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Lack of Sir2 increases acetate consumption and decreases extracellular pro-aging factors

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ABSTRACT

Yeast chronological aging is regarded as a model for aging of mammalian post-mitotic cells. It refers to changes occurring in stationary phase cells over a relatively long period of time. How long these cells can survive in such a non-dividing state defines the chronological lifespan. Several factors influence cell survival including two well known normal by-products of yeast glucose fermentation such as ethanol and acetic acid. In fact, the presence in the growth medium of these C2 compounds has been shown to limit the chronological lifespan. In the chronological aging paradigm, a pro-aging role has also emerged for the deacetylase Sir2, the founding member of the Sirtuin family, whose loss of function increases the depletion of extracellular ethanol by an unknown mechanism. Here, we show that lack of Sir2 strongly influences carbon metabolism. In particular, we point out a more efficient acetate utilization which in turn may have a stimulatory effect on ethanol catabolism. This correlates with an enhanced glyoxylate/gluconeogenic flux which is fuelled by the acetyl-CoA produced from the acetate activation. Thus, when growth relies on a respiratory metabolism such as that on ethanol or acetate, *SIR2* inactivation favors growth. Moreover, in the chronological aging paradigm, the increase in the acetate metabolism implies that *sir2Δ* cells avoid acetic acid accumulation in the medium and deplete ethanol faster; consequently pro-aging extracellular signals are reduced. In addition, an enhanced gluconeogenesis allows replenishment of intracellular glucose stores which may be useful for better long-term cell survival.

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1. Introduction

Aging is a time-dependent progressive and irreversible physiological/functional decline of an organism that is accompanied by an increased vulnerability to both environmental stress and diseases and increased risk of mortality. On the whole, aging is a complex multi-factorial process modulated by interplay between genetic and environmental factors. No single variable can adequately capture the full extent of this complexity since several processes interact simultaneously and operate at different levels of functional organization [1,2]. The budding yeast *Saccharomyces cerevisiae* is one of the most established model systems used for aging-related research which provides, among others, the opportunity to study and compare the aging processes of both proliferating and non-proliferating cells in a simple single-celled organism. In fact, in yeast two aging paradigms have been described: replicative and chronological. In the former, replicative lifespan (RLS) is defined as the number of daughter cells an asymmetrically dividing mother generates in the presence of nutrients before senescence [3]. In the latter, chronological lifespan (CLS) is the mean and maximum survival period of a population of non-dividing cells in stationary phase. Viability over time, in this case, is defined as the ability to resume mitotic growth upon return to rich fresh medium [4]. In the stationary phase, yeast cells display a survival-based metabolism and acquire physiological and morphological features, including a thickened cell wall, accumulation of storage carbohydrates and increased stress resistance, which result from the integrated responses of different signalling pathways. The establishment of a quiescent program allows the cells to survive starvation and resume growth when nutrient conditions become favorable again [5]. CLS can be extended by either inhibition/reduced activity of two major nutrient-sensing pathways such as TORC1-Sch9 and Ras-PKA ones, or by calorie restriction (CR), the practice of limiting nutrient intake which in yeast is generally imposed by reducing the glucose concentration in the growth medium [6–8]. Defects in TORC1-Sch9 or Ras-PKA signaling as well as CR lead to in part common downstream targets (Rim15, Msn2/4 and Gis1) which ultimately by increasing endogenous stress defence mechanisms contribute to enhance cell survival [6,9]. Moreover, during chronological aging, in addition to the well known intrinsic factors such as hydrogen peroxide and superoxide [10], cellular stresses also include extrinsic factors such as ethanol and acidic acid. In fact, in some settings, the presence in the growth medium of these two by-products of the yeast metabolism restricts CLS [11,12].

In the chronological aging paradigm, a pro-aging role is also played by Sir2 [12]. Sir2 is the founding member of Sirtuins, a family which comprises the unique class III of NAD^+-dependent deacetylases

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known to be evolutionary conserved regulators of aging [1,7,13]. Much work in yeast has focused on histone deacetylation, but a wide range of non-chromatin substrates for the other Sirtuins have been identified which are involved in different metabolic processes including energy production, the urea cycle, fatty acid and acetate metabolism [14,15]. Concerning the acetate metabolism, in mammals, acetate derived from both exogenous and endogenous sources, is activated to acetyl-CoA either in the cytoplasm by acetyl-CoA synthetase 1 (AceCS1) or in the mitochondria by AceCS2 [16,17]. The Sirtuins, SIRT1 and SIRT3 deacetylate AceCS1 and AceCS2, respectively, promoting their activity [18]. Both SIRT1 and SIRT3 play an important role during energy-poor diets [19]. In particular, under long-term fasting or CR, acetate is released from the liver and utilized for energy production in extraepatic tissues following AceCS2 activation [16,20]. In this context, SIRT3 might modulate the reprogramming of mitochondria to low energy input [21]. The SIRT1/3-dependent regulation of AceCS1/2 and the implication of the two Sirtuins in aging and in the CR-mediated longevity response [13,22,23], have suggested an involvement of the acetate metabolism in the aging process [16]. Similarly to SIRT1/3, a Sir2 ortholog, CobB, in *Salmonella enterica* activates through deacetylation the acetyl-CoA synthetase (Acs in bacteria and yeast) allowing acetate utilization for acetyl-CoA synthesis and bacterial growth on acetate [17,24]. In *S.cerevisiae* two Acs isoenzymes, Acs1 and Acs2, are present which differ with respect to kinetic properties and cellular localization [25,26]. To date, there is no evidence of a reversible acetylation involved in their enzymatic activation [25]. Interestingly, Acs2 is required for replicative longevity [25], further supporting the notion that the acetate metabolism can play an important role during aging. This is also suggested by data showing that genetic interventions which drive yeast metabolism away from acetic acid production increase CLS [11].

In this study, we provide evidence that in the budding yeast, *SIR2* inactivation actually influences positively the acetate utilization by way of an increased flux of the glyoxylate/gluconeogenic pathway. In the chronological aging paradigm, this implies low levels of toxic extracellular factors (ethanol and acetic acid) and an increase of protective intracellular factors (trehalose) in the *sir2Δ* cultures which all together may favor a better long-term survival and extension of CLS.

2. Materials and methods

2.1. Yeast strains and growth conditions

All yeast strains used in this work are listed in Table S1. All deletion strains were generated by PCR-based methods [27]. Genomically 3HA-tagged strains were obtained as described [28]. The tagged strains were undistinguishable from the congenic untagged ones with respect to overall morphology, cellular volumes, duplication times and CLS. The accuracy of all gene replacements and correct deletions/integrations was verified by PCR with flanking and internal primers. Primer sequences are available upon request. Standard methods were used for DNA manipulation and yeast transformation. Yeast cells were grown in batches at 30 °C in rich medium (YEP, 1% w/v yeast extract, 2% w/v bacto peptone) with the indicated carbon source at 2%. For the acetate-YEP medium (pH 4 or 5.8) precalculated amounts of 0.2 M acetic acid and 0.2 M sodium acetate solutions were mixed and added to YEP medium to obtain the required pH and molarity (0.1 M). pH 5.8 was selected since it was the initial pH of the unbuffered YEP medium. For cells grown in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/l), auxotrophies were compensated for with a four-fold excess of supplements [12]. All strains were inoculated at the same cellular density (culture volume no more than 20% of the flask volume). Growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyzer, as described [29]. Doubling time (Td) was obtained by linear regression of the cell number increase over time on a semilogarithmic plot.

2.2. Ethanol and acetate pulses

Yeast cells were grown in glucose-limited chemostat cultures [30] at a dilution rate of 0.15 h^{-1} and with an airflow of 0.8 l/min. Mineral medium was prepared according to [31], supplemented with glucose at 7 g/l. The pH was kept constant at 4.5 by the automatic addition of 2 M KOH. Once chemostat cultures achieved the steady-state, the medium feed and the effluent pumps were switched off. At this time-point, a concentrated solution of ethanol or sodium acetate (pH 4.5) was injected aseptically giving an initial concentration in the chemostat of about 90 mM of ethanol and 3.3 mM of acetate. Samples were collected at different time-points for analyses of metabolite contents. Cell dry weight was determined as described [32]. Off-gas analysis (O_2 and CO_2) was performed with a BM2001 gas analyzer (Bioindustrie Mantovane, Italy). O_2 consumption and CO_2 production were calculated as in [33]. Each pulse experiment was carried out in triplicate. Trends reported refer to a representative experiment.

2.3. Metabolite measurements

At designated time-points, aliquots of the yeast cultures were centrifuged and both pellets (washed twice) and supernatants were frozen at −20 °C until used. Glucose, ethanol and acetate concentrations in the growth medium were determined using enzymatic assays (K-HKGLU, K-ETOH and K-ACET kits from Megazyme). Intracellular trehalose was extracted and measured as described [34]. After incubation with trehalase (Sigma), the amount of glucose generated from trehalose hydrolysis was determined using the K-HKGLU kit. The pre-existent glucose in each sample was measured in a parallel reaction without trehalase and subtracted from the total glucose. Total protein concentration was estimated using the BCA™ Protein Assay Kit (Pierce).

Final values represent the average of three independent experiments. Differences in measurements were assessed by Student's *t*-test. The level of statistical significance was set at a P value of ≤ 0.05.

2.4. Enzyme assays

Cell extracts were prepared from harvested cells as described [35] except that cells were broken with acid-washed glass beads by shaking on a vortex for several cycles interspersed with cooling on ice. Immediately after preparation of cell-free extracts, Pck1 and Icl1 activities were determined [35].

2.5. Immunoprecipitation and Western analysis

Cellular extracts for anti-HA immunoprecipitation were prepared essentially as described [36] in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride and Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche) and histone deacetylase inhibitors (100 μM Trichostatin A, 50 mM nicotinamide and 50 mM sodium butyrate). A crude lysate aliquot was stored at −20 °C as immunoprecipitation input control. For immunoprecipitation, lysates (about 500 μg) were incubated with 2 μg of anti-HA mAb (12CA5; Roche) at 4 °C overnight, followed by the addition of 50 μl Dynabeads Protein A (Dynal Biotech) for 2 h. After five washes with washing buffer (50 mM Tris, pH 7.4, and 50 mM NaCl) at 4 °C, bound proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE and then subjected to Western analysis. Primary antibodies used were: anti-HA mAb (12CA5; Roche), anti-acetylated-lysine mAb (Ac-K-103; Cell Signaling) and anti-3-phosphoglycerate kinase (Pgk1) mAb (22C5; Invitrogen). Secondary antibodies were purchased from Amersham. Binding was visualized with the ECL Western Blotting Detection Reagent (Amersham). Afterwards ECL detection, films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer and quantified with Scion Image software.

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