. Glucose synthetic medium (SC) contained 2 % glucose, 0.67 % yeast nitrogen base (Difco, without amino acids), the required auxotrophic supplements plus drop-out mixture (Kaiser et al., 1994).

**Western blot analysis.** Cell extracts were obtained as described by Rodriguez-Manzano et al. (1999), separated in SDS-polyacrylamide gels and transferred to PVDF membranes. The following antibodies were used: anti-Adh1 (1 : 400 000 dilution; Chemicon), anti-aconitase (1 : 2000 dilution; a gift from R. Lill, Philipps-Universita¨t Marburg), anti-x-ketoglutarate dehydrogenase (1 : 5000 dilution; Chemicon). Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Comments</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>CML128</td>
<td>MATa, ura3-52 his4 leu2-3,112 trpl</td>
<td>WT (Integration of empty Yiplac128 in CML128 (WT))</td>
<td>Gallego et al. (1997)</td>
</tr>
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<td>GRB2401</td>
<td>CML128 LEU2</td>
<td>Integration of Yiplac128 with ADH1 in CML128 (2 × ADH1)</td>
<td>This study</td>
</tr>
<tr>
<td>GRB2402</td>
<td>CML128 LEU2, ADH1</td>
<td>Deletion of SIR2 in GRB2401 (WT sir2A)</td>
<td>This study</td>
</tr>
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<td>GRB2402, LEU2, ADH1, sir2::kanB</td>
<td>Deletion of SIR2 in GRB2402 (2 × ADH1 sir2A)</td>
<td>This study</td>
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<tr>
<td>GRB2404</td>
<td>GRB2401, LEU2, adh4::URA3-TEL (VII-L)</td>
<td>Integration of adh4::URA3-TEL (VII-L) in GRB2401 (WT-UT)</td>
<td>This study</td>
</tr>
<tr>
<td>GRB2401UT</td>
<td>GRB2402 LEU2, ADH1, adh4::URA3-TEL (VII-L)</td>
<td>Integration of adh4::URA3-TEL (VII-L) in GRB2402 (2 × ADH1-UT)</td>
<td>This study</td>
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RESULTS

Cells with an extra copy of ADH1 display chronological life-span extension

During ageing, oxidation (and inactivation) of a given protein could be either deleterious or protective for the cell. In a previous work (Reverter-Branchat et al., 2004), our group has demonstrated that several glycolytic enzymes (Adh1p, enolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate decarboxylase and fructose-1,6-bisphosphate aldolase) are oxidatively damaged during yeast ageing (both chronological and replicative). In order to investigate the effect of an increased amount of a given protein in the ageing process and stress resistance, we constructed a strain carrying an extra copy of the ADH1 gene. When cells with an extra copy of ADH1 were cultured in YPD media they effectively expressed 70% more Adh1p (Fig. 1a) and, accordingly, the activity was increased by 60% (Fig. 1b) compared to the WT strain carrying the empty plasmid. The introduction of the extra copy did not affect the duplication time, which was 90 min in both strains.

To measure the effect of this extra dose on ageing, chronological life span was determined. Chronological life span in S. cerevisiae is defined as the time for which cells in stationary-phase culture remain viable, and has been proposed as a model for the ageing of post-mitotic tissues in mammals. Resistance to oxidative stress plays a preponderant role in the increase in survival in stationary phase (Harris et al., 2005). The viability of the WT and 2 × ADH1 strains was monitored for 70 days. As shown in Fig. 1(c), survival declined after day 30 for WT cells, while such a decline was not observed until day 40 for the 2 × ADH1 strain.

In order to explain the extended viability in stationary phase, resistance to oxidative stress was analysed in both strains. WT and 2 × ADH1 cells were grown exponentially in YPD, challenged with several H2O2 concentrations for 1 h, and viability assays were performed. Fig. 1(d) indicates that the 2 × ADH1 strain was clearly more resistant than WT. The increased resistance to oxidative stress observed during exponential phase by the 2 × ADH1 cells extended into stationary phase, since cells from 25 days of culture showed higher resistance to H2O2 than WT cells (data not shown). Additionally, cell survival after heat stress (48°C) was also increased in the 2 × ADH1 strain (Fig. 1e).

Growth on a respiratory carbon source such as glycerol (YPD medium) can be used as an internal control, since the activity of Adh1p as a fermentative enzyme is dispensable. When both strains were grown exponentially in YPG, no differences were observed when the cultures were challenged with H2O2, indicating that the different phenotype was due to the increased Adh activity (data not shown).

Antioxidant enzymes are induced in 2×ADH1 cells

We next measured the level of enzymes mainly involved in oxidative stress resistance (Fig. 2a). Catalase activity raised in rabbit as described by Cabisco et al. (2002), anti-mitochondrial porin (1:1000 dilution; Molecular Probes), anti-Ssa (1:600 dilution; EAC), anti-Sb (1:600 dilution; EAC), anti-Hsp104 (1:5000 dilution; StressGen), anti-Hsc90 (1:10000 dilution; a gift of S. Lindquist, Whitehead Institute of Biomedical Research), and anti-Hsp60 (1:8000 dilution; StressGen). Secondary antibodies conjugated to horseradish peroxidase were used as follows: goat anti-mouse antibody (1:2500 dilution; Pierce), donkey anti-rabbit antibody (1:12500 dilution; Amersham) and rabbit anti-goat antibody (1:25000 dilution; Pierce).

Enzyme activities. Cell extracts were obtained using glass beads. Catalase activity was measured as described by Jakubowski et al. (2000). Sod (Sod1 and Sod2) and Adh activities were assayed by chromogenic staining (zymogram), as described in Luk & Culotta (2001) (Sod) and Williamson et al. (1980) (Adh).

Life-span determination. To measure chronological life span, exponential-phase cells (2 × 107 cells ml−1) incubated in YPD medium were regarded as day 0. To determine the age-dependent mortality, cell viability at different days of growth was compared by spreading serial dilutions (1:10) of the cultures on solid YPD plates followed by incubation at 30°C for 3 days. Replicative life-span determinations were performed by micromanipulation, as described elsewhere (Smith & Boeke, 1997; Sinclair & Guarente, 1997), using a Singer MSM system 300 micromanipulator. Life-span analyses were carried out on YPD plates twice independently with 60 cells per strain per experiment. Cells that never budded were excluded from analysis.

Silencing assay. Telomeric URA3 reporter strains GRB2401UT (WT-UT) and GRB2402UT (2 × ADH1-UT) were assayed for Sir2p-silencing activity. Cells were grown at 30°C in SC medium plus uracil (20 μg ml−1) to 2 × 108 cells ml−1. Cells were centrifuged, washed and diluted 1:10 in SC medium lacking uracil, with or without the Sir2p inhibitors splitomicin (5 μM) or nicotinamide (NAM; 1 mM). Growth was measured and the duplication time calculated after a 1 h adaptation period.

Analysis. Ethanol concentration was analysed enzymically in cell-free culture media obtained at different times of growth, from exponential to diauxic phase (2 days) using the Ethanol UV test (Roche Diagnostics). Resistance to H2O2 or heat shock was tested on cells grown on YPD or YPG media. At the middle of exponential phase (2 × 107 cells ml−1), cells were either changed to 48°C and incubated for 10, 20 and 30 min, or treated with H2O2 for 60 min at the required concentration. Viability was measured by plating 0.1 and 0.2 ml of culture dilution on YPD plates and counting survivor colonies after 2 days at 30°C. Levels of NAD+ and NADH were measured as described by Ashraft et al. (2000). Total cellular iron was determined under reducing conditions with bathophenanthroline sulfonate as chelator (Tamarit et al., 2006). Superoxide ion levels were measured in a spectrophotofluorometer (RF-5000, Shimadzu). Exponentially growing cells were washed in water and resuspended in PBS plus 0.1% (v/v) glycerol and the superoxide-specific probe dihydroethidium (DHE; 5 μg ml−1) (Fluka) (Aguilaniu et al., 2003). The rate of oxidation of DHE was calculated from the rate of increase in fluorescence for 30 min (excitation 520 nm, emission 590 nm). Oxygen consumption was measured in a Clark detector, as described by Brown & Cooper (1995). In brief, oxygen uptake was measured in a 4 ml stirred chamber (100 r.p.m.) using a YSI 53 biological oxygen monitor (Yellow Springs Instruments) following the manufacturer’s directions. A volume containing 1 × 107 cells grown in YPD was harvested at mid-exponential phase, washed, resuspended in fresh YPD medium and tested for oxygen consumption. Results shown represent the mean ± SEM of three experimental trials.
was 40% higher in 2×ADH1 cells compared to WT when grown in YPD. Sod1 (cytosolic, Cu/Zn-dependent) and Sod2 (mitochondrial, Mn-dependent) activities measured in these strains were also 30 and 50% higher, respectively. These results could explain the increased resistance to both oxidative and heat stresses, since there is a well-known cross-protection effect mediated by several stress transcription factors (Martínez-Pastor et al., 1996; Schmitt & McEntee, 1996). The increased catalase activity observed in the 2×ADH1 cells when grown exponentially was also observed at stationary phase, since cells from 3 to 25 days of culture showed higher catalase activity.
activity (~30% more) compared to WT cells (data not shown).

An endogenous oxidative stress would be the explanation for the increased antioxidant capacity observed in $2 \times ADH1$ cells. Thus, we measured the steady-state levels of superoxide anions inside the cells. As shown in Fig. 2(b), the rate of oxidation of DHE, a superoxide-specific probe, was increased fourfold in $2 \times ADH1$ cells. Such differences were abrogated when cells were grown in YPG media (data not shown).

The fact that the catalytic activity of Adh1p generates ethanol opened the possibility that these cells carrying an extra copy of $ADH1$ were under ethanol stress (Piper, 1995). In this context, it has been reported that translocation of Msn2 to the nucleus upon environmental ethanol stress triggers the expression of resistance genes (van Voorst et al., 2006). As shown in Fig. 3(a), the ethanol concentration present in the culture media from exponential to diauxic phase was the same in both strains. In addition, quantification by Western blotting of several heat-shock proteins, known to be induced during ethanol stress (Piper, 1995), showed no difference (Fig. 3b).

Mitochondria are the primary site of reactive oxygen species (ROS) generation; thus, an increase in either the activity or the number of mitochondria could be the origin of the stress. Oxygen consumption has been used as a measure of respiratory chain activity and can be checked by using a Clark electrode. As shown in Fig. 4(a), a 25% increase in oxygen consumption was observed in $2 \times ADH1$ compared to the WT strain.

Since the number of mitochondria could affect the oxygen consumption, we analysed the amount of several mitochondrial proteins as an indirect measure of the number of mitochondria. Although a slight increase (around 15%) in mitochondrial Hsp60 was detected, no differences were observed in aconitase, $\alpha$-ketoglutarate dehydrogenase and the mitochondrial outer-membrane porin when compared with the amounts present in WT cells (Fig. 4b). These results ruled out the possibility that an increased number of mitochondria was responsible for increased oxygen consumption.

Strains carrying an extra $ADH1$ copy show extended replicative life span and increased Sir2p activity

Replicative life span has been defined as the number of buds generated by a dividing individual mother. The increased activity of Adh1p in $2 \times ADH1$ cells would vary the NAD$^+$/NADH ratio. This ratio has been demonstrated to have effects on Sir2 activity and, consequently, on replicative life span. The mean and maximum life span can be quantified by micromanipulation experiments. As shown in Fig. 5(a), mean life span increased 30% in the

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**Fig. 3.** Ethanol production in $2 \times ADH1$ cells. (a) The ethanol concentration in cell-free culture media was measured in WT (○) and $2 \times ADH1$ (●) cells at different times of growth. The inset shows the production of ethanol in exponential phase in more detail. (b) Amounts of heat-shock proteins Hsp104, Hsp90 and two types of Hsp70 (Ssa and Ssb) were assessed by immunoblotting analysis.

**Fig. 4.** Effect of an extra copy of $adh1$ on oxygen consumption and amounts of mitochondrial proteins. Cultures of WT and $2 \times ADH1$ were grown exponentially in YPD medium. (a) Oxygen consumption (nmol O$_2$ min$^{-1}$) was measured by a Clark electrode. Data are presented as means ± SD of three independent experiments. Statistical comparison between $2 \times ADH1$ and WT was done using Student’s $t$ test (*P* = 0.0062). (b) The amounts of four mitochondrial proteins [outer-membrane porin, $\alpha$-ketoglutarate dehydrogenase ($\alpha$KGDH), aconitase and Hsp60] were assessed by immunoblotting analysis.
2 x ADH1 cells compared to WT (20 generations for WT and 26 for 2 x ADH1 cells).

As shown in Fig. 5(b), the NAD\(^+\)/NADH ratio in the 2 x ADH1 strain was increased by 20% compared to that of WT cells grown in YPD. Consistently, cells grown in YPG showed a twofold increase in the NAD\(^+\)/NADH ratio with respect to YPD-grown cells, but no differences were observed between the two strains (data not shown). To analyse whether a 20% increase in NAD\(^+\)/NADH ratio could affect Sir2 activity, we constructed the strains GRB2401UT (WT-UT) and GRB2402UT (2 x ADH1-UT). They had the URA3 reporter gene integrated into the subtelomeric region of chromosome VII-L, thus allowing for efficient determination of Sir2-dependent telomeric silencing (Aparicio & Gottschling, 1994). Because Ura3p is required for uracil biosynthesis, the more silenced the telomeric URA3 gene (higher Sir2 activity) the slower the growth in medium lacking uracil. Fig. 5(c) shows that the duplication times of WT-UT and 2 x ADH1-UT grown on SC medium were 196 and 238 min, respectively. To demonstrate that this difference in duplication time was Sir2p-dependent, the addition of two well-known Sir2p inhibitors, splitomicin (split.; 5 \(\mu\)M) or NAM (1 mM) was measured. Data are presented as mean ± SD from three separate experiments. Statistical comparisons between 2 x ADH1 and WT were done using Student’s t test (*P=0.041; **P=0.0096).

**Sir2-mediated oxidative-stress resistance**

Could Sir2p mediate the resistance to oxidative stress observed in 2 x ADH1 cells? In fact, mammalian cells respond to oxidative stress by the formation of complexes between Sirt1p, the orthologue of Sir2p, and members of forkhead transcription factors (FOXOs) (Brunet et al., 2004; Kobayashi et al., 2005). Such interaction allows these factors to control various biological functions, including detoxification of ROS (Kops et al., 2002). To answer the above question, the SIR2 gene was deleted in the WT and 2 x ADH1 background cells to obtain the corresponding sir2\(^D\) derivatives. Fig. 6(a, b) shows that GRB2403 (WT sir2\(^D\)) and GRB2404 (2 x ADH1 sir2\(^D\)) displayed the same resistance to both oxidative and heat stresses. Accordingly, when catalase activity was measured in these strains grown at exponential phase, no significant difference was observed (Fig. 6c). Additionally, catalase activity in both strains showed no significant difference in stationary phase (data not shown). These results indicated that the increased antioxidant capacity (and consequently higher survival in stationary phase) was a SIR2-dependent process in 2 x ADH1 cells.

**DISCUSSION**

Adh1p is a dehydrogenase that catalyses the final step of glucose fermentation, producing ethanol and regenerating
Adh1p and S. cerevisiae life-span extension

Chronic life-span extension has been related to antioxidant activity in stationary phase (Fabrizio et al., 2001; Fabrizio & Longo, 2003). Given the characteristics described for 2×ADH1 cells, survival on stationary phase could be a consequence of such resistance. The data presented here on stress resistance show that cells adapted to increased respiratory conditions are more stress resistant in both exponential and stationary phase, and show extended chronological life span (MacLean et al., 2001).

It has also been reported that SOD2 overexpression (10-fold normal levels) increases yeast chronological life span (Harris et al., 2003), although replicative life span is shortened due to defects in mitochondrial segregation. Such life-span reduction is independent of Sir2p. In our model, these problems were not observed, probably because in 2×ADH1 cells only a mild increase in Sod1p and Sod2p was observed. In addition, these cells induced not only Sod2p but a balanced set of antioxidant enzymes.

The data for DHE oxidation also indicated the existence of an endogenous oxidative stress in 2×ADH1 cells. Iron, as a prooxidant metal, was also measured as a possible inducer of oxidative stress. Total iron values measured in cultures of WT and 2×ADH1 cells grown in YPD at exponential phase as well as during stationary phase were indistinguishable (data not shown). How can the increased Adh1 activity generate such a mild stress situation? An ethanol stress was ruled out by experimental data (Fig. 3a, b). One possible explanation relies on the existence of the ethanol/acetaldehyde shuttle (Lin & Guarente, 2003; Bakker et al., 2001). According to this pathway, some of the ethanol produced by glycolysis diffuses across the mitochondrial membrane. In the matrix, mitochondrial dehydrogenases oxidize ethanol, with a concomitant conversion of NAD+ to NADH, which, in turn, is then oxidized to NAD+ by the electron transport chain. Increased Adh1 activity would therefore raise the activity of this shuttle and, as a result, a rise in oxygen consumption in these cells should be observed (Fig. 4a) as well as increased ROS production (as detected by DHE oxidation). Another possibility to explain the observed phenotype (increased oxygen consumption, antioxidant resistance, and chronological and replicative life span) is that 2×ADH1 cells have lowered catabolite repression. A similar situation is observed under calorie restriction when yeast is switched from 2 to 0.5 % glucose, generating increased respiration and therefore an increased NAD+/NADH ratio (Lin et al., 2002).

In any case, the fact that replicative life span was increased in the 2×ADH1 cells seems, a priori, to be a consequence of the increased NAD+/NADH ratio. It is worth pointing out that replicative life span increased ~30 %, a value quite similar to that reported for cells grown under calorie restriction (Lin et al., 2002). However, since the increase in NAD+/NADH ratio observed in cells with an extra copy of ADH1 was moderate, testing Sir2 activity seemed relevant to support the results obtained. This was carried out by removing silencing in cells in which the URA3 reporter gene was located in the telomere, under Sir2p regulation.

Fig. 6. Sir2 and oxidative stress. Resistance of WT sir2Δ (■) and 2×ADH1 sir2Δ (■) cells grown on YPD medium to oxidative and heat stresses. (a) H2O2 treatment for 60 min; (b) incubation at 48 °C for 10, 20 and 30 min. Viability was measured by counting survivor colonies on YPD plates. (c) Cultures of WT sir2Δ and 2×ADH1 sir2Δ were grown exponentially in YPD medium, and catalase activity (μmol min⁻¹ mg⁻¹) was determined. Data are presented as means ± SD of three independent experiments.

The role of Adh1p on yeast ageing was explored by construction of a strain that carried an additional copy of the ADH1 gene under the control of its own promoter. The strain expressed 70 % more protein than a WT strain carrying the empty plasmid and had ~60 % more activity. Initial results showed that this strain was resistant to both oxidative and heat stresses by moderate increases in catalase, Sod1p and Sod2p. In contrast, strains carrying an extra copy of one of the genes coding for proteins that are also targets of oxidative stress, such as enolase, pyruvate decarboxylase and fructose-1,6-bisphosphate aldolase (Reverter-Branchat et al., 2004), did not display such a phenotype (data not shown).

the oxidized coenzyme NAD+.

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Duplication time was 20% slower in the 2 × ADH1 strain, and this difference was abolished when splitomicin or NAM was added to the culture. The specificity of these inhibitors indicated that the difference was due to higher Sir2p activity.

The results obtained from the sir2Δ strains (WT sir2Δ and 2 × ADH1 sir2Δ) indicate that the response to mild oxidative stress is mediated by Sir2p. In contrast to the differences observed between WT and 2 × ADH1 cells, the derivative strains lacking SIR2 showed the same catalase activity and, accordingly, the same survival after H₂O₂ treatment. How can SIR2 be involved in a response to the mild oxidative stress observed in the 2 × ADH1 strain? In S. cerevisiae, adaptation to H₂O₂ correlates with the rapid induction of a so-called H₂O₂ stimulon of several hundred proteins (Godon et al., 1998) and the repression of many others. This adaptation is orchestrated by the action of several transcriptional regulators, such as Msn2/Msn4 (Hasan et al., 2002), Yap1 and Skn7 (Kuge & Jones, 1994; Krems et al., 1996). To our knowledge, there are no reports connecting Sir2p with the activation of such transcription factors in yeast. However, in mammalian cells, Sirt1p, the mammalian orthologue of Sir2p, interacts with and deacetylates FOXOs (Brunet et al., 2004; Van der Horst et al., 2004). These factors enter the nucleus under stress conditions. As a consequence, they transactivate a series of target genes that have critical roles in the cellular stress resistance and concluded that the effects of SIR2 deletion on chronological life span are opposite to those on replicative life span. One of the consequences of chronological life span are opposite to those on replicative life span. One of the consequences of SIR2 deletion, among others, is a substantial increase in mutation frequency. Probably, the increased stress resistance observed in both sir2Δ and 2 × SIR2 cells is a consequence of the activation of different pathways.

Regarding the question about the relationship between oxidative modification and ageing, it seems clear that oxidative damage of Adh1p contributes to the ageing phenotype. The relevance of maintaining Adh1p activity is to maintain the NAD⁺/NADH ratio, which in turn contributes to the maintenance of Sir2 activity. Although the results shown in this paper have been obtained with an overdose of ADH1, of which the only known function is to participate in the fermentative metabolism of glucose, it is not purely speculative to infer from these results that the impairment of any enzyme that participates in NAD⁺/NADH balancing will actively affect cell ageing.

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