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The yeast *Saccharomyces cerevisiae* as a "road" from aging basic research to interventions for healthy aging

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"Time will heal everything;
but what if time is the illness?"

TABLE OF CONTENTS

Abstract

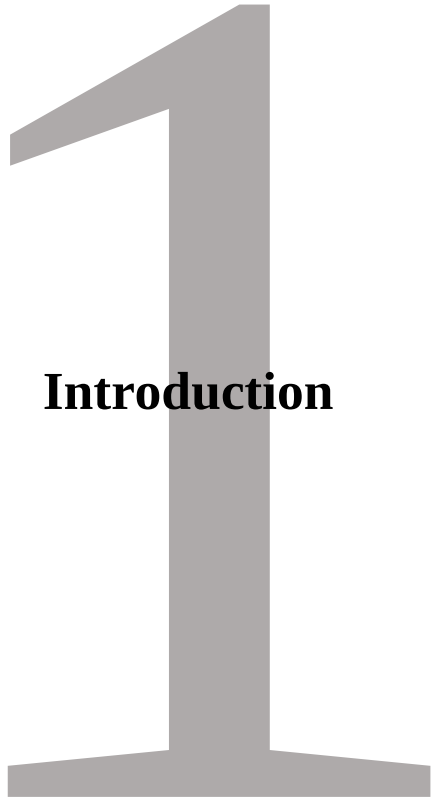
All living organisms undergo a functional/physiological decline with age, which is progressive and irreversible, and it is associated to an increased risk of the development of many diseases. Among the factors involved in aging, TORC1/Sch9 and Ras/PKA nutrient-sensing pathways and Sirtuins, a family of NAD^+-dependent deacetylases, play a prominent role. They are evolutionarily conserved from yeast to humans, and they also mediate some of the effects of Calorie Restriction (CR), an intervention consisting in a reduction in calorie intake without malnutrition, known to extend longevity in many organisms. In the field of aging research, the yeast *Saccharomyces cerevisiae* is a useful experimental system. In particular, the Chronological LifeSpan (CLS), defined as the time that a population of quiescent cells can survive in stationary phase, represents a model for studying aging of post-mitotic mammalian cells, such as neurons and myocytes. Although cells do not proliferate during the stationary phase, they remain metabolically active and responsive to stimuli.

In the first part of this thesis, we investigated the relationship existing between metabolism and chronological aging. In particular, we have elucidated how ethanol and acetic acid, produced during the exponential phase, and, in particular, their metabolism can influence CLS. In fact, the pro-aging effects of these two compounds can be abolished i) inhibiting the metabolism of ethanol by specific inhibition of its reduction to acetaldehyde or ii) preventing acetate uptake through a modulation of the extracellular pH conditions or modulating its transport mediated by the aquaglyceroporin Fps1. We have also shown how the fate of such metabolites influence the long-term survival. In fact, a positive role in CLS is played by the enhancement of anabolic pathways that allow the use of the C2 carbon sources for the production of reserve carbohydrates. A metabolic remodeling towards gluconeogenesis prevents the rapid dissipation of energy reserves and reduces the generation of ROS due to an intense respiratory activity. This results in an increase of the mitochondrial functionality. We have also shown

that an enhanced glyoxylate/gluconeogenic flux characterizes the extreme CR, imposed by transferring cells to water. In this condition, adding ethanol stimulates respiration that competes with gluconeogenesis and reduces the beneficial effect on CLS associated to CR.

In the second part of the work, we have focused on some nutraceutical compounds known to influence RLS with different outcomes, such as NAM, RESV and QUER. Thus, we have investigated whether they could also influence CLS, and, if so, whether Sir2 is involved. It turned out that NAM treatment phenocopies *sir2Δ* in CLS as well as in the case of RLS. NAM increases CLS to the same extent that inactivation of Sir2 did, producing the same metabolic effects such as an increase in gluconeogenesis mediated by the increased enzymatic activity of Pck1 and a more efficient respiratory activity, which preserves mitochondrial function and reduces ROS accumulation. Whereas inducing an extension in RLS, RESV was shown to elicit an opposite effect on CLS which is not associated to variations in gluconeogenic flux, but it is due to an increased respiratory activity, mostly inefficient that leads to ROS accumulation. Basing on our data, we cannot exclude the possibility that RESV affects CLS activating Sir2.

Concerning QUER, we observed that this compound has a positive effect on the CLS in wild-type and also when Sir2 is absent. QUER treatment stimulates gluconeogenesis and improves respiratory efficiency, being this effect additive to that elicited by Sir2. Furthermore, QUER exerts a beneficial effect also on the CLS of cells unable to perform gluconeogenesis because of lack of Pck1, indicating the involvement of other protective mechanisms for cell survival. Which are the factors modulated by QUER and how they are placed in the network of CLS modulators are aspects still to be defined.



Introduction

The yeast *Saccharomyces cerevisiae* has been widely utilized as a biotechnological tool since the dawn of agriculture thanks to its ability to perform alcoholic fermentation - the metabolic process underlying baking, brewing and winemaking - becoming now a real cell factory for the production of fuels and commodity chemicals such as lactate or pharmaceuticals, including human insulin and Human Papilloma Virus (HPV) vaccines.

The single-celled *S. cerevisiae* has also become an important experimental model as it couples technical advantages with fundamental metabolic and regulatory pathways similar to those of multicellular eukaryotes. Indeed, the classification as generally regarded as safe (GRAS), the short generation time (about 1 hour in rich media), convenient and cheap experimental setups, straightforward genetic approaches and high-throughput methodologies [1] contribute to its success in both basic and applied research. It was the first eukaryote whose genome was completely sequenced [2] and since then very efficient techniques have been developed allowing to replace or delete about 6000 genes [3,4]. *S. cerevisiae*, in fact, can be easily and stably transformed and has a high endogenous rate of homologous recombination. Thus, the analysis and cloning of genes in this organism is significantly easier than in other eukaryotes. Therefore, it played a pivotal role in the understanding of many cellular processes such as cell cycle regulation [5], intracellular trafficking [6,7], protein folding regulation [8,9], and others.

Additionally, *S. cerevisiae* has also received a widespread acceptance as a useful model for human diseases [10] and to study the aging process. In this context, in 1959, Mortimer and Johnston were the first scientists to perform a yeast lifespan experiment [11], laying the basis for yeast aging research. Even if *S. cerevisiae*, as a single-celled eukaryote evolutionary distant from humans, seems an unlikely candidate for aging studies, its use had/has a profound impact on our current understanding of the mechanisms involved in human aging.

1.1 Aging

1.1.1 Definition, causes and interventions

Aging is an extremely complex, multifactorial process that is characterized by a progressive biological/physiological decline seen at all levels of organization from cells to tissues and to organisms, leading to a decrease in both survival rate and reproductive capability [12]. In particular, the population of aged people living 85 years and beyond is predicted to increase by 351% in the next 40 years [13]. This is due to different factors, including better nutrition and health care. However, an aging world poses social and economic challenges, as it is accompanied by an increased incidence of many pathologies including cancer, cardiovascular and neurodegenerative diseases [14].

This has given a great impetus to aging research aimed at extending, besides the lifespan, also the healthspan in order to reduce the incidence or progression of diseases associated with aging and promote a healthy aging.

Over the years, given the multifactorial nature of aging, many theories have been proposed in the effort to explain the causes and mechanisms underlying it, which should not be considered as mutually exclusive, but may be complementary of others to describe some or all the features of the normal aging process [15]. Of the many theories of aging that exist, a first distinction is drawn between damage-based and programmed theories.

Damage-based theories identify environmental assaults to living organisms as the cause of aging, inducing cumulative damage at various levels. In particular, this group of theories proposes that aging is the result of the inevitable small random changes that accumulate with time and the failure of the body's repair mechanisms to fix the damage. The accumulated damage ultimately injures cells and tissues, contributing to the age-related declines in an organ's function.

Conversely, according to programmed theories, aging and consequently limited lifespan are genetically programmed and serve some evolutionary purpose: a lifespan longer than some species-specific value conveys disadvantage and

therefore produces evolutionary motivation to develop the aging function. Thus, aging is regulated by hormones, genes and sensing of external conditions, like other necessary biological functions [16].

Despite being polygenic traits for which neither the number of genes involved, their variants and the extent of interactions are known, nor is the complexity of the epigenetic factors influencing them are elucidated at present, aging and longevity are amenable to genetic modulation, even in a highly protected laboratory condition, as proved by studies performed on various experimental models. However, genetic interventions in humans, on the one hand, for slowing down aging and, on the other, for extending healthspan pose obviously serious technical limitations, and ethical and safety concerns [17].

Calorie restriction (CR), namely a reduction in caloric intake without malnutrition, is the only non-genetic and consistent non-pharmacological intervention that extends lifespan in model organisms, and protects against the deterioration of biological functions, delaying or reducing the risk of many age-related diseases. However, the applicability of a CR regimen to humans is uncertain as it is unlikely that many would be willing or able to maintain it.

Preventive actions based on nutritional interventions for healthy aging may be seen as a safer and more feasible strategy compared to the other ones. It is well known, in fact, that healthspan can be extended by practicing good health behaviors, among which getting proper nutrition is a determinant factor. Since ages, food has been used for the treatment and prevention of various disorders - it should be sufficient to recall the use of iodine-enriched table salt and wheat flour fortified with iron/folic acid for iodine deficiency goiter and anemia prevention, respectively [18] - but, only recently, scientifically supported evidence has permitted the so-called “nutraceuticals” to emerge as being potentially effective [19]. The term “nutraceutical” was coined by Dr Stephen DeFelice merging the words “nutrition” and “pharmaceutical” [20]. Nutraceuticals, also named as functional foods, provide the amount of essential

nutrients necessary for healthy survival. When a functional food assists in the prevention and/or treatment of disease(s) other than deficiency conditions it is called a nutraceutical [21]. Some of the most common ways of classifying nutraceuticals can be based on food sources, mechanism of action, chemical nature, etc. The food sources used as nutraceuticals are all natural and can be categorized as dietary fibres, probiotics, prebiotics, polyunsaturated fatty acids, antioxidant vitamins, polyphenols, and spices [22]. More broadly, nutraceuticals can be classified as potential and established [23]. A potential nutraceutical could become an established one only after clinical data of its health and medical benefits are obtained. However, it is to be noted that much of the nutraceutical products remains only potential.

1.1.2 Aging in humans

In humans, the age-associated functional decline tends to begin after the sexual peak and proceed in a linear fashion. The more general human aging phenotype comprises a gradual reduction in height due to loss of muscle and bone mass, graying hair, sagging and wrinkling skin, joint stiffness, osteoporosis, memory loss, decline in hearing, olfaction, and vision, declines in kidney, pulmonary, and immune functions, multiple endocrine changes, and the slowing of sexual responsiveness [24].

Human aging studies at cellular level started with Hayflick and Moorhead who first observed that a culture of human skin fibroblasts *in vitro* underwent a limited number of divisions (about 50-60), the so-called ‘‘Hayflick limit’’, and then reached an irreversible growth arrest [25]. This process, called replicative senescence, was linked initially only to the progressive shortening of telomeres, a process that leads to chromosomal instability and promotes tumorigenesis, supporting the original hypothesis that senescence guards against unrestricted growth of damaged cells [25, 26].

*C*ellular senescence *in vitro* is defined as an irreversible cell cycle arrest due to loss of proliferative potential. It happens in two steps: cell cycle arrest followed by gerogenic conversion (geroconversion). Geroconversion is a form of futile growth during cell cycle arrest. It converts reversible arrest to irreversible senescence [27]. In the adult organism, most of the cells are arrested but not senescent. Non-senescent arrest in cell culture can be induced by withdrawal of serum, growth factors and nutrients. In these conditions, cells become quiescent. A quiescent cell neither grows nor cycles, yet it retains the proliferative potential and its re-stimulation leads to proliferation.
Senescence in culture has sped up aging studies and has been informative, providing insights into cell cycle control, differentiation, and cancer. However, its relevance to organismal aging remains a highly debated topic [28].

Whereas it was possible to identify many rare mutations having negative effects on longevity, as in the case of Segmental Progeroid Syndromes that mimic prematurely aging, such as Werner Syndrome and Hutchinson-Guilford Syndrome [29], epidemiological studies indicate that common genetic variants associated to exceptional longevity in humans are unlikely to exist. Indeed, one of the most astonishing results from studies of centenarians is how diverse they are [30] and the reason why some humans live to extreme ages, some even in relatively good health, remains unknown. This phenotypic trait is very complex and probably its genetic architecture involves many rare variants with small effects.

1.1.3 Yeast as a model of aging

Studying aging directly in humans is prohibitively long and complex, due to the length of lifespan, genetic heterogeneity and vast differences in environmental influences. Thus, researchers were obliged to resort to model organisms, whose

survival curves resemble those of humans quite remarkably, although they display significantly different lifespans. The mechanisms underlying biological aging have been extensively studied in the past 20 years with the avail of mainly four model organisms: the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the domestic mouse *Mus musculus*, and the yeast *S. cerevisiae*.

As said before, *S. cerevisiae* has proven to be a qualified model for different kinds of research. Nevertheless, at the beginning more than a doubt arose whether it was also a good model to study the aging process. Signs of aging, in fact, are evident in humans and most of other multicellular organisms, whereas unicellular species have long been mistaken as being immortal.
Actually, yeast can be propagated in culture indefinitely. In particular, *S. cerevisiae* divides by asymmetric budding, with the daughter cell being smaller than the mother from which it derives. Besides the difference in size, the mother cell is clearly distinguishable by the bud-scars left after separation of the daughters. Taking advantage of this asymmetry, when single mother yeast cells were followed by removing every daughter cell produced, they were reported to eventually die [31] The analysis of cell cohorts indicated also that mortality increases during the life, a hallmark of aging [11]. Furthermore, cultures inoculated with the first or last daughters of the same mother cell were both able to grow [31]. These early experiments demonstrated that yeast cells are mortal, but their daughters do not inherit what kills the mother cells and are therefore born with a reset lifespan. This also speaks for the finite lifespan of yeast cells being the product of an aging process.

In *S. cerevisiae*, two aging models have been described: replicative and chronological aging. Replicative LifeSpan (RLS) is defined as the number of divisions an individual mother cell undergoes in the presence of nutrients before senescence [32]. Normally after 25-35 divisions, replicatively aged mother cells start to die. RLS can model aging of human actively proliferating cells, such as

fibroblasts and leukocytes [32,33]. RLS studies in yeast, as already stated, date back more than 50 years when Mortimer and Johnston wondered how many times one cell can divide. The assay they conceived is conceptually simple: daughter cells were isolated on a solid media substrate, and, once they started dividing, all progeny was removed and tabulated [11].

The latter aging paradigm in yeast, the Chronological LifeSpan (CLS), represents the mean and maximum survival period of a population of quiescent cells in stationary phase, with survival being measured as the ability to resume mitotic growth upon return to rich fresh medium [34]. In stationary phase, yeast cells do not proliferate, but they remain metabolically active and responsive to stimuli and could be a model for post-mitotic mammalian cells, such as neurons and myocytes [33].

CLS experiments are generally performed by monitoring survival in the synthetic complete medium containing 2% glucose (SDC), a four-fold excess of the supplements required for auxotrophies, yeast nitrogen base, ammonium sulphate (nitrogen source), sodium phosphate, vitamins, metals and salts [34]. After approximately 10 hours of exponential growth, the glucose concentration in the medium reaches very low levels and yeast switch from a fermentation- to a respiration-based metabolism. After this switch, called "diauxic shift", yeast catabolizes the ethanol accumulated during the fermentative phase and obtain most of the energy from mitochondrial oxidative phosphorylation. When yeast organisms are incubated in SDC, the diauxic shift is followed by a post-diauxic phase, in which growth continues slowly until approximately 48 h, and then stops. In the post-diauxic phase, metabolic rates remain high until day 5-6. The final density reached at day 3 varies from strain to strain (for example, about $1 \cdot 10^8$ cell/mL for W303-1A wild-type strain). The mean survival of wild-type strains depends on their genetic background and ranges from 6-7 days (DBY746/SP1) to 15-20 days (S288C/BY4700). The post-diauxic phase differs for organisms incubated in SDC and those incubated in rich YPD medium [35]. In fact,

incubation in YPD promotes a 6-7 day post-diauxic phase characterized by slow growth and low respiration, followed by entry into a non-dividing hypometabolic stationary phase in which organisms are highly resistant to multiple stresses and survive for up to 3 months. By contrast, incubation in SDC triggers the entry into an alternative post-diauxic phase in which only minimal growth occurs after 48 h and metabolic rates remain high until the population begins to die.

CLS is usually determined by measuring the clonogenic survival of yeast cultures upon chronological aging. The majority of cells stops dividing within 2-3 days from the starting of the culture and viability is assayed by colony forming units (CFUs) measurement beginning on day 3 (conventionally referred to as the 100% survival point) until survival reaches 1-5% of the day 3 CFUs [36].

1.2 Yeast stationary phase

1.2.1 Characteristics of stationary-phase yeast cells

In free-living yeasts, the main factor that leads to a reversible, quiescent state is nutrient scarcity, which triggers a shift from a growth-based to a survival-based metabolism. Carbon and nitrogen frequently become limiting under natural conditions, with yeast viability depending on which nutrient is limited first [37]. *S. cerevisiae*, like most of the other yeasts, is facultative-fermentative, and may display either a fully respiratory or a fermentative metabolism or even both in a mixed respiratory-fermentative metabolism, depending on the growth conditions: the type and concentration of carbon sources and/or oxygen availability are the two main environmental conditions having a strong influence on yeast metabolic physiology. The leading example of this is the Crabtree effect, which is the occurrence of alcoholic fermentation under aerobiosis at high glucose concentrations, which are the conditions of a standard CLS experiment. In this context, the metabolism is characterized by a high glycolytic flux, glucose fermentation, and a negligible aerobic respiration.

Upon glucose depletion, the diauxic shift occurs which results in a shift from fermentation to respiration of the fermentation by-products, ethanol and acetic

acid. At the diauxic shift, a massive reprogramming of gene expression occurs in the cell: the mRNA level of around 700 genes increases, whereas the mRNA level of approximately 1000 genes declines [38]. Among them, there are genes involved in the gluconeogenesis, the TCA cycle and the glyoxylate cycle [39]. Besides, mitochondrial mass and respiration are increased, both causing substantial changes in metabolites crucial for cellular function and survival [40]. Also the genes encoding antioxidant defenses that allow scavenging of Reactive Oxygen Species (ROS) are up-regulated, probably as a result of the switch to the respiratory metabolism [41, 42].

In the post-diauxic phase, cells undergo one or two slow doublings, consuming ethanol and acetate as carbon/energy sources. Finally, when these latter are fully exhausted, cells cease proliferation and enter the stationary phase [43].

Stationary-phase yeast cells have the following features:

- they are unbudded and contain morphologically distinct condensed chromosomes [44].
- They are characterized by a decreased metabolism and biosynthesis. The overall transcription rate is about 3 to 5 times lower than in cells growing exponentially [45]. Genes encoding ribosomal proteins are expressed at lower levels [46], and translation is reduced to about 0.3% [47].
- They are surrounded by a thickened cell wall [48], which partially results from the increased expression of the cell wall synthesizing enzyme β-1,3-glucan synthase and the localized synthesis of its substrate UDPG during the post-diauxic growth phase [49].
- The mitochondrial tubular network is less interconnected [50].
- They are more resistant to a variety of stresses [35], such as heat and osmotic shocks, as well as to treatment with toxic drugs.
- They accumulate storage molecules, such as glycogen and trehalose. The fraction of dry weight represented by glycogen can be as high as 8%, while the fraction represented by trehalose can be as high as 20% [51]. Whereas

glycogen accumulation occurs before depletion of glucose and peaks at the diauxic shift, trehalose is accumulated after the diauxic shift early in the stationary phase [52]. Glycogen stores are partially utilized to fuel the metabolic adaptations to respiratory-based growth on glucose-derived fermentation products and the synthesis of trehalose [51]. Then, they are refilled to ultimately serve as an energy depot during extended periods of starvation. However, trehalose has been indicated as the carbohydrate of choice for surviving starvation and for fast cell cycle re-entry, as cells lacking it exit quiescence more slowly. Trehalose, in fact, may be preferred over glycogen as the cleavage of one glycosidic trehalose bond rapidly provides two glucose molecules, whereas cleavage of a single glycosidic bond in a glycogen molecule provides only one glucose molecule. Supporting this hypothesis, when stationary phase yeast cells are provided with fresh, rich medium, trehalose stores are depleted rapidly, and activity of the trehalase enzyme increases [53]. Besides, it is widely accepted that yeast capacity to cope with environmental stresses correlates with intracellular trehalose content [54]. This sugar, in fact, acts also as a stress protectant in response to heat shock, cold shock [55] and especially, desiccation [56]. The stress protectant role of trehalose is due to its intrinsic property to substitute for water molecules so to stabilize membrane structure. The ability of this disaccharide to exclude water enables it also to protect proteins from denaturation and to suppress the aggregation of the denatured ones. Strikingly, trehalose is also capable of protecting proteins against oxidative damage caused by exposure to ROS [57]. Indeed, it can prevent oxidative carbonylation of proteins by interacting with their carbonylation-prone aberrantly folded species, thus having an indirect inhibitory effect on the aggregation of oxidatively damaged proteins [58].

- They activate autophagy. Autophagy is the highly evolutionarily conserved process responsible for the lysosome-mediated degradation of damaged proteins and organelles. At a low basal level, it is constitutively active in order

to sustain cellular integrity, homeostasis, and survival, while it is strongly up-regulated by diverse stimuli, such as nitrogen and, to a lesser extent, carbon starvation [59,60], organelle deterioration, stress, and pathogen infection. The hallmark morphological feature of autophagy is the formation of the double-membrane vesicle known as the autophagosome containing either bulk cytoplasm or selected cargo, depending on the inducing condition. In yeast, flux through the pathway culminates in autophagosome-vacuole fusion, and the subsequent degradation of the resulting autophagic bodies and cargo by vacuolar hydrolases and stationary phase-induced proteases [61], followed by efflux into the cytosol of the breakdown products and their subsequent metabolic reutilization, contributing significantly to cells survival during starvation [62].

During chronological aging, unfit cells may undergo apoptosis. In yeast, apoptosis closely resembles the intrinsic or mitochondrial derived one in multicellular organisms [63]. There are many stimuli, either externally or internally derived, able to induce apoptosis in yeast, such as extreme pH environment [64], osmotic stress [65], defects in actin dynamics [66], acetic acid [67], the presence of lipid hydroperoxides [68] and, as already stated, aging itself [69]. Although these stressors are different, many have in common the ability to generate internal ROS, including the superoxide anion, hydroxyl ion, and hydrogen peroxide [70]. Oxygen radicals are defined as molecular derivatives of oxygen with one or more unpaired electrons (Fig. 1.1). Because the chemical properties of ROS are different, they target different cellular components. The highly reactive hydroxyl radical is indiscriminate. Negatively charged superoxide primarily targets iron-sulfur clusters, while neutrally charged hydrogen peroxide seems to react preferably with cysteine thiols. ROS also collaborate with metals in multi-step reactions, yielding high molecular-weight products, such as carbonylated protein aggregates [71]. Internal ROS concentrations higher than a certain threshold

induce oxidative stress that is characterized by accumulation of oxidized lipids, carbonylated proteins, damaged DNA and mitochondria [72].

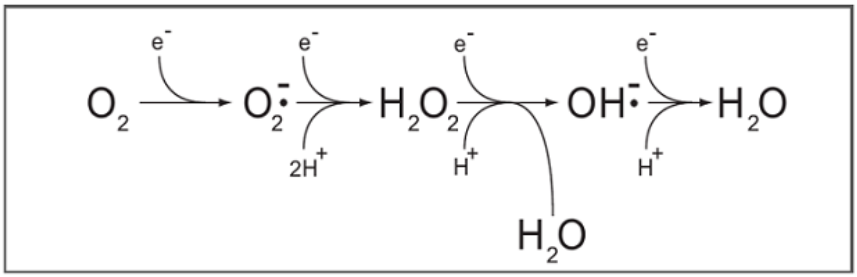


Fig. 1.1. Stepwise reduction of oxygen to subsequently superoxide, hydrogen peroxide, hydroxyl radicals and finally water.

Internal ROS are mostly derived from organelles performing their normal functions. In Fig. 1.2, the main sources of ROS triggering apoptosis are shown. The best studied and perhaps most important of these are mitochondria, which can produce ROS through the leakage of electrons from the Electron Transport Chain (ETC) during ATP synthesis. Mitochondrial dysfunction via mutations in ETC components, compounds that inhibit ETC function, or loss of mitochondrial inner membrane integrity, can generate sufficient ROS concentrations to induce the oxidative stress response [73]. Moreover, damaged mitochondria are in turn more prone to further ROS generation. In addition to mitochondria, other organelles also contribute to oxidative stress, including the endoplasmic reticulum (ER) [74] and peroxisomes [75].

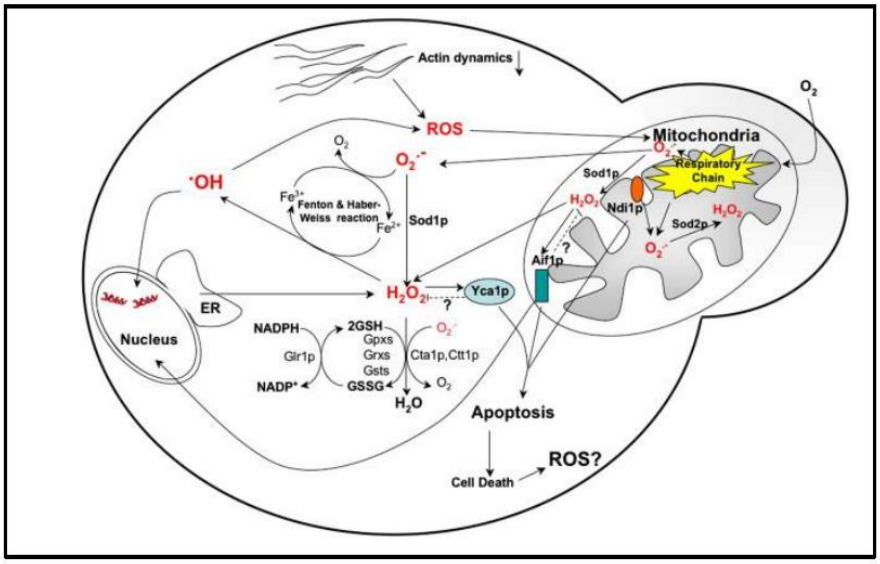


Fig. 1.2. Potential sources of ROS relevant to apoptosis in yeast. Intracellular sources of ROS that are involved in apoptosis are in red. The respiratory chain in the mitochondrion produces superoxide anions which are converted to H_2O_2 by Sod2 in the mitochondria matrix or by Sod1 in the cytosol and the mitochondrial inter-membrane space. Ndi1 represents the internal mitochondrial NADH dehydrogenase which transfers electrons to complex III of the respiratory chain. The apoptosis inducing factor (Aif1) is normally located in the mitochondrion and may have antioxidant activity. As part of the apoptotic process, Aif1 is translocated to the nucleus where it initiates DNA breakage. Deletion of the yeast metacaspase Yca1 may lead to increased ROS-induced damage. During protein folding, protein disulphide bond formation machinery in the ER produces H_2O_2. Hydrogen peroxide is detoxified to water by catalases (Ctt1 and Cta1) or glutathione-dependent enzymes including glutathione peroxidases (Gpxs), glutaredoxins (Grxs) and glutathione transferases (Gsts). In the latter processes reduced glutathione (GSH) is converted to oxidised glutathione (GSSG). GSSG is reduced to GSH by glutathione reductase (Glr1) using NADPH as electron donor. Hydroxyl radical (·OH) is generated via the Fenton and Haber–Weiss reactions. Actin dynamics involves changes in the actin cytoskeleton, if converted to stabilized actin filaments, leads to increased accumulation of ROS (from [63]).

Mitochondrial outer membrane permeabilization represents the commitment step to apoptosis execution [76]. The loss of inner and outer mitochondrial membrane integrity is required for release of pro-apoptotic factors such as cyt c and two nucleases, Aif1 and Nuc1. Deleting these nucleases increases resistance to ROS-induced cell death, whereas their overexpression causes hypersensitivity [77]. Aif1 and Nuc1 enter the nucleus and fragment chromatin. Yeast genetic studies indicate that cyt c is partially required for efficient apoptosis [67] and have identified several proteases necessary for apoptosis. Similarly to the caspase cascade in mammalian cells, the metacaspase Yca1 is activated by proteolysis and

is required for H_2O_2 and acetic acid-induced apoptosis [78]. Esp1 cleaves the cohesin Mcd1 in response to H_2O_2 treatment [79]. Nma111, an ortholog of the human HtrA protease [80], cleaves Bir1, the yeast ortholog of the mammalian inhibitor of apoptosis factor [81]. Interestingly, these proteases exhibit full, partial, or no role in apoptosis execution depending on the stress [82]. These results suggest that different stimuli utilize specific caspases to execute the cell death pathway. The cell has multiple avenues to counteract ROS-induced apoptosis. During oxidative damage, the stress-dependent transcription factor, Yap1, present in the cytoplasm under normal growth conditions, accumulates in the nucleus where it drives the transcription of many genes, among which those encoding proteins involved in ROS scavenging such as superoxide dismutases (SOD), catalases as well as some genes involved in DNA repair or cell cycle check point control [83-85]. Besides, the chronologically aged cells induce the NADP-dependent glutamate dehydrogenase Gdh3 that detoxifies ROS and prevents apoptosis initiation [86]. Trehalose and small molecules such as glutathione, vitamin E and vitamin C are also known for their involvement in ROS protection [87].

Why would yeast maintain a cell death pathway? Altruism has been argued to provide a selective pressure to maintain apoptosis based on the normal colony mode for yeast growth. For example, colonies contain regions of young and old cells [88] with the death of older cells no longer capable of cell division providing metabolites for the younger cells. Therefore, recycling the components of severely damaged or non-replicative cells within a colony would maximize growth chances for younger, reproductive cells.

1.2.2. Carbon metabolism: fermentation and respiration

Yeast cells grow on a wide variety of compounds as sources of energy and as carbon-containing precursors of anabolic metabolism and biomass accumulation [39]. However, *S. cerevisiae* uses glucose in preference to other sugars. This

phenomenon, defined as catabolite repression, is achieved in part by inhibition of the synthesis of enzymes required for the utilization of alternative carbon/energy sources, such as those involved in the gluconeogenesis, the glyoxylate cycle, the TCA cycle or the respiratory chain [89, 90], as well as of proteins required for the active transport system across the plasma membrane of acetate, lactate and pyruvate [91]. This repression is mainly due to the glucose repressor Mig1, which, in its non-phosphorylated form, binds to upstream repression sequences found in numerous target genes [92]. However, when glucose is unavailable, alternative sugars such as galactose, sucrose, maltose, and melbiose as well as non-sugar substrates such as ethanol, acetate, pyruvate, lactate, glycerol, or even fatty acids may be used for energy and biomass production. Thus, the yeast life cycle can integrate metabolic characteristics that are typical for rapid growing cells, storage cells, or highly metabolizing cells depending on nutrient supply.

As previously stated, in a standard CLS experiment, during the exponential phase, glucose is preferentially metabolized via alcoholic fermentation. Pyruvate is the end-product of glycolysis and is a key node in the branching point between alcoholic fermentation and respiratory metabolism as well as assimilatory and dissimilatory metabolic reactions [93]. At the branching point, it can follow three major fates (see Fig. 1.3): (i) decarboxylation to acetaldehyde which generates acetyl-CoA by the pyruvate dehydrogenase (PDH) bypass; (ii) anaplerotic carboxylation to oxaloacetate and (iii) the direct oxidative decarboxylation to acetyl-CoA by the pyruvate dehydrogenase (PDH) complex, which is located in the mitochondrial matrix. Pyruvate can cross the outer mitochondrial membrane while the passage across the inner mitochondrial membrane requires the Mitochondrial Pyruvate Carrier (MPC) [94,95]. This carrier effectively represents a link between cytosolic pyruvate metabolism and the TCA cycle.

In the cytosol, pyruvate can be converted to acetyl-CoA by the so-called PDH-bypass pathway [93] that requires the activity of three different enzymes: (i) pyruvate decarboxylase, which converts pyruvate to acetaldehyde; (2) acetaldehyde dehydrogenase (Ald), converting acetaldehyde to acetate; and (3)

acetyl-CoA synthetase (Acs), which activates acetate to cytosolic acetyl-CoA that can then be transported unidirectionally into the mitochondrion via the carnitine acetyltransferase system [93]. In *S. cerevisiae*, carnitine is involved in a process referred to as the carnitine shuttle which allows the transport of acetyl-CoA to the mitochondria. This transport system which is non-functional unless carnitine is supplied with the medium [96,97], involves the transfer of the acetyl moiety of acetyl-CoA to carnitine and the subsequent transport of the acetylcarnitine to the mitochondria. Here, a mitochondrial carnitine acetyltransferase catalyses the reverse reaction generating carnitine and acetyl-CoA which enters the TCA cycle [96,98]. *ACS1* and *ACS2* encode the two yeast acetyl-CoA synthetases which catalyze catalyzes the formation of acetyl-CoA from acetate and CoA. Acs2 is known as the glycolytic isoform [99] and besides its role in carbon metabolism it is required for histone acetylation [100]. Acs1 (the gluconeogenic isoform), instead, is responsible for the generation of the mitochondrial acetyl-CoA pool, together with the acetyl-CoA hydrolase 1 (Ach1) which catalyzes the transfer of the CoASH moiety from succinyl-CoA to acetate [101]. Acetyl-CoA is a key metabolite that links metabolism with signalling, chromatin structure, and transcription. It is produced by other catabolic pathways besides glycolysis, such as fatty acids β-oxidation, and also used as a precursor in anabolic processes, including sterols and amino acids synthesis [93,102].

Pyruvate might be directly converted to acetyl-CoA by the mitochondrial multi-enzyme complex PDH after its transport into the mitochondria through the MPC carrier. This complex comprises Mpc1 and Mpc2 subunits during fermentative growth, or Mcp1 and Mpc3 during respiratory growth. One of the mechanisms underlying the partitioning of pyruvate through the different routes is the regulation of the enzymes involved and their kinetic properties [93]. From an energetic point of view, PDH-bypass is less efficient owing to the consumption of an ATP molecule, which is converted into AMP by Acs1 [103].

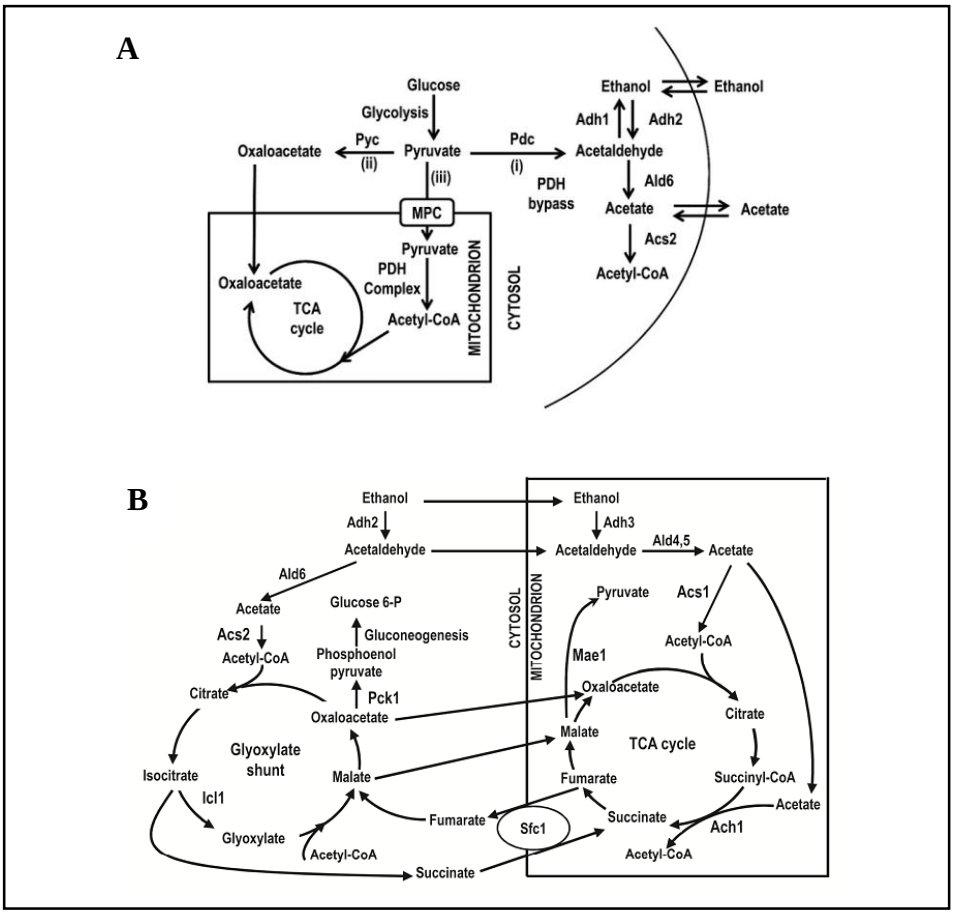


Fig.1.3. (A) Scheme of metabolic pathways allowing pyruvate utilization. The three pathways which originate from pyruvate after (i) decarboxylation to acetaldehyde, (ii) carboxylationto oxaloacetate and (iii) oxidative decarboxylation to acetyl-CoA are schematically shown. Acs, acetyl-CoA synthase; Adh, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; MPC, mitochondrial pyruvate carrier; Pdc, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; Pyc, pyruvate carboxylase. (B) Scheme of the TCA cycle and of the glyoxylate shunt. Ach1, acetyl-CoA hydrolase 1; Acs, acetyl-CoA synthase; Adh, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; Icl1, isocitrate lyase 1; Mae1, malic enzyme; Pck1, phosphoenolpyruvate carboxykinase 1; Sfc1 succinate-fumarate carrier (From [104]).

As previously stated, following the diauxic shift, cells switch to a respiration-based metabolism, in which cells start to use ethanol/acetate, the main glucose fermentation by-products, as carbon/energy sources via the TCA cycle and the glyoxylate shunt. TCA cycle, also termed Krebs cycle or citric acid cycle, is an amphibolic pathway since it fulfils both catabolic and anabolic functions. As a

catabolic pathway, the TCA cycle provides reducing equivalents to the mitochondrial respiratory chain through the oxidation of acetyl-CoA. In addition, the TCA cycle also functions in furnishing several precursors for various biosynthetic processes, in fact every TCA cycle intermediate is commonly used by other metabolic reactions. Thus the presence of anaplerotic pathways that replenish the cycle with intermediates is indispensable to keep it functioning. An important anaplerotic pathway is the glyoxylate cycle that is required for growth in minimal medium with carbon sources with less than three carbon atoms, as in the case of ethanol and acetate. In addition another main anaplerotic reaction is represented by the conversion of pyruvate into oxaloacetate catalyzed by pyruvate carboxylase localized in the cytosol [105].

The eight enzymes of the TCA cycle are encoded by 15 nuclear genes in *S. cerevisiae* [106] and are subjected to regulation by glucose levels. In *S. cerevisiae*, the depletion of glucose increases 3-10 times the TCA messenger RNAs [38]. Oxygen limitation could also induce a shift in the TCA pathway, which operates as a cycle during aerobic growth and as a two-branched pathway under oxygen limitation, sustaining only carbohydrates and amino acids synthesis. ATP synthesis by oxidative phosphorylation must be continuously adapted to changes in the cell energy demand to sustain growth and/or homeostasis. During respiratory metabolism, both cytosolic and mitochondrial NADH are reoxidized by the mitochondrial respiratory chain. *S. cerevisiae*, in contrast to other eukaryotic cells lacks the multi-subunit complex-I-type NADH dehydrogenase [107]. Instead, it contains a single-subunit NADH:ubiquinone oxidoreductase (encoded by *NDI1*), referred to as the "internal NADH dehydrogenase", which catalyzes the transfer of two electrons from intra-mitochondrial NADH to ubiquinone [108]. Nonetheless, yeast mitochondria contain also a mitochondrial external NADH dehydrogenase activity. *S. cerevisiae* has two genes encoding external NADH dehydrogenase isoenzymes, *NDE1* and *NDE2*, both of them typical aerobic expressed genes [109]. Like the internal NADH dehydrogenase, the external isoenzymes do not pump protons [110].

1.3 Regulation of yeast chronological aging

In accordance with the complex nature of chronological aging, molecular factors from virtually all cellular locations have been implicated in its regulation, including mitochondrial, nuclear, vacuolar, peroxisomal and cytoplasmic effectors. Here we present the main factors involved in the regulation of CLS.

1.3.1 TORC1/Sch9 and Ras/PKA pathways

Chronological aging is regulated by the two highly conserved nutrient-sensing pathways TORC1/Sch9 and Ras/PKA. They are both positive key regulators of cell growth that participate in the cell's decision whether or not to enter into quiescence. In the presence of nutrients, these pathways promote cell division and growth while inhibiting the general stress response and autophagy [111, 112]. When yeast cells are starved for glucose, instead, they arrest cell division and promote stationary phase entry, while down-regulating global transcription and translation, and inducing the expression of genes that aid in the survival during nutrient absence, such as those involved in metabolism reprogramming, reserve carbohydrate accumulation, autophagy, cell wall remodeling and general stress resistance. In particular, the TORC1/Sch9 pathway acts primarily as a nitrogen/amino acid starvation response circuit [113]. The rapamycin-sensitive kinase complex TORC1 is composed of the regulatory subunits Lst8, Kog1 and Tco89, and either the Tor1 or Tor2 kinase [114]. Although the effects of TORC1 signalling on yeast physiology are ample, only a few direct targets/effectors of this complex have been identified. Among them, the AGC protein kinase Sch9, ortholog of the mammalian ribosomal protein S6 kinase (S6K). Phosphorylation of Sch9 by TORC1 occurs on multiple serine and threonine residues in its C-terminal region and was shown to be required for Sch9 to be active [115]. Under optimal growth conditions, TORC1 is active, resulting in Sch9 activation. Sch9 is the major effector by which TORC1 signalling impacts on yeast lifespan. Indeed, *SCH9* deletion, as well as *TORC1* deletion, increases CLS [111]. Upon nutrient starvation conditions, the subsequent reduction of TORC1/Sch9 signalling

triggers nuclear translocation of the protein kinase Rim15, which stimulates the activity of the zinc-finger transcription factors, Msn2, Msn4, and Gis1 [116]. While Msn2 and Msn4 activate transcription of genes containing stress-responsive promoter elements, STRE [117,118], Gis1 drives the expression of post-diauxic shift (PDS) element-controlled genes [119]. Overall, this results in an increased cellular protection against oxidative stress through the activation of *SOD2*, encoding mitochondrial superoxide dismutase [42]. The promoter of *SOD2* contains both STRE and PDS elements. In addition to stress, these factors regulate metabolism and the accumulation and utilization of intracellular and extracellular carbon sources [120,121]. In yeast, a fourth related zinc finger protein, Rph1 was identified, whose sequence is 34% similar to that of Gis1. Rph1 as well plays a role in growth phase-dependent gene expression; more specifically, Gis1 and Rph1 function both as repressors and activators on overlapping sets of genes as well as on distinct targets [122].

The extended CLS of both *tor1Δ* and *sch9Δ* strains depends to a large extent on the presence of a functional Rim15 protein kinase, as well as on its downstream effectors Msn2/4 and Gis1 [111]. Intriguingly, this function of Rim15 seems to be conserved among eukaryotes. Alternatively, a recent study proposes a Rim15-independent mechanism for lifespan extension in reduced TORC1/Sch9 signalling [123]. It was shown that both *tor1Δ* and *sch9Δ* strains, due to an augmented translation of mtDNA-encoded OXPHOS complex subunits [121,124], were characterized by an increased respiration and a more elevated superoxide production during active growth phase that might elicit an adaptive response (mitohormesis), responsible for their enhanced survival in stationary phase.

Rim15 activity is modulated by multiple nutrient signalling pathways [125], affecting either its intracellular location or its kinase activity. In response to glucose, the protein kinase A (PKA) directly phosphorylates and thereby inactivates the kinase activity of Rim15 [126]. PKA in yeast cells, like in all other eukaryotes, is a heterotetramer, composed of two catalytic subunits and two regulatory subunits. It occupies a crucial position in cell response to glucose and

couples cell cycle progression and growth, having a key inhibitory role in transition from exponential growth to diauxic shift and stationary phase. Cells with increased PKA activity fail to acquire the characteristics typical of stationary-phase cells, such as resistance to high temperature and osmotic stress, and die precociously. Conversely, cells with decreased PKA signalling display the stationary-phase characteristics even when glucose is present [121]. PKA negatively regulates transcription of Msn2-dependent genes by phosphorylating Msn2 and keeping it in the cytosol [127].

1.3.2. Sirtuins

Sirtuins are a family of NAD^+-dependent protein deacetylases [128]. Sir2 (silent information regulator 2) of *S. cerevisiae* is the founding member of the family and regulates silencing at the mating-type loci, telomeres, and ribosomal DNA (rDNA) loci [129]. There are seven mammalian Sirtuins, termed SIRT1-7, which share the sequence homology of catalytic domain with Sir2 [130].

The link between Sirtuins and aging was first made in yeast. In fact, Sir2 is required for RLS being this latter dependent on the stability of the rDNA repeats and correct telomeric silencing [131]. In mice, at least two Sirtuins are involved in lifespan extension: SIRT6 overexpression extends the lifespan of male mice when overexpressed in the whole body [132], and SIRT1 extends the lifespan of mice when overexpressed in the brain [133]. In general, Sirtuins play regulatory roles in transcription, metabolism and genome maintenance and seem to be implicated in carcinogenesis through mechanisms yet to be defined completely. Indeed, Sirtuins like SIRT2 and SIRT6 are thought to be tumor suppressor, on the contrary, SIRT1, for example, acts as both tumor suppressor and oncogenic factor depending on the context and the study condition [134-136].

All the Sirtuins are characterized by their requirement for beta-nicotinamide adenine dinucleotide (NAD^+) as a co-substrate [137, 138]. Deacetylation by

Sirtuins involves the consumption of NAD^+ and acetylated protein substrate to produce nicotinamide (NAM), 2 O'-acetyl-adenosine diphosphate-ribose (O-AcADPR), and deacetylated substrate. In the first step of the reaction (see Fig.1.4), ADP-ribose is covalently attached to the acetyl moiety of the substrate, accompanied by release of free NAM. Hydrolysis of the acetyl-lysine bond then occurs, liberating O-AcADPR. NAM acts as an inhibitor of the reaction, and thus provides negative feedback inhibition of the Sirtuins *in vivo* [137]. The peculiar requirement for NAD^+ of these deacetylases potentially makes their activity controlled by NAD^+ homeostasis and responsive to different metabolic states of the cell.

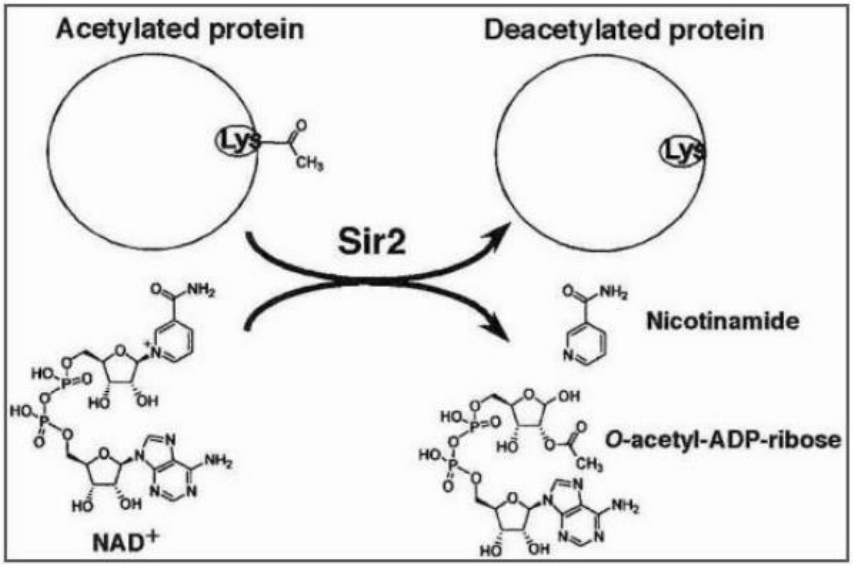


Fig.1.4. Sir2/Sirtuins deacetylation reaction. Sir2 and Sirtuins catalyze NAD^+ dependent deacetylation of histone tails or other non-histone acetylated proteins; the reaction transfers the acetyl group from acetylated lysine residues to the ADP -ribose moiety of NAD^+, generating deacetylated histone tails, NAM and the novel metabolite O-AcADPR.

For all eukaryotes, including *S. cerevisiae*, regulating chromatin structure both locally and globally is essential for controlling cellular processes through transcriptional activation and, more importantly in terms of the Sirtuins, through

transcriptional repression. Transcriptional repression can be highly localized and transient, such as at the promoters of specific genes, or more widespread across large regions of the genome that remain in a repressive and condensed state for extended periods. These latter domains tend to be heterochromatic and stable, sometimes even through multiple generations. In yeast, HML, HMR, and telomeres are generally considered heterochromatin regions in this organism [138], and are characterized by hypoacetylation of the N-terminal tails of histones H3 and H4 [139]. The repetitive rDNA in yeast also has characteristics of heterochromatin, including transcriptional silencing of Pol II transcription within the tandem array and suppression of homologous recombination between the repeats [140].

At *HML* and *HMR*, Sir2 associates with Sir1/3/4. The SIR complex is recruited to cis-acting sequences flanking the silent domains known as E and I silencers (Fig 1.5). The silencer elements contain binding sites for Rap1 and Abf1, which together act as binding platforms [141]. Following the recruitment to silencers, the general model for yeast heterochromatin formation is that targeted Sir2 locally deacetylates an adjacent nucleosome on H3 and H4 tails, which then promotes continual spreading of the SIR complex across the silenced domains in between the silencers [142]. Spreading outside the silencers is blocked by boundary/insulator elements, one of which is well defined as a $tRNA^{Thr}$ gene [143].

For telomeric silencing, Sir2 and Sir4 are recruited to telomeres, then Sir3 associates and the complex can spread into sub-telomeric regions promoting silent chromatin assembly.

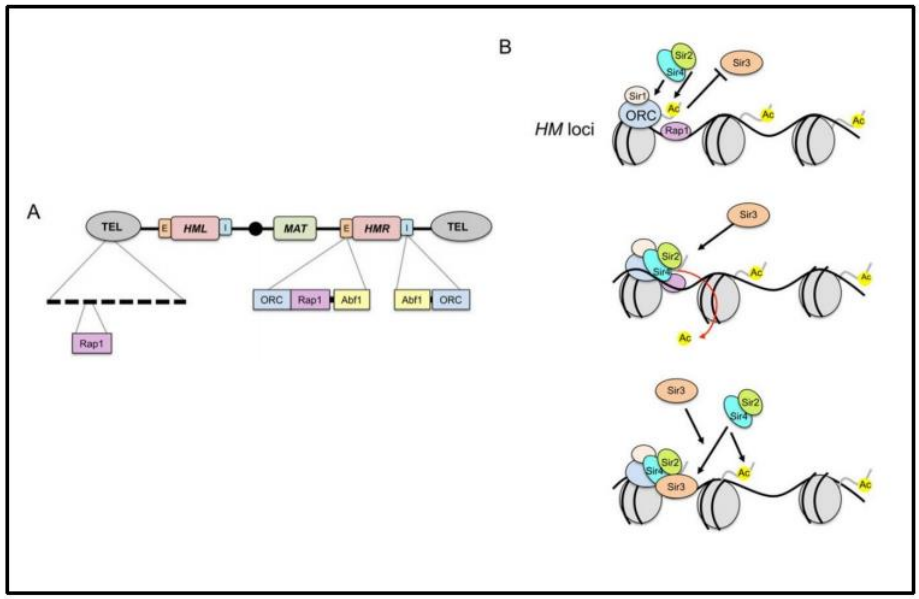


Fig. 1.5. Classic silencing targets of Sir2 on chromosome III. A) Schematic diagram showing Rap1 binding sites at the telomeric repeats. The Rap1, origin recognition complex (ORC), and Abf1 binding sites within the E and I silencer elements flanking HMR are also shown. B) Updated model for Sir2-mediated histone deacetylation at silent chromatin (via the SIR complex). A Sir2/Sir4 sub-complex is recruited by ORC, Sir1, and Rap1 at the silencers. H4K16 is then deacetylated, which promotes Sir3 binding to form a SIR holocomplex, and induce spreading. Silencing spreads as more Sir2/4 is recruited to adjacent nucleosomes, resulting in further histone deacetylation and the binding/stabilization of additional Sir3 units. (From [144]).

For silencing at the rDNA repeats, Sir2 does not function as part of the SIR complex. In early experiments, it was shown that although deleting other components of the SIR complex (Sir3 or Sir4) eliminated silencing at telomeres and the silent mating type loci, silencing at the rDNA increased, implying that cofactors for silencing at the rDNA were distinct from the other silent loci [145]. These other cofactors were found to be Net1 and Cdc14, and together they form the RENT complex (Fig.1.6), which is critical for formation of silent chromatin at the rDNA [146]. The RENT complex is recruited to the intergenic spacer through interactions with Fob1 at *IGS1* and Pol I at the rDNA promoter in *IGS2*. Consequently, loss of Fob1 or Pol I causes severe DNA silencing defects [147].

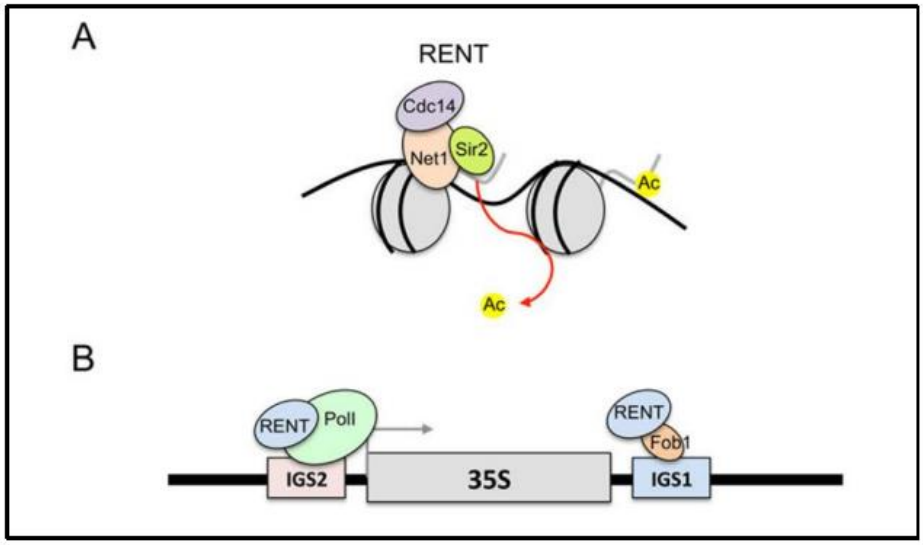


Fig. 1.6. The RENT complex, recruitment to the rDNA and rDNA stability in yeast aging. A) The RENT complex consists of Sir2, Net1, and Cdc14, and functions as a histone H3 and H4 deacetylase within the nucleolus. B) RENT is recruited to the intergenic spacers of the rDNA repeats at either IGS1 via interactions with Fob1, or at IGS2 via interactions with RNA polymerase I at the rDNA gene promoter (Pol I) (From [144]).

As already mentioned, the reaction catalyzed by Sir2 has a unique feature that distinguishes it from the other classes of histone deacetylases, that is the requirement for NAD^+, which was unexpected for thermodynamic reasons. The deacetylation reaction catalyzed by the other histone deacetylases, a simple hydrolysis reaction, is energetically favorable. Sir2, instead, actually consumes NAD^+, therefore its constant replenishment is essential for maintaining cellular fitness. Cells have developed complex interconnecting biosynthetic and signalling pathways to regulate intracellular NAD^+ levels. However, factors regulating NAD^+ metabolism and homeostasis remain unclear due to the dynamic and complex nature of the NAD^+ synthesis pathways.

In yeast, NAD^+ is synthesized from tryptophan and the three vitamin precursors of NAD^+, nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR) (Fig.1.7). NAD^+ homeostasis is maintained by a combination of biosynthesis and salvage pathways, and balanced secretion/import of the vitamin precursors

[148]. In the absence of any vitamin precursors in the growth medium, NAD^+ is synthesized *de novo* from tryptophan using the Bna1-Bna6 proteins [149], which ultimately produce NA mononucleotide (NAMN). The NAMN is then adenylated by the redundant nicotinic acid/nicotinamide adenylyltransferases Nma1 or Nma2 to generate nicotinic acid adenine dinucleotide (NaAD) [150], followed by conversion to NAD^+ by the NAD synthetase (Qns1) [151]. NA is imported from the growth medium by the NA permease Tna1 and then converted by nicotinic acid phosphoribosyltransferase (Npt1) into NAMN, thus merging with the last two steps of the *de novo* pathway. This is historically known as the Preiss-Handler pathway. In commonly used yeast growth media containing NA as the vitamin precursor, Npt1 is the rate-limiting step for NAD^+ production, and deleting *NPT1* results in a 2- to 3-fold reduction in the intracellular NAD^+ concentration, sufficient to inhibit Sir2 function in transcriptional silencing and shorten RLS [152]. The Npt1, Nma1, and Nma2 proteins are each concentrated in the nucleus [150], and their overexpression extends RLS without increasing the overall NAD^+ level, suggesting that flux through the Preiss-Handler pathway is important for maintaining proper Sir2 activity in the nucleus [150].

NR is imported by the thiamine/NR transporter Nrt1 [153], and then phosphorylated by nicotinamide riboside kinase (Nrk1) to produce nicotinamide mononucleotide (NMN), which is adenylated by Nma1 or Nma2 to produce NAD^+ [153]. NR supplementation suppresses the short RLS and transcriptional silencing defects of an *npt1Δ* mutant by restoring normal NAD^+ concentration. NR can also be degraded into NAM by several nucleoside hydrolases and phosphorylases [154]. The nicotinamidase Pnc1 scavenges any NAM generated by NR hydrolysis, Sir2 and Hst activity, or other NAD^+ consuming reactions by deamidating it into NA. This not only prevents the accumulation of NAM to high concentrations that could inhibit Sir2 but also pushes it into the Preiss-Handler pathway to be recycled into NAD^+ via Npt1 [155]. High NAM concentrations (5mM) in the growth medium are inhibitory for Sir2 activity, and result in shortened RLS that is similar to the effects of a *sir2Δ* mutant [137]. Overexpressing *PNC1* suppresses this

inhibition by detoxifying the NAM through deamidation [155], and *PNC1* overexpression extends RLS even when NAM is not added to the growth medium [156], probably because of its dual role in promoting flux through the Preiss-Handler pathway and preventing NAM accumulation. The downstream effect is most likely Sir2-mediated enhancement of rDNA silencing and stability, though other Sir2 and Hst-dependent processes could certainly also be at play [144].

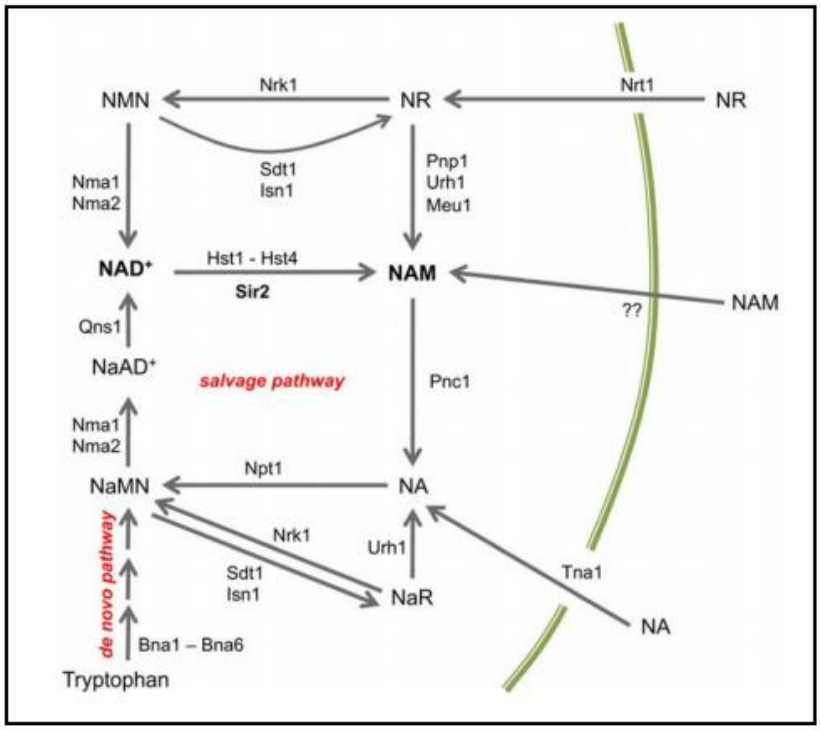


Fig.1.7. Overview of NAD^+ biosynthesis and metabolism in *Saccharomyces cerevisiae*. NAD^+ is synthesized *de novo* by the Bna1-Bna6 enzymes using tryptophan as the starting substrate. The vitamin precursors nicotinic acid (NA), nicotinamide (NAM), and nicotinamide riboside (NR) are imported and then enter a set of salvage pathways that ultimately feed into nicotinic acid mononucleotide (NaMN) or nicotinamide mononucleotide (NMN). These mononucleotides are further adenylated to by Nma1 or Nma2 to form the dinucleotide forms, which for nicotinamide, is actually NAD^+. Precursors from the nicotinamide branch of the salvage pathways can be shifted to the nicotinic acid branch through deamidation of nicotinamide by Pnc1. The Sirtuins produce nicotinamide during the deacetylation reaction. The mechanism of nicotinamide import is unknown. Other abbreviations: NaAD, deamido NAD; NaR, nicotinic acid mononucleotide (From [144]).

Whereas Sir2 has an anti-aging role in replicative lifespan, it plays a pro-aging one in CLS. Depending on the strain background, *sir2*Δ either has no effect or

modestly increases CLS when cells are grown in YPD or SC medium [157-159]. When cells are transferred to water after reaching stationary phase they have a long CLS (considered an extreme form of CR), and deleting *SIR2* dramatically extends the lifespan even more [157]. Similar extreme CLS extension is observed when *sir2Δ* is combined with *sch9*Δ or Ras/cAMP/PKA mutations that reduce nutrient signaling [157]. Given that both of these pathways extend CLS through the activation of stress response genes [132], it is possible that Sir2 may prevent full CLS extension by partially repressing these same genes. This would be consistent with the observation that *sir2*Δ also confers greater resistance to oxidative and heat stresses [157]. Sir2 has been found to deacetylate and subsequently inactivate the phosphoenolpyruvate carboxykinase (Pck1) [160]. In short, the loss of Sir2 activity pushes cellular metabolism from glycolysis towards gluconeogenesis and glycogen and trehalose production during chronological aging [160]. Both of these pathways contribute to the survival by increasing stress resistance of the non-dividing cells and allowing them to quickly reenter mitosis upon reintroduction of nutrients to the media [43,53].

1.3.3 Calorie restriction

Diet is one of the lifestyle components capable of affecting the quality and the duration of life in a wide range of living organisms. CR, namely a reduction in calorie intake without malnutrition, to date is the only nutritional intervention known to promote longevity in many species [161].

CR was first investigated in the thirties by McCay and collaborators, who reported that feeding rats a diet of reduced content throughout their lives significantly extended their mean as well as the maximum lifespan [162]. CR and its potential application in humans as a means of increasing lifespan and healthspan has gained

considerable interest [163]. Lifespan extending effects of CR has yet to be demonstrated conclusively in humans (for a review see [164]).

In yeast, CR is obtained by limiting the initial glucose concentration in the media from the standard 2% up to 0.5% [161,165] or by transferring post-diauxic cells to water, which is viewed as an extreme form of CR [34]. CR, as a nutrient stress, activates stress-resistance transcription factors, whose targets are genes involved in protection against oxidative and heat stress, DNA repair, and metabolism [111,161]. These protective factors are the same implicated in CLS elongation following the down-regulation of TORC1/Sch9 and Ras/PKA pathway (see Fig. 1.8). Indeed, the effects of CR appear to be largely mediated by the two highly conserved nutrient signalling pathways TORC1/Sch9 and Ras/PKA [161].

Many metabolic changes are associated with CR and some of them must be responsible for its effect on longevity. CR accelerates ethanol and neutral lipids catabolism as well as gluconeogenesis, promoting trehalose and glycogen storage [120,166]. Neutral lipids regulate energy homeostasis as well as membrane stability. Moreover, CR, inhibiting TORC1 activity, should increase CLS in part by an adaptive mitochondrial longevity signal involving increased ROS levels [167,168]: the idea is that these effects are mediated by a metabolic shift toward mitochondrial respiration induced by CR. It was also reported that CR cells exhibit a lower respiratory rate in stationary phase with respect to wild-type cells and that lower overall mitochondrial effort could be the reason why CLS is extended [169]. In addition, CR also induces autophagy: enhanced degradation of damaged mitochondria, proteins or other macromolecules could reduce the overall burden of cell damage.

Given the uncertain applicability of a CR regimen to humans, research efforts have been devoted to mimic its beneficial effects on health and longevity with drugs. Natural or synthetic pharmacological agents could theoretically be used to induce the beneficial effects of CR without provoking its discomfort.

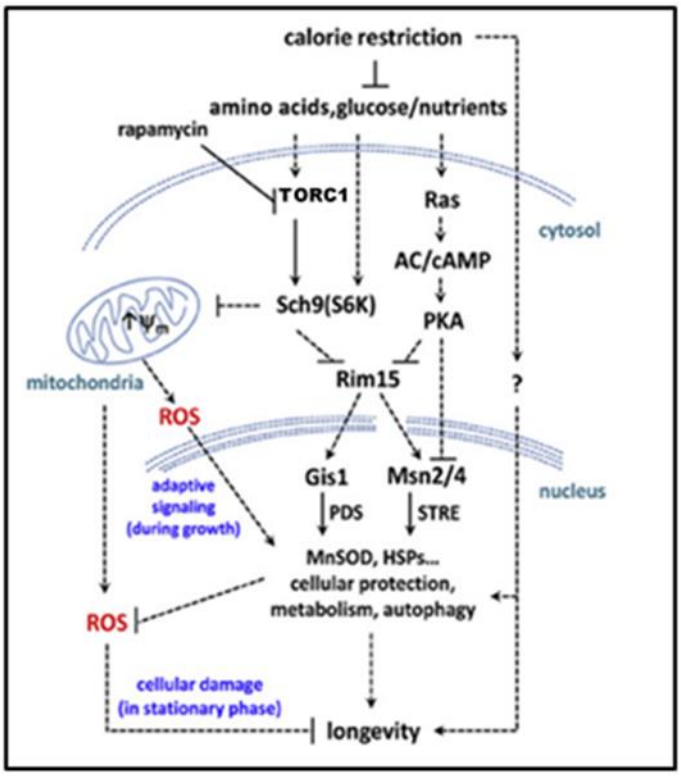


Fig. 1.8. Yeast chronological lifespan major regulatory pathways The nutrient-sensing pathways controlled by Sch9, TORC1, and Ras converge on the protein kinase Rim15. A major portion of the effect of CR on longevity appears to be mediated by the down-regulation of the Ras//PKA and TORC1/Sch9 pathways and consequent activation of the Rim15-controlled Msn2/4 and Gis1 stress-responsive transcription factors. Reduced TORC1/Sch9 signalling (genetic mutations or rapamycin) also increases coupled mitochondrial respiration and membrane potential during growth phase, which leads to an adaptive mitochondrial ROS signal. During chronological aging, TORC! and Sch9 deficiencies and adaptive mitochondrial ROS signalling decrease ROS production and enhance cellular stress responses, culminating in lifespan extension (adapted from [34]).

1.4 Scope of this thesis

The population of aged people is constantly increasing. This poses social and economic challenges, as it is accompanied by an increased incidence of many pathologies including cancer, cardiovascular and neurodegenerative diseases. Therefore, great interest in now focused on aging research aimed at extending, besides the lifespan, also the healthspan in order to reduce the incidence or progression of diseases associated with aging and promote a healthy aging. In particular, many efforts are currently centered on the identification/improvement of interventions based on dietary restriction or supplementations that can represent a safe and a feasible strategy to achieve the improvement of healthy aging. In the field of aging and aging-related research, the yeast *Saccharomyces cerevisiae* is a useful experimental system. In yeast, a clear correlation exists between chronological aging and metabolism.

Chapter 1 gives a general overview of aging and underline how *S. cerevisiae* and the Chronological LifeSpan (CLS) model can offer valuable insights into the aging process, translating this knowledge in interventions applicable to humans. The main factors involved in the regulation of CLS are briefly presented.

Chapter 2 is focused on the relation between chronological aging and metabolism, in particular we provided evidence that a general re-assessment of the carbon metabolism toward gluconeogenesis correlates with CLS extension.

In Chapter 3, we evaluated the effects of Nicotinamide, Resveratrol and Quercetin, considered as potential nutraceuticals, on yeast CLS and metabolism.

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2

Ethanol and acetate acting as carbon/energy sources negatively affect yeast chronological aging

2.1 Abstract

In *Saccharomyces cerevisiae*, the chronological lifespan (CLS) is defined as the length of time that a population of nondividing cells can survive in stationary phase. In this phase, cells remain metabolically active, albeit at reduced levels, and responsive to environmental signals, thus simulating the postmitotic quiescent state of mammalian cells. Many studies on the main nutrient signaling pathways have uncovered the strong influence of growth conditions, including the composition of culture media, on CLS. In this context, two byproducts of yeast glucose fermentation, ethanol and acetic acid, have been proposed as extrinsic proaging factors. Here, we report that ethanol and acetic acid, at physiological levels released in the exhausted medium, both contribute to chronological aging. Moreover, this combined proaging effect is not due to a toxic environment created by their presence but is mainly mediated by the metabolic pathways required for their utilization as carbon/energy sources. In addition, measurements of key enzymatic activities of the glyoxylate cycle and gluconeogenesis, together with respiration assays performed in extreme calorie restriction, point to a long-term quiescent program favoured by glyoxylate/gluconeogenesis flux contrary to a proaging one based on the oxidative metabolism of ethanol/acetate via TCA and mitochondrial respiration.

2.2 Introduction

Human aging is associated with a host of time-dependent changes which are the clear manifestation of the progressive decline in cognitive and physical functions of an organism. Albeit extremely complex, aging has turned out to be influenced by mechanisms and nutrient/energy sensing signaling pathways that show strong evolutionary conservation. In this context, the single-celled yeast *Saccharomyces cerevisiae*, exploited as a model system, has provided valuable insight by making it possible to adopt experimental approaches that are not always feasible in higher eukaryotic systems. For example, the nutritional and metabolic status of yeast cells can be diversely coordinated by the simple choice of cultural conditions. Glucose is the preferred carbon and energy source, but in its absence other substrates such as glycerol, ethanol, acetate, or even fatty acids can be used [1]. Thus, the yeast life cycle can integrate metabolic characteristics that are typical for rapid growing cells, storage cells, or highly metabolizing cells depending on nutrient supply.

In the field of aging-related research, replicative and chronological lifespan models have been described which offer the opportunity to study the aging process of both proliferating and postmitotic quiescent mammalian cells, respectively [2-4]. The chronological lifespan (CLS) is defined as the length of time that a population of nondividing cells survives in stationary phase. Viability over time is measured as the ability to resume mitotic growth upon return to rich fresh medium [5]. In a standard CLS experiment, yeast cells are usually grown in synthetic defined media containing 2% glucose [6] where the metabolism is characterized by a high glycolytic flux, glucose fermentation, and a negligible aerobic respiration. Upon glucose depletion, the diauxic shift occurs which results in a shift from fermentation to respiration of the C2 compounds previously produced. This shift involves a massive reprogramming of gene expression including genes which encode enzymes involved in gluconeogenesis, the glyoxylate and TCA cycles. Moreover, overall growth rate is dramatically

reduced. Finally, when nutrients are fully exhausted, cell division stops, and the yeast culture enters a quiescent stationary phase [7, 8]. In the stationary phase, yeast cells display a survival-based metabolism characterized by low metabolic rates and upregulation of stress resistance resulting from the integrated responses of different signaling pathways [9].

CLS can be increased by calorie restriction (CR) which, in yeast, is generally imposed by reducing the glucose concentration in the initial growth medium [10-12] or by transferring postdiauxic cells to water (extreme CR) [5]. Moreover, inhibition/reduction activity of two pathways which sense nutrient availability, namely, TORC1-Sch9 and Ras-PKA ones, also extends CLS [13-16]. These signaling pathways lead in part to common downstream targets which include the protein kinase Rim15 and the transcriptional factors Msn2/4 and Gis1 [17-19]. These factors, besides regulating directly or indirectly stress defence mechanisms, control the accumulation/utilization of intracellular and extracellular carbon sources [20-23]. In particular, Gis1 regulates the accumulation of acetate, a metabolite involved in chronological aging [24]. Interestingly, lack of the NAD^+-dependent deacetylase Sir2, the founding member of Sirtuins, further extends the CLS of long-lived mutants such as *sch9*Δ, as well as the CLS in water indicating that the sole presence of Sir2 can serve as a "blocker" of extreme longevity extension [25]. In addition, *SIR2* inactivation induces stress resistance and affects positively the metabolism of extracellular carbon sources such as ethanol and acetate [25, 26]. These two by-products of glucose fermentation which are metabolised by yeast cells during the post-diauxic phase have been proposed as extrinsic factors promoting chronological aging [25, 27]. In fact, in some long-lived mutants, as well as in short-lived ones, an inverse correlation between the amount of extracellular ethanol or acidic acid and their CLS has been found [25, 26, 28-30]. In line with this, genetic or metabolic (CR) interventions which drive yeast metabolism away from acetic acid production increase CLS [27, 31]. Furthermore, although some connections have been established between nutrient-

sensing pathways and the proaging effect of acetic acid involving superoxide anion accumulation which inhibits quiescence [32], the mechanisms by which this compound (and also ethanol) reduces the CLS are still controversial [33].

Here we present results showing that both ethanol and acetic acid contribute to chronological aging. In this context, these compounds are not simply extrinsic toxic factors, but it is their metabolic utilization as carbon/energy sources which results in proaging effects. In particular, in extreme CR, their oxidative metabolism increasing respiration impairs mitochondrial functionality and negatively affects long-term cell survival.

2.3 Materials and methods

2.3.1 Yeast strains and growth conditions

All yeast strains with null mutations were generated by PCR-based methods in a W303-1A background (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*): *fps1*Δ (*fps1*Δ::*KlLEU2*), *sir2*Δ (*sir2*Δ::*URA3*) [34], *icl1*Δ (*icl1*Δ::*KlLEU2*), and *pck1*Δ (*pck1*Δ::*KlLEU2*) [26]. Yeast cells were grown in batches at 30°C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/L), supplemented with 2% w/v glucose. Auxotrophies were compensated for with a fourfold excess of supplements [25]. 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 Analyser, as described [35]. Doubling time (Td) was obtained by linear regression of the cell number increase over time on a semilogarithmic plot. For pH measurements, small aliquots of expired media were removed from the culture, and pH was determined using a pH meter.

2.3.2. Metabolite measurements and enzymatic assays

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). Immediately after preparation of cell-free extracts, Pck1 and Icl1 activities were determined as previously described [26]. 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.

2.3.3. CLS determination

Survival experiments in expired medium were performed on cells grown in minimal medium (with a fourfold excess of supplements) of 2% glucose as described by [25]. During growth, cell number and extracellular glucose, ethanol, and acetic acid were measured in order to define the growth profile (exponential phase, diauxic shift, post-diauxic phase, and stationary phase) of the culture. Cell survival was monitored by harvesting aliquots of cells starting 72 h (Day 3, first age-point) after the diauxic shift (Day 0). CLS was measured according to [25] by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 3 was considered the initial survival (100%). Survival was also monitored in the presence of 50 mM pyrazole (Sigma) which was added in the expired medium at Day 1 after the diauxic shift.

For survival experiments in water, at Day 1 cells were harvested, washed with sterile distilled water, and resuspended in a volume of water equal to the initial culture volume. Every 48 h, cells were washed with water and resuspended in

fresh water to remove nutrients released by dead cells [5]. The pH of the water was adjusted to 3.2 since it was the pH value measured in the expired medium or to 5.6. Survival experiments in water containing ethanol, acetic acid, or both were performed essentially as described [25, 26, 29]. Treatments are outlined in the text.

For CLS determination in media-swap experiments, cells were grown in minimal medium of 2% glucose (with a fourfold excess of supplements) and at Day 1 after the diauxic shift, harvested by centrifugation. Cell pellets were washed and then resuspended in the filtered original medium or equivalently conditioned one of the indicated strain. Resuspension in media collected at Day 1 was also performed in the presence of 50 mM pyrazole. Viability was measured as previously described.

2.3.4. Respiration assays

The oxygen consumption of intact cells was measured at 30°C using a "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System, Hansatech Instruments, Norfolk, UK). For all respiration assays, 2 mL of cell suspension at a concentration of 5×10^6/mL were quickly transferred from the flask to the oxygraph chamber, and routine respiration was recorded. Data were recorded at sampling intervals of 1 s (Oxygraph Plus software, Hansatech Instruments, Norfolk, UK). Respiratory rates were determined from the slope of a plot of O_2 concentration against time, divided by the cellular concentration. All assays were conducted in biological triplicate.

Index of respiratory competence (IRC) was also measured according to [36] by plating identical samples on YEPD plates and on rich medium of 3% glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.

2.4 Results and discussion

2.4.1 Lack of Fps1 channel increases CLS

Ethanol and acetic acid are two normal by-products of glucose fermentation, transiently accumulated in the yeast culture medium, which restrict CLS [25, 27]. Moreover, given the low concentration reached by acetic acid in the medium of chronologically aging cells and its faster exhaustion compared to that of ethanol, its physiological relevance as an extracellular factor promoting chronological aging is a matter of debate [33]. In this context, as a first step, we examined the effects on CLS of abolishing the major route of entry into the cell of the undissociated acetic acid such as the Fps1 channel. Uptake of acetate is linked to an active transport for the dissociated form of the acid through the Jen1 and Ady2 transporters accompanied by passive/facilitated diffusion of the undissociated acid through the Fps1 aquaglyceroporin [37, 38]. The former is inducible and subjected to glucose repression [39, 40] while the passive transmembrane flux is strongly influenced by the pH of the medium. In fact, the acetic/acetate couple forms a buffer system in a dynamic equilibrium: at low pH the equilibrium increasingly favours the protonated form while at pH above the pKa of acetic acid (4.75) charged acetate anions prevail. As shown in Figures 2.1(a) and 1(b), measurements of extracellular ethanol and acetate revealed that, at the diauxic shift, in the *fps1*Δ culture the amount of these C2 compounds was slightly higher than that in the wild type (wt) culture, in line with exometabolome data obtained during glucose fermentation [41]. However, after the diauxic shift (respiratory metabolism) a significant effect was observed on the depletion of both ethanol and acetic acid which was reduced in the mutant. In particular, as opposed to the expected fast exhaustion of acetic acid in the wt medium (Figure 2.1(b) and [29]), in the *fps1*Δ mutant this compound decreased very slowly, and it was still present 6 days following the entry in the post-diauxic phase (Figure 2.1(b)), which is in agreement with the role for Fps1 in facilitating the diffusion of the undissociated acid. In fact, during this phase in which the pH of the medium dropped to values of 2.70 for the wt and 2.55 for the *fps1*Δ mutant at Day 6 (Table 2.1), acetic acid

is substantially undissociated, and the diffusional entry into the cells is elevated. Upon *FPS1* deletion, mutant cells can only rely on the uptake of the low fraction of acetate anions by the active transporters. Interestingly, chronologically aging *fps1*Δ cells lived longer than wt (Figure 2.1(c)) despite a prolonged exposure to acetic acid and ethanol.

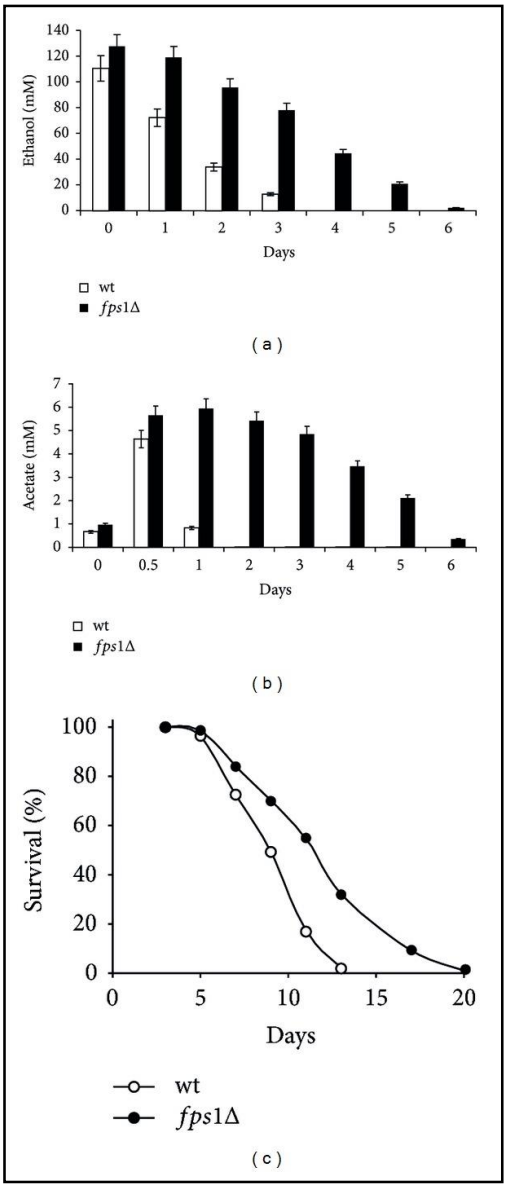

Fig. 2.1. *FPS1* inactivation increases CLS in concert with a decreased uptake of ethanol and acetate. Bar charts of extracellular ethanol (a) and acetate (b) concentrations measured at the indicated time-points in wild type (wt) and *fps1*Δ mutant cultures during chronological aging. Day 0, diauxic shift. Data were obtained from mean values determined in three independent experiments. Standard deviations are indicated. (c) CLS of wt and *fps1*Δ mutant cells. At every time-point, viability was determined by counting CFUs on YEPD plates. 72 h after the diauxic shift (Day 3) was considered the first age-point. One representative experiment is shown.

Table 2.1: pH values of exhausted media.

Days	wt	*fps1*Δ	*icl1*Δ	wt + pyrazole	*icl1*Δ + pyrazole
0	3.21 ± 0.07	3.20 ± 0.06	3.11 ± 0.06		
1	3.18 ± 0.04	3.16 ± 0.03	3.08 ± 0.05	3.18 ± 0.05	3.08 ± 0.07
2	3.13 ± 0.06	3.08 ± 0.04	2.98 ± 0.05	3.11 ± 0.04	2.89 ± 0.04
3	2.97 ± 0.05	2.90 ± 0.04	2.86 ± 0.06	2.93 ± 0.06	2.75 ± 0.07
4	2.81 ± 0.03	2.68 ± 0.06	2.68 ± 0.04	2.76 ± 0.07	2.63 ± 0.06
5	2.72 ± 0.06	2.56 ± 0.06	2.53 ± 0.06	2.65 ± 0.04	2.48 ± 0.07
6	2.70 ± 0.06	2.55 ± 0.05	2.49 ± 0.07	2.63 ± 0.07	2.43 ± 0.07

pH of the exhausted media was measured starting from diauxic shift, Day 0. Data presented are the mean values of three biological replicates. Standard deviations are indicated.

2.4.2 Inhibition of ethanol metabolism increases CLS

During chronological aging, after the diauxic shift, ethanol which is the main by-product of glucose fermentation, is metabolised by virtually the same pathway as acetate. In fact, after its oxidation to acetaldehyde by alcohol dehydrogenase 2 (Adh2), it is converted to acetate. Subsequently, acetate is activated into acetyl-CoA which is used to fuel the glyoxylate and TCA cycles (Figure 2.2(a)) [42, 43]. Consequently, we wondered whether blocking the main pathway for acetate production might influence the chronological survival of wt cells in their exhausted medium. To this end, after the diauxic shift when cells began to utilize the excreted ethanol, pyrazole which is an irreversible inhibitor of Adh2 [44] was added to the culture medium and CLS monitored. As depicted in Figure 2.2(b), pyrazole treatment led to CLS extension. A similar salutary effect took place also when pyrazole was added to the culture medium of postdiauxic *icl1*Δ cells (Figure 2.2(c)). *ICL1* encodes isocitrate lyase (Icl1), which is one of the unique enzymes

of the glyoxylate cycle. During growth on C2 compounds, this cycle plays an essential role for anaplerosis of oxaloacetate which is the substrate of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Pck1) (Figure 2.2(a)) [43]. In the context of a CLS standard experiment, *ICL1* deletion results in a short-lived phenotype and impairment in acetate utilization [26]. Furthermore, pyrazole treatments led to a very slight acidification in the expired media of the wt and *icl1*Δ cultures (Table 2.1) indicating that the extracellular acidic pH alone is not sufficient to chronologically age yeast cells. Since we had already observed that pyrazole was able to abrogate the shortening effect of ethanol on CLS extension following extreme CR such as incubation in water [26], this confirms that some aspects of ethanol metabolism and not its mere presence (it enters the cells by passive diffusion) negatively affect CLS. We next performed some media-swap experiments between wt and *icl1*Δ cultures. Both strains were grown in minimal medium, and, at Day 1 after the diauxic shift, cultures were centrifuged and media were exchanged. The *icl1*Δ preconditioned medium, which contained more ethanol and acetic acid compared with the wt preconditioned one (Figures 2.2(d) and 2.2(e)) shortened the CLS of wt cells (Figure 2.2(b)). This detrimental effect on wt viability was abolished upon pyrazole addition, and CLS increased to the same extent as that of chronologically aging wt cells in their original medium in the presence of pyrazole (Figure 2.2(b)). Moreover, the wt preconditioned medium extended the CLS of the short-lived *icl1*Δ mutant (Figure 2.2(c)). Inhibition of ethanol oxidation by pyrazole further extended the CLS of the mutant which resulted, also in this case, similar to that of the chronologically aging mutant in its original medium supplemented with the Adh2 inhibitor (Figure 2.2(c)). Together these findings may point to proaging signaling effects of the metabolic pathways involved in the utilization of ethanol/acetate as carbon and energy source(s) by chronologically aging cells. This is consistent with the proposed role for acetic acid as a physiological trigger of growth signaling pathways which by promoting entry into S phase in unfavorable conditions would lead, among other effects, to replication stress in chronologically aging cells [45].

A DNA replication stress would negatively affect CLS [32, 46], and in this context experimental manipulations inducing such a stress have been recently shown to determine the loss of the reproductive capacity of chronologically aging cells [47]. Moreover, replication stress promotes apoptosis [48, 49]: a highly regulated cellular "suicide" program which is also activated during chronological aging [50]. In addition, acetic acid represents a compound which triggers apoptosis in the presence of glucose [51-53] and in glucose-derepressed *ach1*Δ cells [29]. Ach1 is an enzyme involved in acetate metabolism, and its lack decreases CLS [29, 54]. Consequently, stimulation of growth induced by acetic acid after the diauxic shift in the lack of favorable conditions required for cell cycle progression would ultimately cause apoptosis.

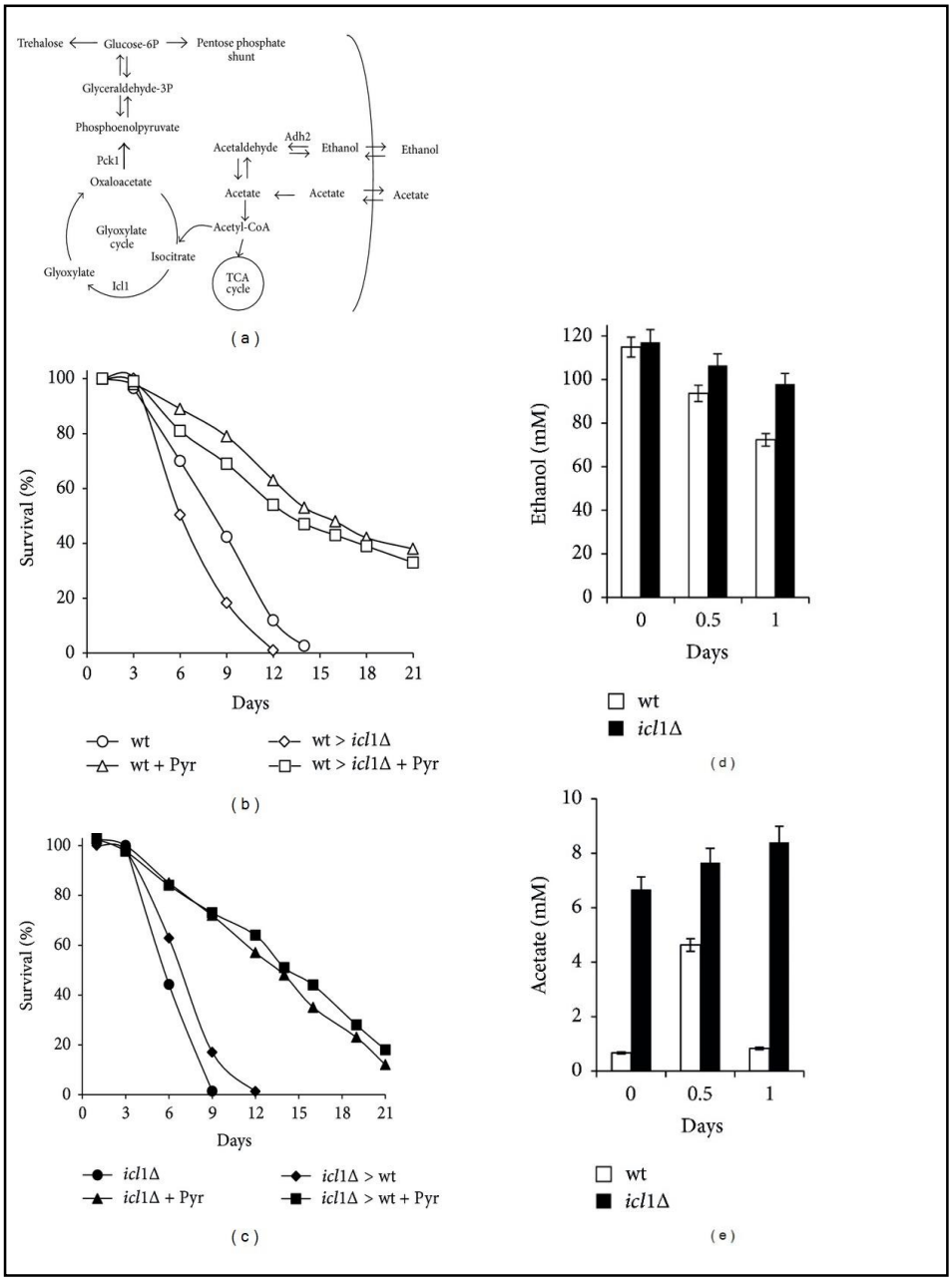

Fig. 2.2. Pyrazole prevents the CLS shortening effect of ethanol. (a) Scheme of metabolic pathways allowing ethanol and acetate utilization. Only relevant reactions are shown. Adh2: alcohol dehydrogenase 2, Icl1: isocitrate lyase 1, Pck1: phosphoenolpyruvate carboxykinase 1. At Day 1 after the diauxic shift, pyrazole (50 mM) was added to the expired media of wt (b) and *icl1Δ* mutant (c) cells. In parallel, aliquots of cells were harvested and subjected to cell-free media swap with or without pyrazole. At every time-point, viability was measured as in Figure 2.1(c). One representative experiment is shown. Extracellular ethanol (d) and acetate (e) concentrations determined in the wt and *icl1Δ* cultures at Day 1. Day 0, diauxic shift. Standard deviations are indicated.

2.4.3 Physiological amount of acetic acid reduces CLS

Next, we evaluated whether the physiological amount of acetic acid accumulated as a by-product of glucose fermentation could influence the chronological survival of yeast cells associated with their transfer to water, which is the extreme condition of CR known to extend CLS [25]. Therefore, we monitored the CLS of wt cells that, after the diauxic shift, were switched from expired medium to water supplemented with the amount of acetic acid (5 mM) we had detected in the expired medium (Figure 2.1(b) and [26, 29]). Treatments were performed in water whose pH was adjusted to 3.2 (the pH of the expired medium we measured) and in water buffered to pH 5.6. In the former condition the uptake of acetate is facilitated compared with that at pH 5.6 where the amount of the acetate anion considerably increases. As shown in Figure 2.3(a), the addition of 5 mM acetic acid to low pH water reduced CLS, but to a lesser extent than that elicited by ethanol [25, 26] which also in this case was supplied in amount comparable with that found in the expired medium. It is noteworthy that the addition of these C2 compounds together prevented CLS extension associated with transfer to low pH water resulting in a CLS similar to that of chronologically aging cells in their exhausted medium (Figure 2.3(a)). This suggests that it is a combined proaging effect of both metabolites which influences the CLS. Buffering water to pH 5.6 did not result in a CLS substantially different from that observed at pH 3.2 while the negative effect on chronological survival linked to the presence of acetic acid, ethanol, or both these compounds together was reduced (Figure 2.3(b)). Thus, buffering the extracellular medium alone is not sufficient to induce the fully extension of CLS observed in water, in line with data showing that an acidic environment alone is not sufficient to suppress the CLS extension associated with a CR regimen of growth which reduces acetic acid production [27, 29]. This further confirms that acidification accelerates chronological aging by influencing acetic/acetate equilibrium and consequently acetate uptake.

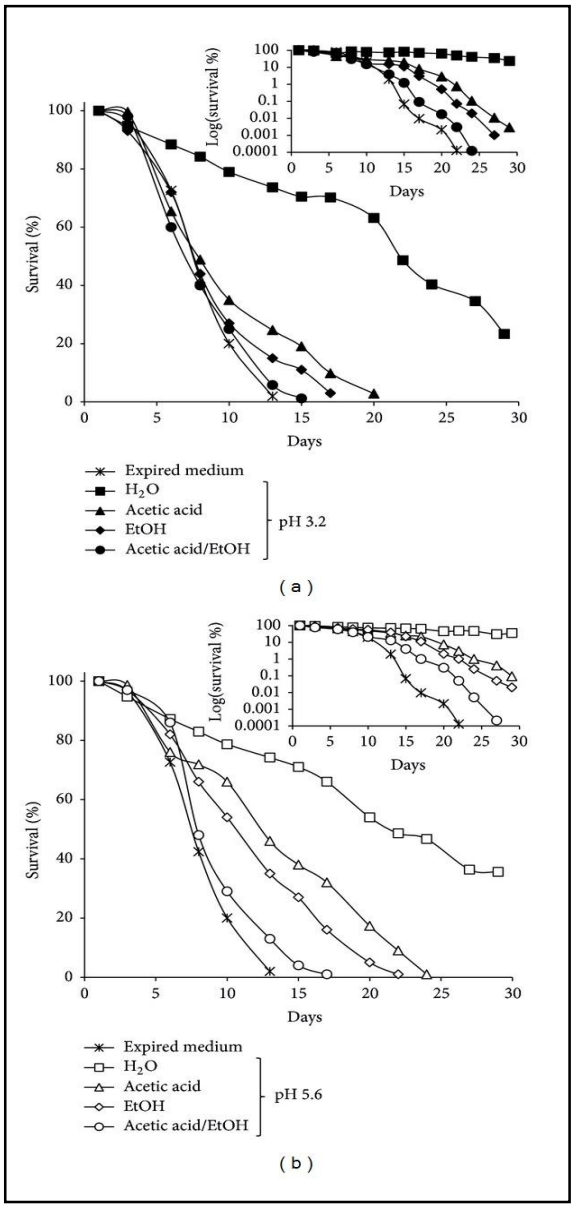

Fig. 2.3. The glyoxylate-requiring gluconeogenesis plays a positive role in extreme life-span extension. At Day 1 after the diauxic shift, wt cells were switched to water adjusted to pH 3.2 (a) and to pH 5.6 (b) and challenged with ethanol (6 g/L), acetic acid (5 mM), or both. Every 48 h, cultures were resuspended in fresh water, and each time ethanol and acetic acid were added when indicated. At every time-point, viability was measured. Survival of cells in their expired medium was also monitored as control. Insets: CLS plotted on a log scale. One representative experiment is shown. (c) CLS of wt, *icl1*Δ, *pck1*Δ, and *sir2*Δ cells switched to pH 3.2 water at Day 1 after the diauxic shift. (d) In parallel, the indicated cultures were challenged with 5 mM acetic acid as in (a). Survival of *icl1*Δ cells in their exhausted medium was also monitored. One representative experiment is shown.

In the chronological aging paradigm, a proaging role is played by Sir2 which has as nonchromatin substrate the Pck1 enzyme. *SIR2* inactivation increases acetylated Pck1 in concert with increased enzymatic activity [26, 28]. Since this correlates with an enhanced glyoxylate/gluconeogenic flux and with a more efficient acetate utilization [26], we analyzed whether the addition of 5 mM acetic acid could influence the CLS of *sir2*Δ cells that, after the diauxic shift, were incubated in low pH water. In parallel, the same analysis was performed for the *icl1*Δ mutant. As shown in Figure 2.3(c), the effect produced by the single *SIR*2 and *ICL1*deletions on the CLS in water was the opposite. In fact, lack of Sir2 significantly extended the CLS compared with that of wt cells in agreement with [25-28] while lack of Icl1 reduced it. Interestingly, a similar decrease in cell survival has been observed following *PCK1* deletion ([28] and Figure 2.3(c)). Moreover, acetic acid-back *sir2*Δ cultures lived longer than acetic acid-back wt ones (Figure 2.3(d)). On the contrary, chronological survival of *icl1*Δ cells was affected dramatically by the same amount of acetic acid (Figure 2.3(d)), indicating that acetic acid, at this concentration, becomes extremely toxic for cells with an impaired glyoxylate cycle activity. Taken together these data suggest that the glyoxylate-requiring gluconeogenesis and the cell ability to metabolize acetate play positive roles in the CLS extension linked to extreme CR.

2.4.4 Ethanol reduces glyoxylate/gluconeogenesis and enhances respiration of cells in extreme CR

Starting from the aforementioned results, for the purpose of investigating the connection between the glyoxylate-requiring gluconeogenesis and chronological longevity we measured the enzymatic activity of Pck1 and Icl1 in chronologically aging wt cells in their expired medium or transferred to water. In parallel, we also examined cellular respiration. In fact, it is well known that in the former experimental condition, when glucose is depleted, cells consume the earlier produced ethanol/acetate via gluconeogenesis (Figures 2.4(a) and 2.4(b)), and

concomitantly they increase their respiration (Figure 2.4(c)). In the extreme condition of CR, once cells were switched to water, the levels of Icl1 and Pck1 enzymatic activities increased and remained higher than those detected during aging in the expired medium (Figures 2.4(a) and 2.4(b)). In addition, they barely respired (Figure 2.4(c)). It is noteworthy that when these cells were challenged with ethanol, Icl1 and Pck1 enzymatic activities were reduced (Figures 2.4(a) and 2.4(b)), and the cellular respiration increased (Figure 2.4(c)). Similar results (with reduction and increase to a lesser extent) were obtained when acetate substituted for ethanol (data not shown) indicating that both C2 compounds are metabolised by the CR cells. Since the ability to respire relies on functional mitochondria and a direct correlation between reduced CLS and dysfunctional mitochondria has been reported [6, 55], we decided to analyze the index of respiratory competence (IRC). This index measures the percentage of viable cells which are competent to respire [36]. At Day 1, the IRC was about 100% for chronologically aging cells in the exhausted medium, in water, and in water/ethanol (Figure 2.4(d)) indicating that all the cells are respiration competent. Starting from Day 12, this value began to decrease progressively for the cells in the exhausted medium and for those in water/ethanol reaching about 20% and 50%, respectively by Day 21 which is indicative of a time-dependent loss of mitochondrial functionality. On the contrary, in the extreme CR condition the IRC was still about 80% (Figure 2.4(d)) indicating, on the one hand, that the low level of respiration is not due to impairment in mitochondrial functionality and, on the other hand, that resuspension in water exerts a protective role on mitochondria which become more prone to damage following ethanol addition.

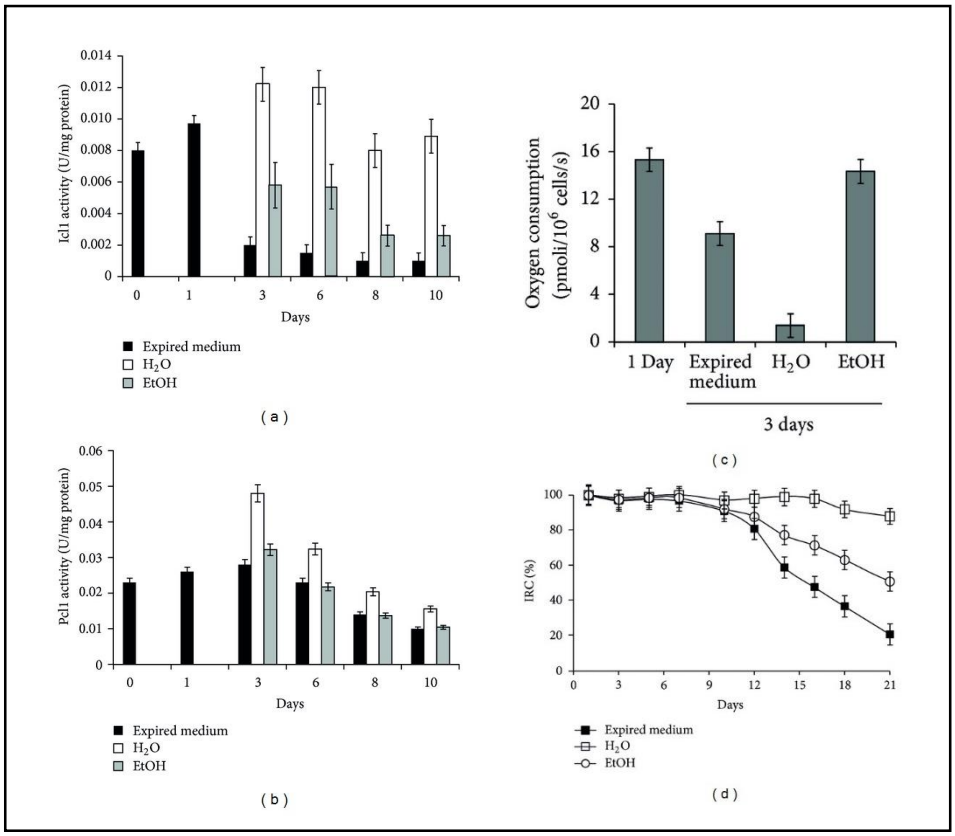

Fig. 2.4. Ethanol affects the glyoxylate-requiring gluconeogenesis and the respiration of cells in extreme CR. At the indicated time-points, Icl1 (a) and Pck1 (b) enzymatic activities were measured in wt cells during chronological aging in their expired medium and after the switch to water or water/ethanol as in Figure 2.3. Day 0, diauxic shift. (c) Cellular respiration of the same cells in the indicated experimental conditions. Error bars are the standard deviation of three replicates. (d) Chronologically aging wt cultures at the indicated time-points were serially diluted, plated onto YEPD and YEPG plates, and the index of respiratory competence (IRC) was determined. Standard deviations of three independent experiments are indicated.

To this effect, a causative role in inducing mitochondrial dysfunction is played by reactive oxygen species (ROS), and, at the same time, mitochondrial dysfunction leads to increased ROS formation [56]. Moreover, mitochondria are the major intracellular source of potentially harmful ROS such as the superoxide anion. This radical can directly induce oxidative damage or can be converted to other ROS which, in turn, induce aging-associated damage [57]. Chronological aging in the absence of any extracellular nutrient, namely, water, which correlates with an increased CLS, implies that cells have to establish a survival-based metabolism

where energy is conserved by shutting down expensive growth-promoting pathways and concomitantly stress resistance and access to alternate energy stores are provided. In addition, cells have to limit damage to cellular components. In this context, reducing respiration may be beneficial since, although highly efficient in producing ATP, the oxidative metabolism produces the superoxide anion which is generated in the electron transport chain.

The other feature of cells in extreme CR discovered was an increase in the enzymatic activities of Pck1 (the main flux-controlling step of gluconeogenesis) and Icl1. This feature, combined with the fact that loss of their function blocks CLS extension, further supports the notion of a positive crucial role of glyoxylate/gluconeogenesis in the control of this form of longevity [28]. Increasing glyoxylate/gluconeogenesis may be advantageous to improve survivability during chronological aging in water since gluconeogenesis switches the direction of metabolite flow towards the biosynthetic precursor, glucose-6-phosphate, which is also needed for glucose stores (Figure 2.2(a)). In particular, trehalose has been proposed as the carbohydrate of choice for surviving starvation and upon cell cycle reentry from quiescence [58]. Moreover, hexoses generated from gluconeogenesis can be used via the pentose phosphate pathway generating additional NADPH which is essential for the activity of antioxidant defenses [59]. On the other hand, with regard to the glyoxylate pathway, it is important to recall that it does not only have the function of fueling gluconeogenesis but can contribute to NADH production [60].

This metabolic scenario may give some explanation why the CLS extension in water is intensified following *SIR2* inactivation [25]. In fact, the increase in the acetylated active form of Pck1 due to the lack of the Sir2-targeted deacetylation enhancing the glyoxylate/gluconeogenic flux [26] might further favour the establishment of a long-term quiescent program. On the contrary, the oxidative metabolism of ethanol/acetate via the TCA and mitochondrial electron transport chain increasing respiration may generate harmful ROS which impair mitochondrial functionality. This, in concert with induced growth signals in the

lack of favorable conditions required for cell cycle progression [32], most likely negatively affects cell survival. Bearing in mind that the relationship between respiration, ROS, and CLS is very complex, how can the proaging effect induced by ethanol in nutrient starvation conditions fit with the ability of pregrowth on the same respiratory carbon/energy source to extend CLS [61, 62]? In fact, in addition to the role played by a mitochondrial respiratory threshold in regulating CLS [63], mitochondrial respiration affects chronological survival through ROS generation. They can be either deleterious or beneficial depending on the biological context/phases of the yeast cell cycle in which they are produced [57]. Although mitochondrial ROS have been associated with damaging effects which promote and/or accelerate chronological aging [64], they also function as signaling molecules with hormetic effects on longevity [65, 66]. In particular, elevating mitochondrial ROS during yeast exponential growth elicits an adaptive response which promotes CLS extension [67]. Similarly, the effects on CLS observed following growth on ethanol [61, 62] are also in line with an adaptive mitochondrial longevity signal generated during active growth which contributes to establishment of a better quiescent program.

2.5 References

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3

Influence of Nicotinamide, Resveratrol and Quercetin on CLS

3.1 Introduction

In aging research, many efforts are currently focused on the identification/improvement of interventions based on dietary restriction or supplementations that can represent a safe and a feasible strategy to achieve the improvement of healthy aging.

Until just recently, analysis of food has been limited to the flavor of food (sensory taste and texture) and its nutritional value (composition of carbohydrates, fats, proteins, vitamins and minerals). However, there is growing evidence that other components of food can play an integral role linked to health. Concomitantly, also consumers are increasingly interested in the health benefits of foods and have begun to consider food not only for their basic nutritional benefits, but also their use in disease prevention and health [1]. At the same time, many opportunities for the development of novel dietary products have been created. With all new fields of study come new terms. "Nutraceuticals" and "functional foods" that are two new terms used to describe health-promoting foods or their extracted components. Although debate continues regarding the exact meaning of these definitions, nutraceuticals can be considered as healthful products that are formulated and taken in dosage form (capsules or tablets). Functional foods, on the other hand, can be defined as products that are consumed as foods and not in dosage form [2]. Nutraceuticals assists in the prevention and/or treatment of disease(s) other than deficiency conditions [3]. The food sources used as nutraceuticals are all natural and can be categorized as dietary fibres, probiotics, prebiotics, polyunsaturated fatty acids, antioxidant vitamins, polyphenols, and spices [4].

In particular, among the most common nutraceuticals, the water-soluble vitamin B3 and its major forms, nicotinamide (NAM) and nicotinic acid (NA), collectively called niacin, have received great interest since are precursors of nicotinamide adenine dinucleotide, NAD^+, and, as such, plays a role in longevity regulation [5,6]. NAM is the amide form of vitamin B3 and can be synthetized directly by the organism or can be supplied as dietary source [7]. NA, although also present from animal sources, is the principal form of niacin in dietary plant sources that

is rapidly absorbed through the gastrointestinal epithelium [8]. NAM is subsequently generated through the conversion of NA in the liver or through the hydrolysis of NAD^+. Then NAM functions as the precursor for NAD^+ [9,10] and also is essential for the synthesis of nicotinamide adenine dinucleotide phosphate ($NADP^+$) [11]. Nicotinamide and its derivative are forms of vitamin B3 (niacin) used interchangeably to self-treat a number of conditions including anxiety and osteoarthritis. No less important is the evidence of a link between NAM metabolism and stress resistance in mammals [12].

Other substances that potentially act as nutraceuticals are Resveratrol (RESV) and Quercetin (QUER). They are both polyphenols that are relatively abundant in foods and beverages and display potential therapeutic and disease-preventing usefulness [13-16]. First isolated from the roots of white hellebore (*Veratum grandiflorum O. Loes*) in 1940 [17], RESV (3,5,4′-trihydroxystilbene) is a phytoalexin found in some fruits, such as blueberries and blackberries, and in peanuts [18,19]. However, red wine is the main source of RESV in the Mediterranean diet. RESV content in red wine comes from grapes. In particular, the richest sources are the skin, seeds, petioles, and woody parts [20]. Initially, much of the interest in this compound came from the observation that in north France there was a high intake of saturated fat diet associated with low mortality from coronary heart disease compared to other countries with the same high saturated fat intake. The high consumption of red wine was proposed as a possible explanation of the so-called French paradox, being the cardio-protective effects of red wine attributable to RESV [21]. Besides possessing the intrinsic capacity of unpaired electrons uptake like most other polyphenols, RESV was shown to exert an antioxidant activity in isolated rat brain mitochondria also through the inhibition of complex III of the mitochondrial electron transport chain, one of the main source of ROS [22]. Moreover, it is thought to induce the expression of antioxidant enzymes and other stress-related targets in the cell, contributing to an overall reduction in oxidative stress [23]. Meanwhile, also anti-cancer [24] and anti-inflammatory properties were attributed to RESV [25,26].

Great interest is currently centered also on the flavonoid QUER (3,3′,4′,5,7-pentahydroxyflavone), one of the most often studied dietary polyphenol, ubiquitously present in various vegetables, such as onions, broccoli, tomatoes, as well as in tea and red wine [27]. QUER is considered to be a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions [28]. In particular, as accounted by some research teams, QUER is the most potent scavenger of ROS, including the superoxide anion O_2^- [29]. By scavenging free radicals, it can also reduce inflammation [30]. QUER has been tested in mammalian models and also to some extent in humans [31] Also noteworthy are both its anti-proliferative activity and extensive autophagy and cell death-inducing effect, being this latter mediated by the inhibition of mTOR signalling, in various cancer cell lines [32].

Strikingly, NAM, RESV and QUER are all *SIRT1*-modulating compounds, with NAM having an inhibitory action, whereas RESV and QUER exerting a stimulatory effect on its activity [33]. It has been shown that NAM inhibits human SIRT1 activity, both *in vitro* and *in vivo* [5]. Howitz and colleagues reported RESV, instead, as the most potent of the natural SIRT1 activators (STACs) *in vitro*, lowering the Km value for acetylated substrate and to a much lesser extent that of NAD^+ [33]. Successively, RESV was shown to induced SIRT1 activity *in vivo*, in particular in mice [34]. Also QUER have been shown to activate SIRT1 either directly or indirectly *in vitro* [35] and *in vivo*, specifically in mice [36].

SIRT1 is the closest mammalian Sirtuin to yeast Sir2 in terms of sequence. Sir2 is the founding member of Sirtuins, the evolutionary conserved family of NAD^+-dependent deacetylases. Deacetylation by Sirtuins involves the consumption of NAD^+ and acetylated protein substrate to produce NAM, 2 O'-acetyl-adenosine diphosphate-ribose (O-AcADPR), and deacetylated substrate. Thus, NAM is a by-product generated during Sir2-mediated deacetylation, which is also a non-competitive inhibitor. NAM supplementation reduces yeast RLS, whereas RESV extended it [17]. QUER effect on RLS have still to be elucidated.

The roles of Sir2 in stress resistance and longevity vary depending on the growth phase: in contrast to its anti-aging role in RLS [37], it plays a pro-aging role in CLS [38]. Intriguingly, the significant CLS elongation following loss of Sir2 also correlates with a fast ethanol depletion and a less acetate accumulation, the main byproducts of glucose fermentation, and an increased intracellular accumulation of trehalose [38,39], the carbohydrate of choice for allowing survival of starvation and a quick recovery from quiescence [40]. This is ascribed to the lack of Sir2-targeted deacetylation of its non-chromatin substrate phosphoenolpyruvate carboxykinase (Pck1), which catalyzes the rate-limiting step of gluconeogenesis, and the subsequent increase of its acetylated active form [39]. Pushing cellular metabolism towards glyoxylate/gluconeogenesis and trehalose stores abrogates the CLS-shortening effects associated to ethanol/acetate catabolism via TCA cycle and respiration, which is likely to shorten cell survival through ROS generation and the consequent impaired mitochondrial functionality [41].

In this Chapter, we tested in vivo the effects of NAM, RESV and QUER with putative health beneficial properties on CLS and determined whether these effects are associated to Sir2.

3.2 Materials and methods

3.2.1. Yeast strains and growth conditions

All yeast strains used are listed in Table 3.1. All deletion strains were generated by PCR-based methods. Standard methods were used for DNA manipulation and yeast transformation.

The pmito-roGFP1 plasmid carrying the roGFP1 fused to the Mitochondrial-Targeting Signal Sequence (MTSS) and under the GPD promoter [42] was kindly provided by Dr. Liza A. Pon.

Table 3.1. Strains used in this study.

Strains	Relevant genotype	Source
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	P.P. Slominski
YVU22	W303-1A *sir2Δ::HIS3*	[43]
YVU83	W303-1A *pck1Δ::KlLEU2*	[44]
YVU92	W303-1A [pmito-roGFP:URA3]	This study
YVU93	W303-1A *sir2Δ::HIS3* [pmito-roGFP:URA3]	This study

Cells were grown in batch cultures at 30°C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/l) with 2% w/v glucose and the required supplements added in excess as described [45]. Cell growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser [46] and, in parallel, the extracellular concentrations of glucose and ethanol were measured in medium samples collected at different time-points in order to define the growth profile (exponential phase, diauxic shift, post-diauxic phase and stationary phase). Duplication times were obtained by linear regression of the cell number increase over time on a semi-logarithmic plot.

Treatments were performed at the diauxic shift (Day 0) by adding nicotinamide (NAM, Sigma) at the final concentrations of 1 and 5mM; Resveratrol (RESV, Sigma) at 10 and 100 μM and Quercetin (QUER, Sigma) at 26 and 300μM.

3.2.2. CLS determination.

Cell survival in expired medium was monitored by harvesting aliquots of cells starting with 72 h (Day 3, first age-point) after the diauxic shift (Day 0). CLS was measured according to [47] by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 3 was considered as 100% of survival.

3.2.3. Metabolite measurements and enzymatic assays.

At designated time-points, aliquots of the yeast cultures were centrifuged and both pellets (washed twice) and supernatants were frozen at -80 °C until used. Glucose, ethanol and acetic acid 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 in [48]. The released glucose was measured using the K-HKGLU kit.

Immediately after preparation of cell-free extracts, Pck1 and Icl1 activities were determined as previously reported in [49]. Total protein concentration was estimated using the BCA™ Protein Assay Kit (Pierce).

3.2.4. Respiration assays and fluorescence microscopy

The basal oxygen consumption of intact cells was measured at 30°C using a "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System, Hansatech Instruments, Norfolk, UK) as described in [50]. Data were recorded at sampling intervals of 1s (Oxygraph Plus software,

Hansatech Instruments, Norfolk, UK). Triethyltin bromide (TET, Sigma) at 37.5 mM was added to the oxygraph chamber and the decrease in oxygen concentration was followed online [51]. The basal oxygen consumption rate (J_R) and the non-phosphorylating oxygen consumption rate (J_{TET}) were determined from the slop of a plot of O_2 concentration against time, divided by the cellular concentration. The net respiration (netR) was calculated subtracting J_{TET} from J_R.

The Index of Respiratory Competence (IRC) was measured according to [52] by plating identical samples on YEPD plates and on rich medium of 3% glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.

ROS were detected with dihydroethidium (DHE, Sigma) according to [53]. The mitochondrial membrane potential and morphology were assessed by staining with the fluorescent dye $DiOC_6$ (Molecular Probes, Invitrogen), according to [54]; cells were also counterstained with propidium iodide to discriminate between live and dead cells.

A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350 F ccd camera was used. Digital images were acquired using FW4000 software (Leica).

3.2.5. Statistical analysis of data.

All values are presented as the mean of three independent experiments with the corresponding Standard Deviation (SD). Three technical replicates were analyzed in each independent experiment. Statistical significance was assessed by one-way ANOVA test. P value of ≤ 0.05 was considered statistically significant.

3.3 Results and conclusions

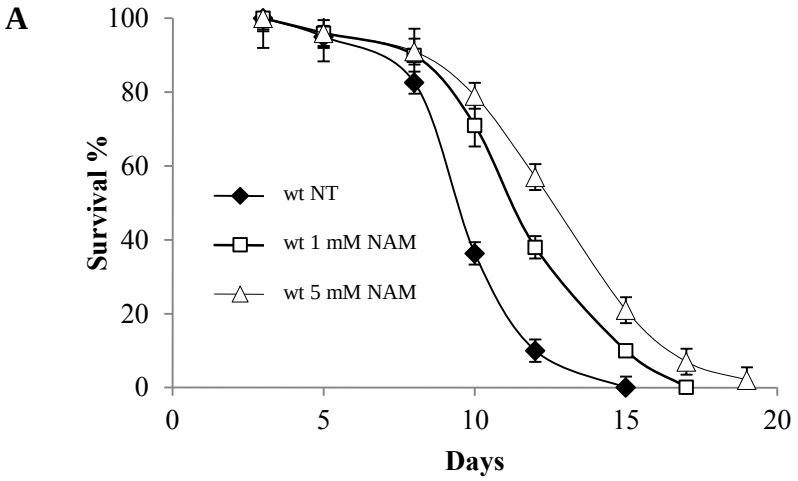
3.3.1. NAM, RESV and QUER supplementations have different effects on CLS

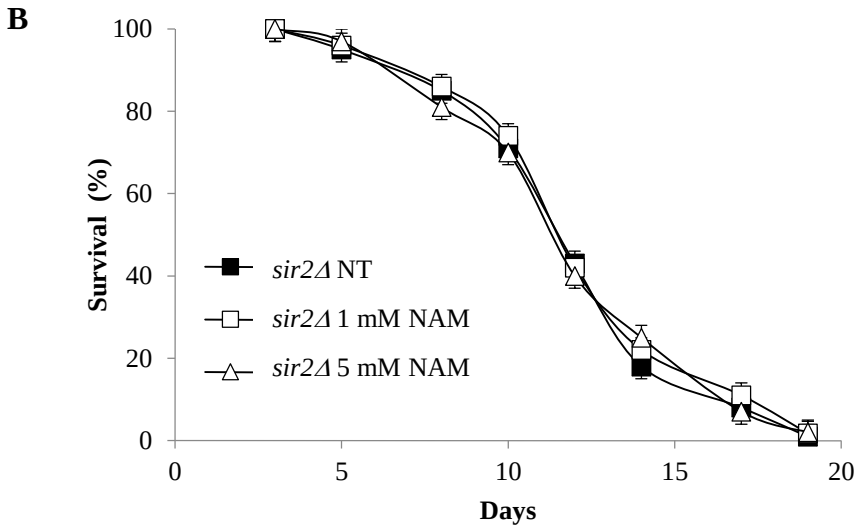
Initially, we investigated whether the influence of NAM, RESV and QUER supplementation could have any effects on CLS. All the treatments were performed at the diauxic shift in order to avoid the influence of these substances on RLS. Moreover, the experiments were performed on wild-type (wt) and *sir2Δ* cells, given the relationship between the 3 substances and the Sirtuin family. In fact, it is known that NAM is a strong non-competitive inhibitor of Sir2 activity *in vitro* and *in vivo* and decreases RLS [5]. NAM was added at 1 and 5 mM, being the latter concentration proven to inhibit Sir2 activity *in vivo* [5,55]. As shown in Fig. 3.1A and Table 1, NAM supplementation increased the CLS of wt in a dose-dependent manner, with the highest concentration tested being able to extend the CLS to the same extent as *SIR2* inactivation did (Fig. 3.1A-B and Table 1). No effect was observed in the *sir2Δ* mutant even when supplemented with 5 mM NAM (Fig. 3.1B and Table 1).

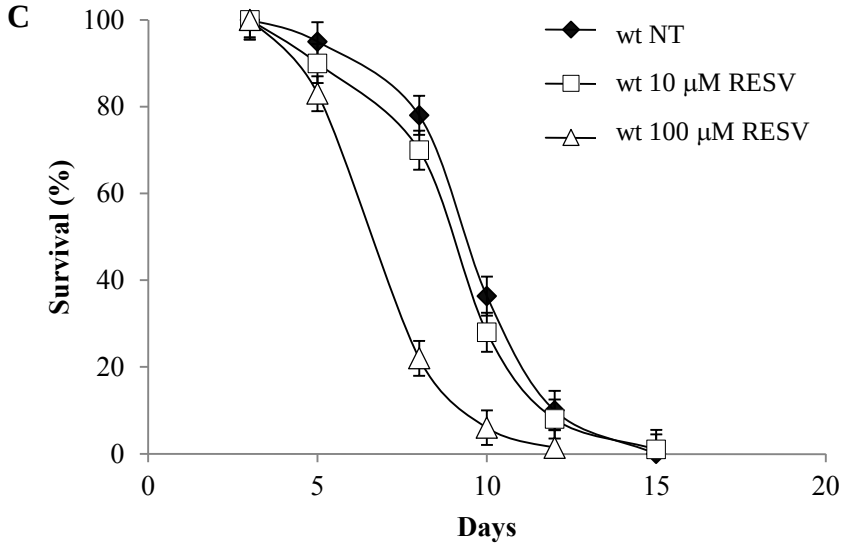
In contrast to NAM, RESV was shown to extend RLS [33,56], but whether it activates Sir2 *in vivo* remains a matter of debate. In fact, the strain used was the only case where overexpression of Sir2 did not increase RLS and no data on Sir2 activity on silencing was reported [57]. The concentrations tested were 10 and 100 μM: the former corresponds to that normally found in natural macerated red grape juice [58], whereas the higher was shown to extend RLS [33]. As shown in Fig. 3.1C, RESV had a negative effect on the cell viability over time only at the higher concentration tested. In particular, RESV supplementation decreased both the mean and maximum CLS of wt cells (Table 1) while no effect was observed in *sir2Δ* mutant (Fig. 3.1D and Table 1).

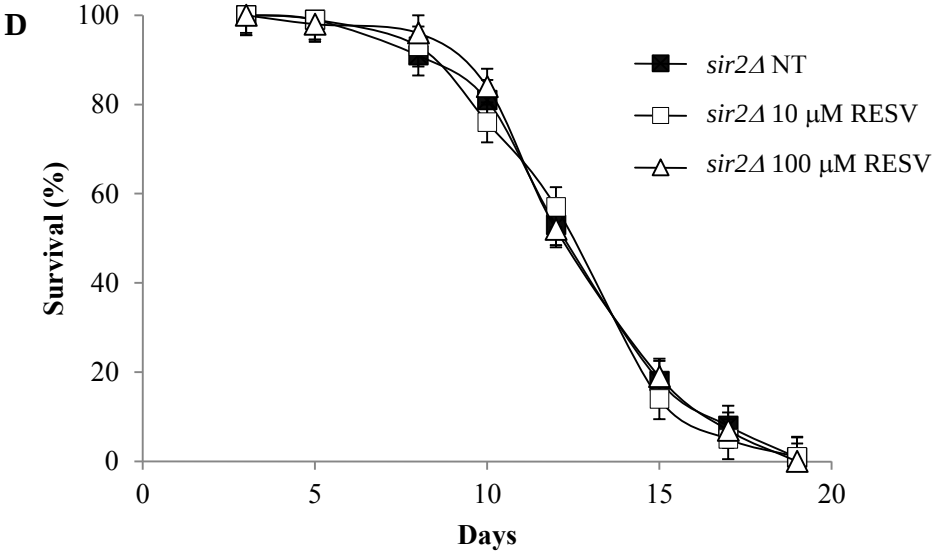
QUER treatments were performed at 26 μM, the concentration that naturally occurs in natural macerated red grape juice [58] and 300 μM. The latter already reported to increase oxidative stress resistance of yeast cells when supplied during the exponential phase [59]. The lower concentration resulted ineffective on CLS

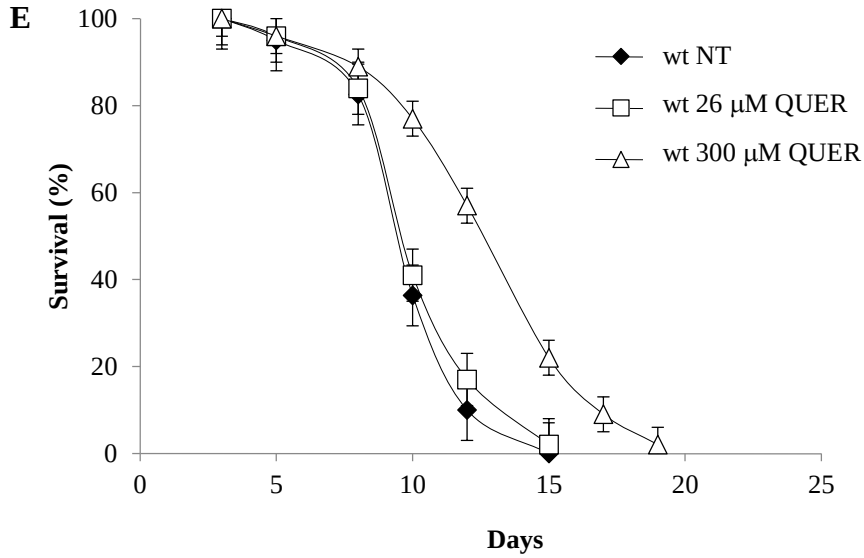
of wt, while 300 μM QUER extended both the mean and maximum CLS (Fig.3.1E and Table 1). Interestingly, 300 μM QUER treatment further extended the mean and maximum CLS also of the long-lived mutant *sir2Δ* (Fig.3.1 F and Table 1).






C
100
80
60
40
20
0
Survival (%)
0
5
10
15
20
Days
wt NT
wt 10 μM RESV
wt 100 μM RESV


D
100
80
60
40
20
0
Survival (%)
0
5
10
15
20
Days
sir2Δ NT
sir2Δ 10 μM RESV
sir2Δ 100 μM RESV



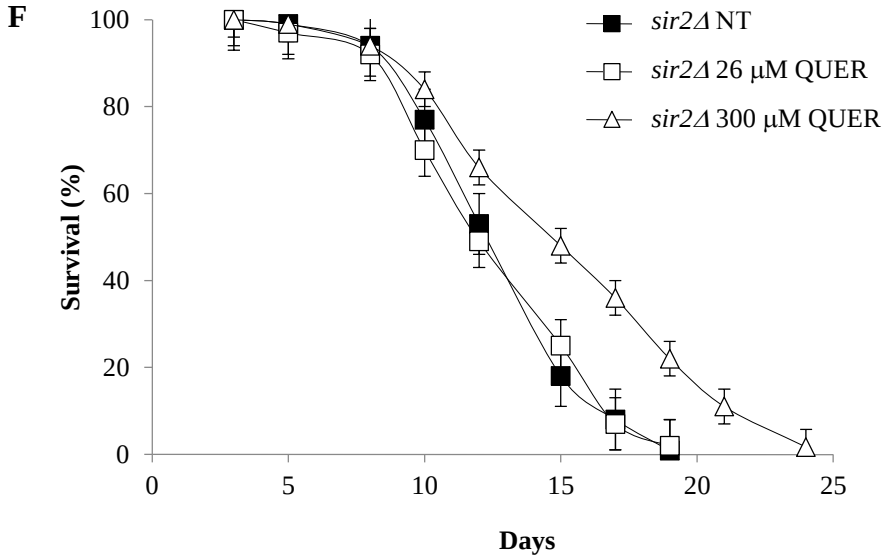
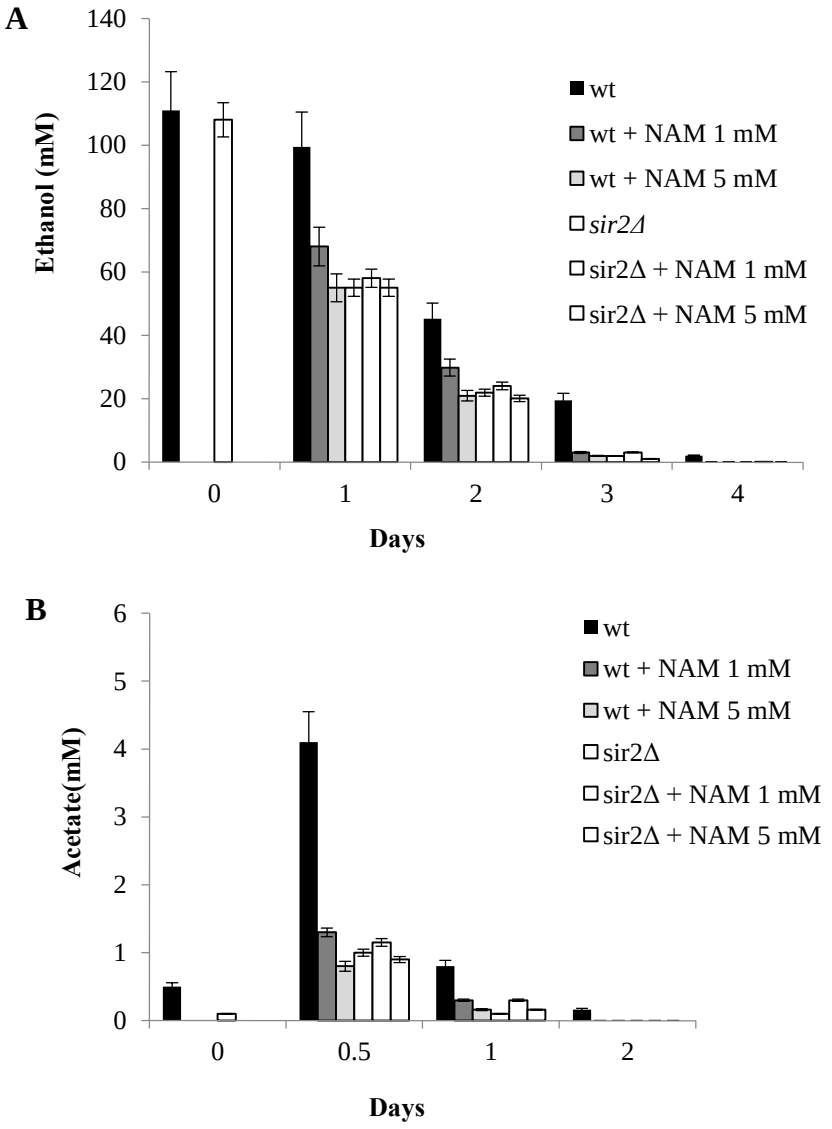


Fig. 3.1 Effects of Nicotinamide (NAM), Resveratrol (RESV) and Quercetin (QUER) on CLS. (A-C-D) Wild-type (wt) and *sir2Δ* (B-D-E) cells were grown in minimal medium/2% glucose and the required supplements in excess. At the diauxic shift (Day 0), NAM, RESV and QUER were added to the expired media at the indicated concentrations. At each time point, survival was determined by colony-forming capacity on YEPD plates. 72 h after the diauxic shift (Day 3) was considered the first age-point. In parallel, survival of cells in their expired medium without NAM, RESV and QUER was monitored. Data refer to mean values of three independent experiments. Standard deviations (SD) are indicated.

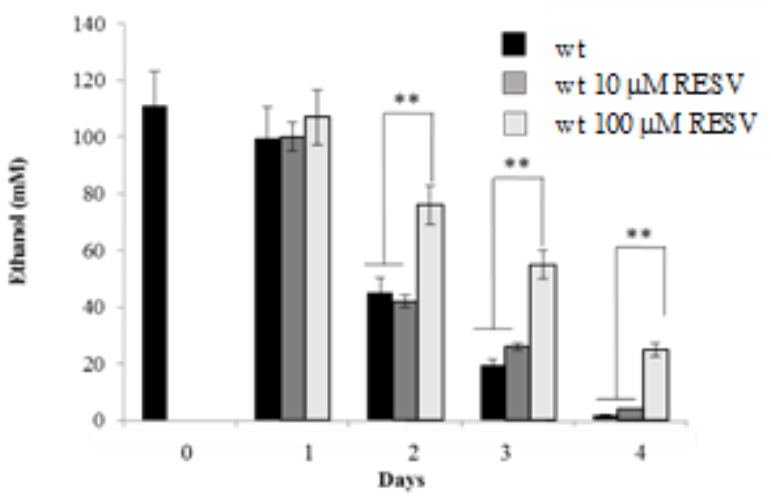
CLS	Mean (days)	Max (days)
wt NT	8,87 +/- 0,53	11,61 +/- 0,31
wt + 1 mM NAM	10,69 +/- 0,4	13,85 +/- 0,47
wt + 5 mM NAM	12,12 +/- 0,6	17,42 +/- 0,35
wt + 10 μM RESV	8, 71 +/- 0,47	11,05 +/- 0,34
wt + 100 μM RESV	5,83 +/- 0,37	8,87 +/- 0,71
wt + 26 μM QUER	9,1 +/- 0,67	11,78 +/- 0,42
wt + 300 μM QUER	13,39 +/- 0,46	18,96 +/- 0,37
***sir2Δ* NT**	12,37 +/- 0,42	18,29 +/- 0,47
***sir2Δ* + 1 mM NAM**	12,09 +/- 0,51	18,45 +/- 0,31
***sir2Δ* + 5 mM NAM**	12,42 +/- 0,26	18,52 +/- 0,21
***sir2Δ* + 10 μM RESV**	12,53 +/- 0,19	18,33 +/- 0,70
***sir2Δ* + 100 μM RESV**	12,37 +/- 0,22	18,26 +/- 0,53
***sir2Δ* + 26 μM QUER**	12,18 +/- 0,27	18,29 +/- 0,65
***sir2Δ* + 300μM QUER**	15,08 +/- 0,35	21,25 +/- 0,16

Table 1. Data refer to the time-points where stationary cultures survived 50% (mean CLS) and 10% (maximum CLS) of survived.

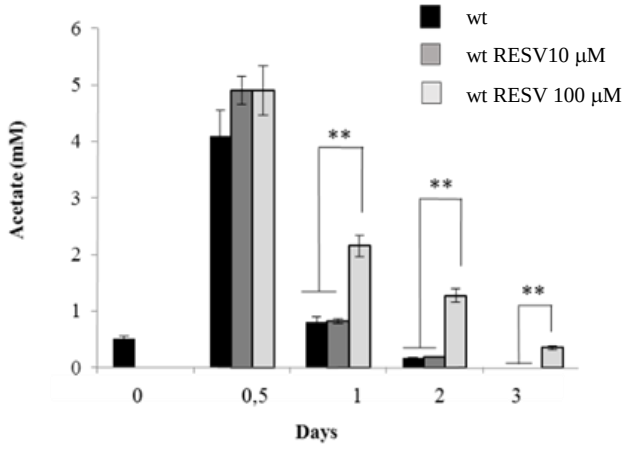
Since the long-lived phenotype of the *sir2Δ* mutant correlates with a faster ethanol depletion and an almost absent acetate accumulation [38,39], we determined the extracellular levels of these C2 compounds following the treatment with all the substances. NAM administration increased ethanol depletion and decreased the level of extracellular acetate in wt cultures (Fig. 3.2 A-B); whereas in *sir2Δ* mutant culture, either treated or not, the expected lower levels of the C2 compounds were measured [38,39] (Fig. 3.2 A-B). Also in this case, 5 mM NAM supplementation affected these parameters to the same extent as lack of Sir2 did (Fig. 3.2 A-B). An opposite behavior was exhibited by RESV in wt cultures, which delayed the disappearance of both the C2 compounds were detected (Fig. 3.1 C-D). No effects, instead, were observed on ethanol and acetate extracellular levels of *sir2Δ* mutant (Fig. 3.2 G-H). Following QUER administration, the extracellular levels of the C2 compounds detected were lower than the untreated culture both in wt (Fig. 3.2 E-F) but also in *sir2Δ* (Fig. 3.2 G-H).


A
Ethanol (mM)
140
120
100
80
60
40
20
0
0
1
2
3
4
Days
wt
wt + NAM 1 mM
wt + NAM 5 mM
sir2Δ
sir2Δ + NAM 1 mM
sir2Δ + NAM 5 mM
B
Acetate(mM)
6
5
4
3
2
1
0
0
0.5
1
2
Days
wt
wt + NAM 1 mM
wt + NAM 5 mM
sir2Δ
sir2Δ + NAM 1 mM
sir2Δ + NAM 5 mM

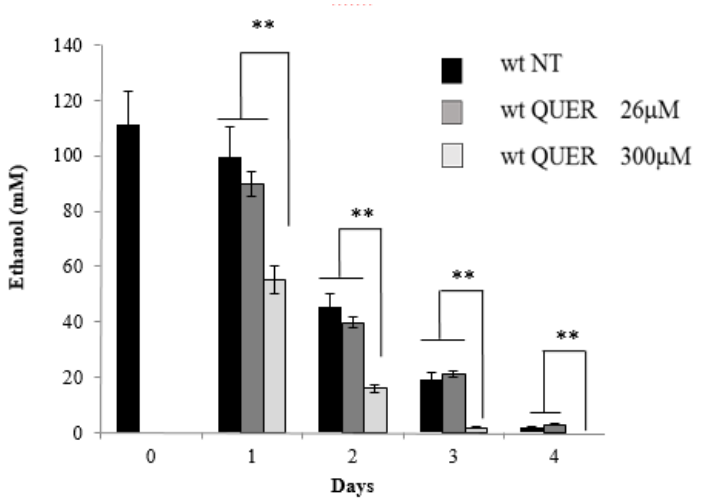
C


Ethanol (mM)
140
120
100
80
60
40
20
0
0
1
2
3
4
Days
wt
wt 10 µM RESV
wt 100 µM RESV
**
**
**

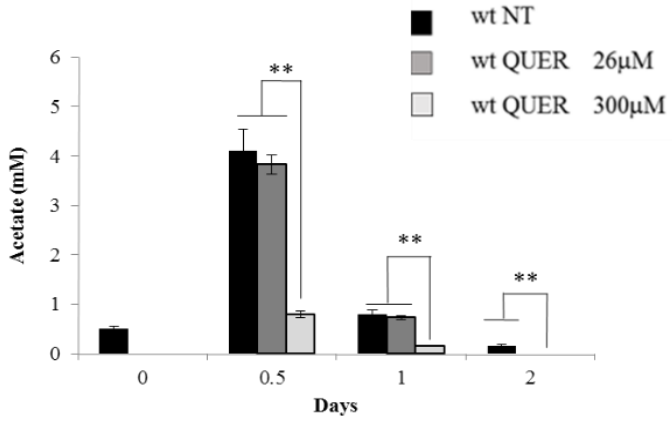
D


Acetate (mM)
6
5
4
3
2
1
0
0
0,5
1
2
3
Days
wt
wt RESV10 µM
wt RESV 100 µM
**
**
**

E


Ethanol (mM)
140
120
100
80
60
40
20
0
0
1
2
3
4
Days
**
**
**
**
wt NT
wt QUER 26µM
wt QUER 300µM

F


Acetate (mM)
6
5
4
3
2
1
0
0
0.5
1
2
Days
**
**
**
wt NT
wt QUER 26µM
wt QUER 300µM

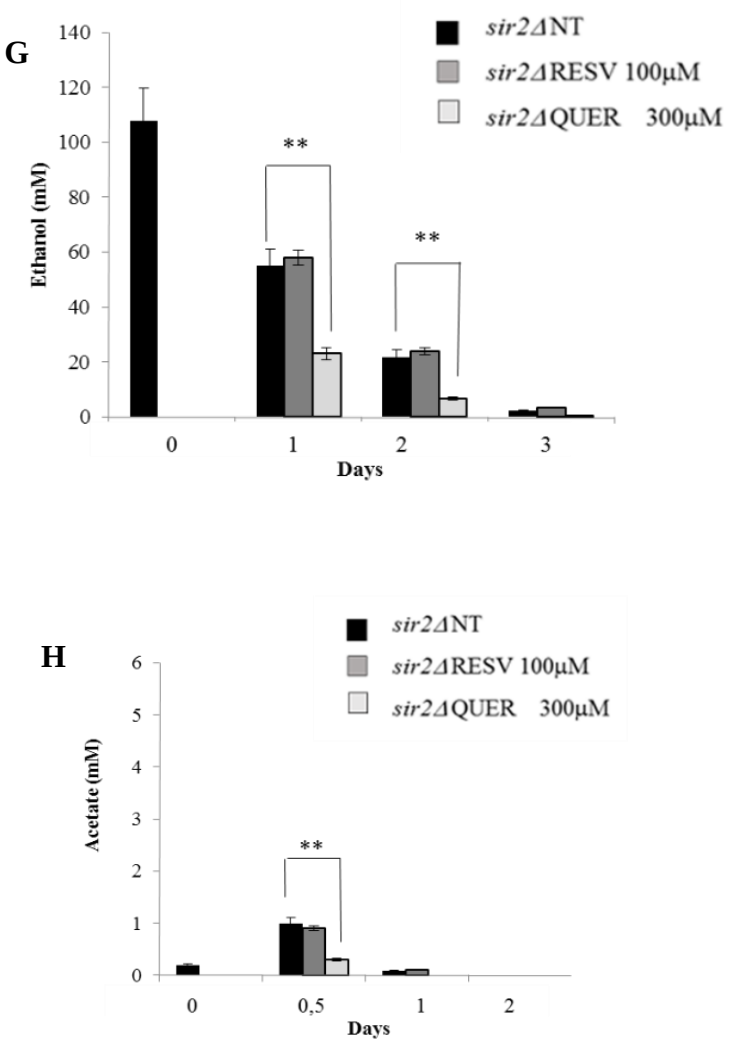
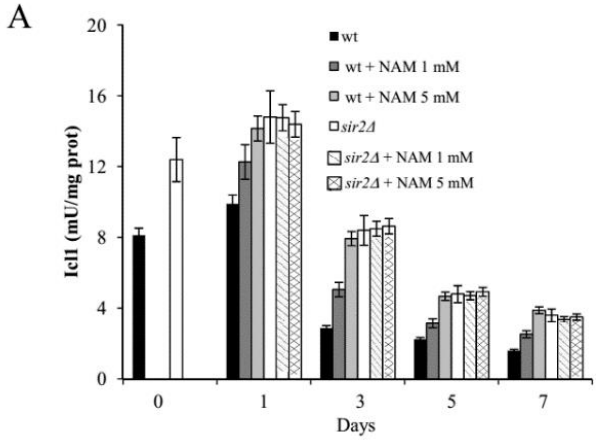
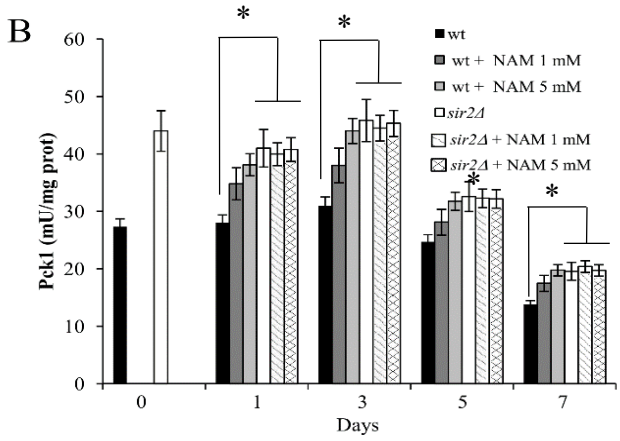


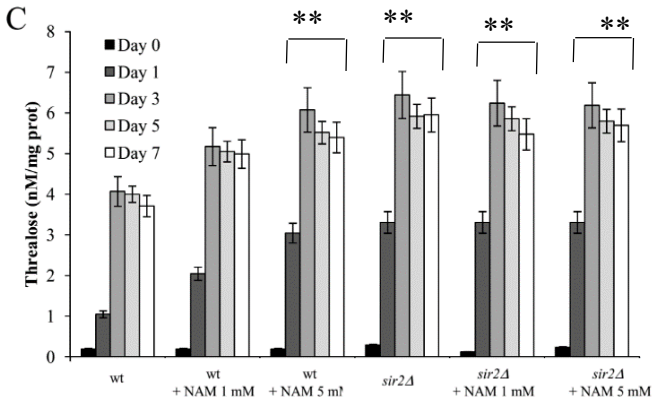
Fig. 3.2 Effects of NAM, RESV and QUER on the levels of extracellular C2 compounds. Bar chart of extracellular ethanol (A-C-E-G) and acetate (B-D-F-H) concentrations of wt and sir2□ of Fig. 3.1 were measured at the indicated time points after the diauxic shift (Day 0). Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by one-way ANOVA test is indicated (*P ≤ 0.05 and **P ≤ 0.01).

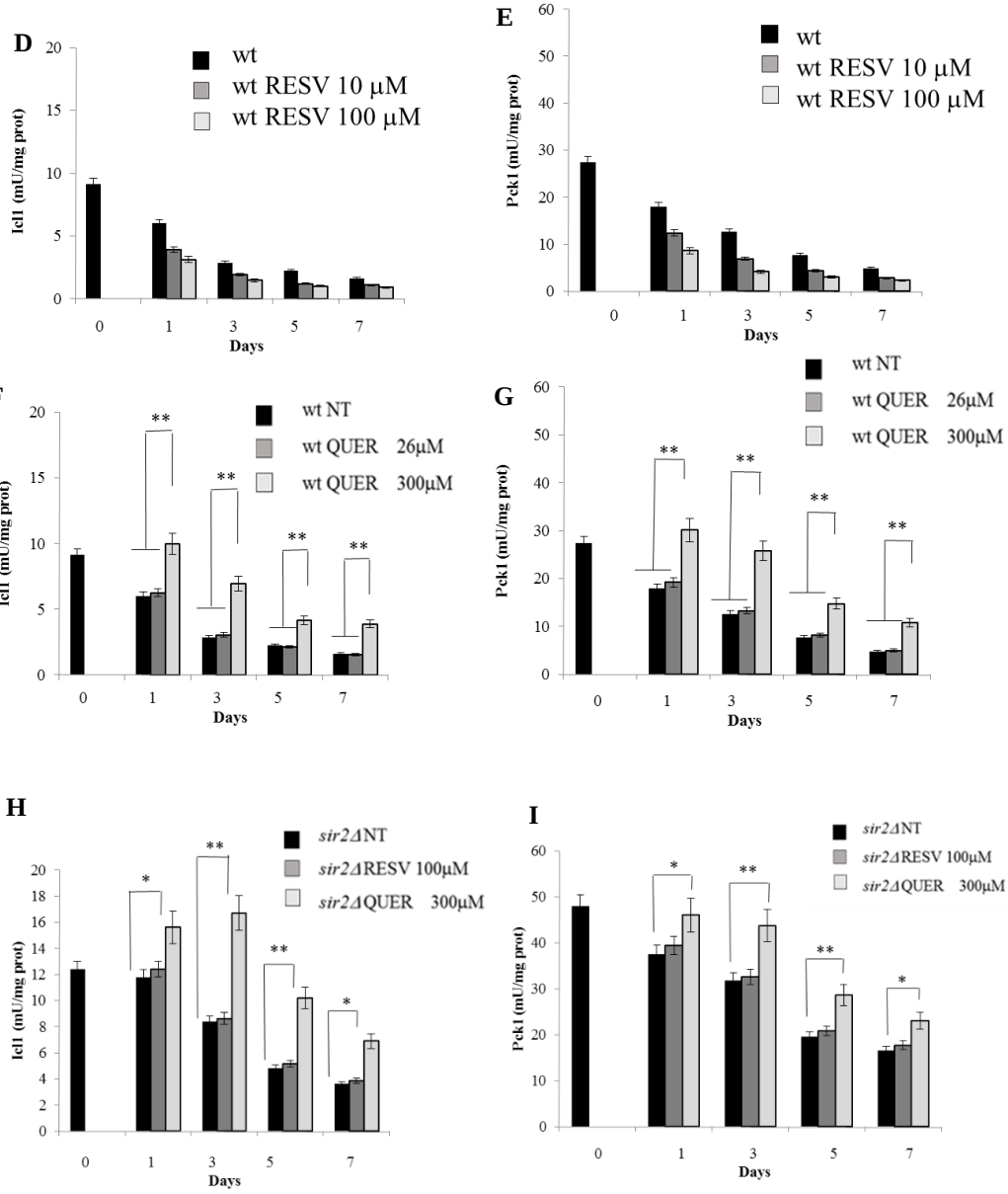
At the diauxic shift following glucose depletion, the catabolite repression "turns off" and yeast cells utilize ethanol and acetate, the main glucose fermentation by-products, as carbon/energy sources. Consequently, given the differences observed of these C2 compounds in the medium following NAM, RESV and QUER treatment, we decided to analyze the metabolic pathways implicated in their utilization and, as a first step, we focused on their anabolic fate, that is to say the glyoxylate/gluconeogenic pathway. Gluconeogenesis gives raise to the biosynthetic precursor glucose-6-phosphate, which in turn leads also to the production of trehalose, proposed as the carbohydrate of choice for surviving the starvation occurring throughout chronological aging, allowing a faster cell cycle re-entry upon refeeding [40]. In addition, trehalose is a well-known stress protectant, preserving proteins from oxidative damage [60]. In the post-diauxic phase, when ethanol is used, the glyoxylate cycle becomes necessary for the replenishment of oxaloacetate, the substrate of phosphoenol pyruvate carboxykinase (Pck1), catalyzing the rate-limiting step of gluconeogenesis. So, we measured the enzymatic activity of Pck1 and also of isocitrate lyase (Icl1), one of the unique enzyme of the glyoxylate cycle. In parallel, we determined the intracellular trehalose content, too.

In wt, NAM supplementation increased the levels of both the enzymatic activities analyzed, which remained constantly higher than those detected in the untreated culture (Fig. 3.3 A-B), and correlated with a higher trehalose accumulation (Fig. 3.3 C). On the other hand, the *sir2Δ* mutant, which is *per se* characterized by higher levels of Icl1 and Pck1 enzymatic activities and of trehalose than wt [39], was unaffected by NAM treatment (Fig. 3.3 A-C). A negligible reduction in both Icl1 and Pck1 enzymatic activities and of trehalose content was observed upon 100 μM RESV addition to wt cells (Fig. 3.3 D-E-L). On the contrary, following QUER administration, Icl1 and Pck1 activities were constantly higher than the corresponding of untreated cells (Fig. 3.3 F-G) and, in concert, a greater amount of trehalose both in wt and *sir2Δ* cultures was detected (Fig. 3.3 M-N).


A
Icl1 (mU/mg prot)
Days
wt
wt + NAM 1 mM
wt + NAM 5 mM
sir2Δ
sir2Δ + NAM 1 mM
sir2Δ + NAM 5 mM


B
Pck1 (mU/mg prot)
Days
wt
wt + NAM 1 mM
wt + NAM 5 mM
sir2Δ
sir2Δ + NAM 1 mM
sir2Δ + NAM 5 mM


C
Threalose (nM/mg prot)
Day 0
Day 1
Day 3
Day 5
Day 7
wt
wt + NAM 1 mM
wt + NAM 5 mM
sir2Δ
sir2Δ + NAM 1 mM
sir2Δ + NAM 5 mM


D
wt
wt RESV 10 µM
wt RESV 100 µM
Icl1 (mU/mg prot)
Days
E
wt
wt RESV 10 µM
wt RESV 100 µM
Pck1 (mU/mg prot)
Days
F
wt NT
wt QUER 26µM
wt QUER 300µM
Icl1 (mU/mg prot)
Days
G
wt NT
wt QUER 26µM
wt QUER 300µM
Pck1 (mU/mg prot)
Days
H
sir2ΔNT
sir2ΔRESV 100µM
sir2ΔQUER 300µM
Icl1 (mU/mg prot)
Days
I
sir2ΔNT
sir2ΔRESV 100µM
sir2ΔQUER 300µM
Pck1 (mU/mg prot)
Days

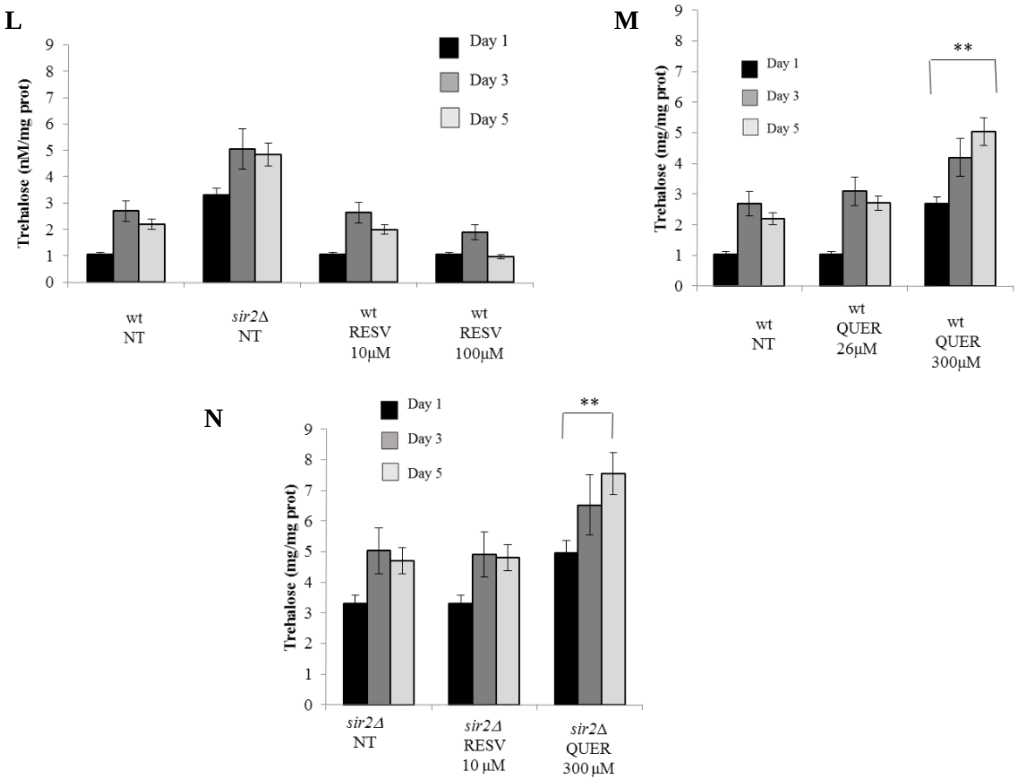


Fig. 3.3 Effects of NAM on glyoxylate/gluconeogenesis. Bar charts of Icl1 (A-C-E) and Pck1 (B-D-F) enzymatic activities and of intracellular trehalose content (C-L-M-N) measured at the indicated time points for wt and sir2Δ cells grown as in Fig. 3.1 and treated at the diauxic shift (Day 0) with NAM at the indicated concentrations. Icl1, Pck1 enzymatic activities and trehalose determined for both untreated cultures are also reported. Data refer to mean values determined in three independent experiments. SD is indicated. *P ≤ 0.05 and **P ≤ 0.01.

Thus, we determined the influence of the three compounds also on the CLS of the *pck1Δ* mutant. As shown in Fig. 34, loss of Pck1 correlates with a shorter CLS than wt, in line with published data [39, 61].Both NAM and RESV did not elicit any effects on the CLS of the mutant, which was, instead, extended upon QUER treatment.

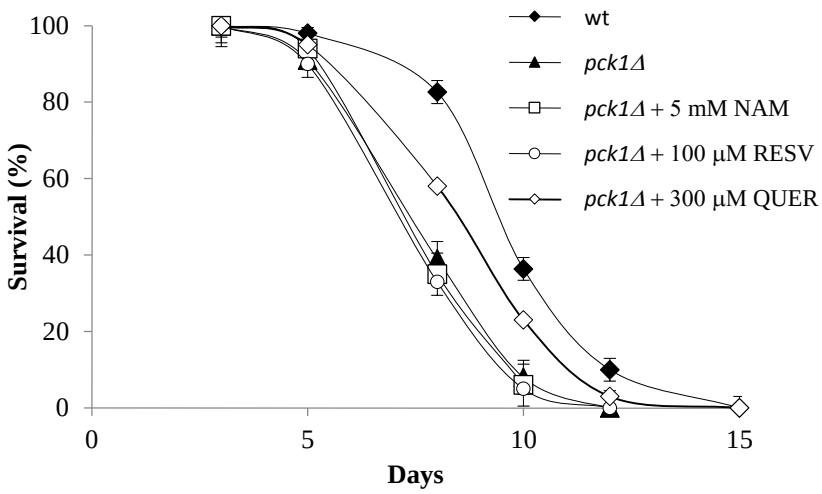


Fig. 3.4 *pck1Δ* cells grown as in Fig. 3.1 were treated at the diauxic shift (Day 0) with NAM, RESV or QUER at the indicated concentrations. At each time-point, survival was determined as in Fig. 3.1. Survival of wt cells in their exhausted medium was also monitored. Data refer to mean values determined in three independent experiments. SD is indicated. *$P \leq 0.05$ and **$P \leq 0.01$.

3.3.2 Effects of NAM, RESV and QUER supplementation on cellular respiration and mitochondrial functionality

When yeast cells have used up all the available glucose, they switch to oxidative metabolism. Consequently, we examined cellular respiration, determining the net respiration (netR), which represents the quote of coupled respiration, since it is obtained by subtracting the non-phosphorylating respiration rate (J_{TET}), assessed by the ATPase inhibitor TET, from the basal oxygen consumption (J_R). In wt, the addition of NAM resulted in an increased netR, with the effect of 5 mM supplementation being comparable to that elicited by lack of Sir2 (Table 2A). In this mutant, NAM supplementation had no effect (Table 2B). Upon RESV treatment, a strong decrease in netR was observed at Day 3 for the higher concentration (Table 2C). In contrast, following 300 μM QUER addition, netR values were higher than those of the untreated cultures (Table 2D). No effect was detected in *sir2Δ* subsequently to the supplementation of RESV at 100 μM. Whereas, 300 μM QUER supplementation further increased coupled respiration (Table 2E).

A

	Day 0	**Day 1**	**Day 3**	**Day 5**
wt NT	7.4 ± 0.3	10.26 ± 0.5	3.41 ± 0.2	0.17 ± 0.1
wt 1 mM NAM		10.37 ± 0.5	4.1 ± 0.2	0.57 ± 0.1
wt 5 mM NAM		9.32 ± 0.4	4.25± 0.3	1.4 ± 0.2

B

	Day 0	**Day 1**	**Day 3**	**Day 5**
***sir2Δ* NT**	6.7 ± 0.3	9.62 ± 0.5	4.34 ± 0.3	1.41 ± 0.2
***sir2Δ* 1 mM NAM**		9.5 ± 0.4	4.26 ± 0.4	1.4 ± 0.1
***sir2Δ* 5 mM NAM**		9.47 ± 0.4	4.19 ± 0.3	1.37 ± 0.2

C

	Day 0	Day 1	Day 3	Day 5
wt NT	7.4 ± 0.3	10.26 ± 0.5	3.41 ± 0.2	0.17 ± 0.1
wt 10 μM RESV		9.9 ± 0.4	3.2 ± 0.2	0.2 ± 0.1
wt 100 μM RESV		8.2 ± 0.3	1.5 ± 0.1	0.1 ± 0.1

D

	Day 0	Day 1	Day 3	Day 5
wt NT	7.4 ± 0.3	10.26 ± 0.5	3.41 ± 0.2	0.17 ± 0.1
wt 26 μM QUER		9.55 ± 0.4	3.3 ± 0.3	0.2 ± 0.2
wt 300 μM QUER		11.05 ± 0.3	5.3 ± 0.2	2.5 ± 0.4

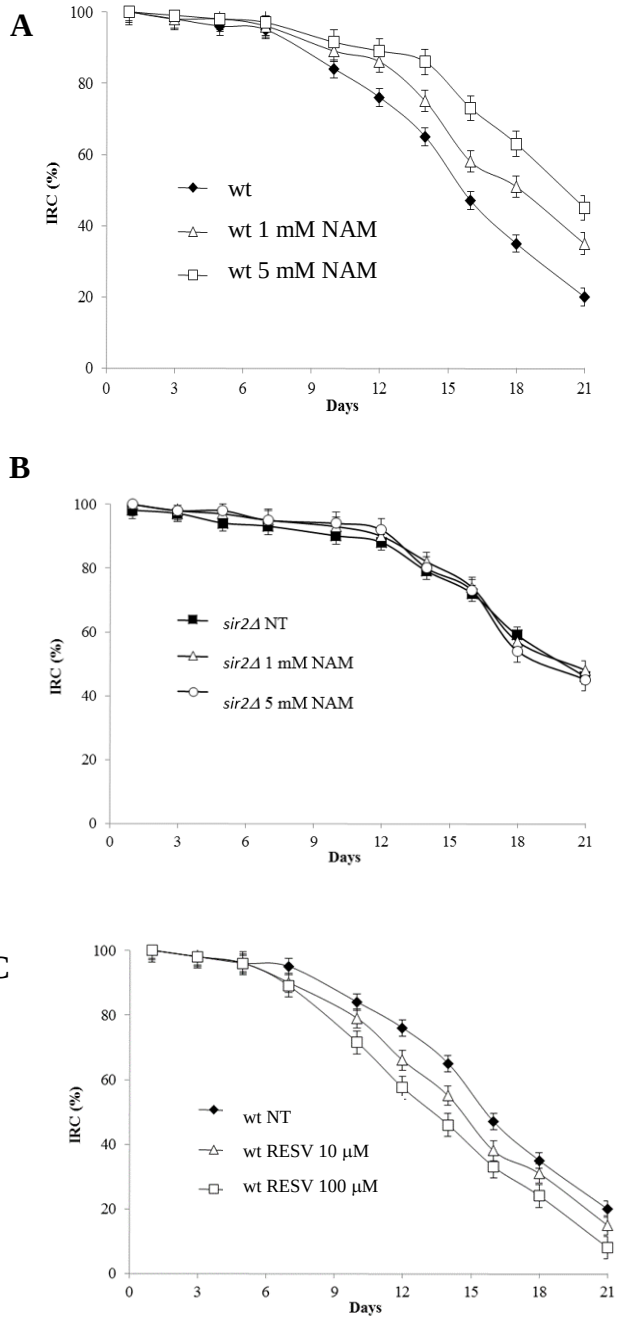
E

	Day 0	Day 1	Day 3	Day 5
***sir2Δ* NT**	6.7 ± 0.3	9.62 ± 0.5	4.34 ± 0.3	1.41 ± 0.2
***sir2Δ* 100 μM**		9.11 ± 0.4	4.95 ± 0.5	1.15 ± 0.1
***sir2Δ* 300 μM**		10.04 ± 0.4	5.7 ± 0.4	2.7 ± 0.1

Table 2. netR values obtained by substracting J_{TET} from J_R of wt and *sir2Δ* grown as in Fig. 3.1 and treated with NAM, RESV and QUER at the indicated concentrations. Data were obtained from mean values determined in three independent experiments. Standard deviations are indicated.

These variations observed in cellular respiration prompted us to analyze the index of respiratory competence (IRC), representing the percentage of viable cells which are competent to respire [52]. As shown in Fig. 3.5 A-B, NAM supplementation, as well as lack of Sir2, exerted a protective role on mitochondria, which retained a higher mitochondrial functionality than wt cells. In fact, at Day 21 the percentage of cells respiration-competent in wt cells was about 20% while in NAM-treated and *sir2Δ* cultures was about the double (Fig. 3.5 A-B). Following RESV addition, instead, IRC values were lower than those of wt untreated cells (Fig. 3.5 C); whereas no effect was observed in *sir2Δ* mutant (Fig. 3.5 E). QUER

supplementation improved mitochondrial functionality over time in wt (Fig. 3.5 D) as well as in *sir2Δ* mutant cells (Fig. 3.5 E).



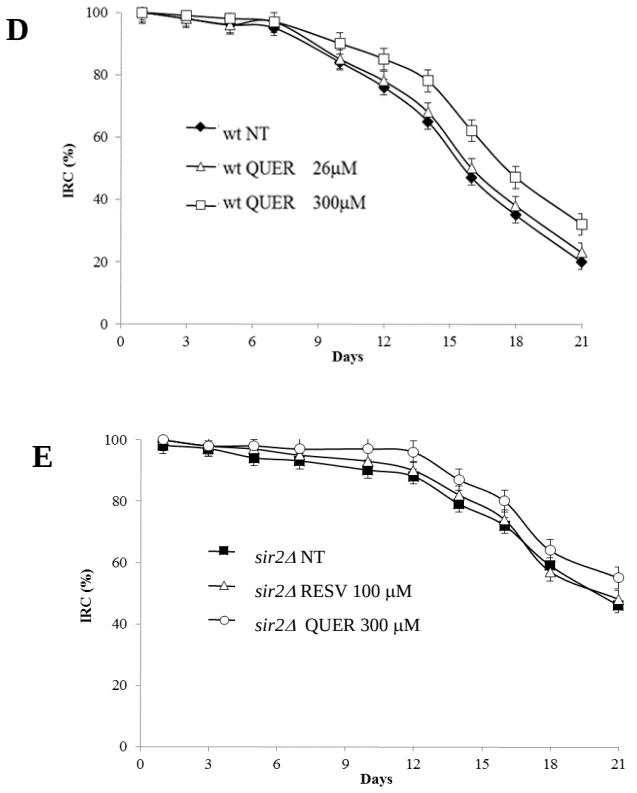


Fig. 3.5 Chronologically aging wt (A-C-D) and *sir2Δ* (B-E) cultures of Fig. 3.3 treated at the diauxic shift with the indicated concentrations of NAM, RESV and QUER were serially diluted, plated onto YEPD and YEPG plates, and the index of respiratory competence (IRC) was determined at the indicated time-points. Standard deviations of three independent experiments are indicated.

In parallel, we analyzed also the mitochondrial morphology and membrane potential by using the fluorescent dye $DiOC_6$. In fact, this dye accumulates specifically at mitochondrial membranes and can be observed by fluorescent microscopy. Balanced fusion and fission of mitochondria results in tubular mitochondrial morphology and reflects the functional condition of mitochondria. As shown in Fig. 3.6, in NAM and QUER-treated wt as well as *sir2Δ* cells, either treated or not, mitochondria were still organized in a tubular network at Day 7 whereas RESV-treated cells mitochondria appeared organized in punctiform structures that are indicative of mitochondrial fragmentation and consequently of mitochondrial dysfunction.

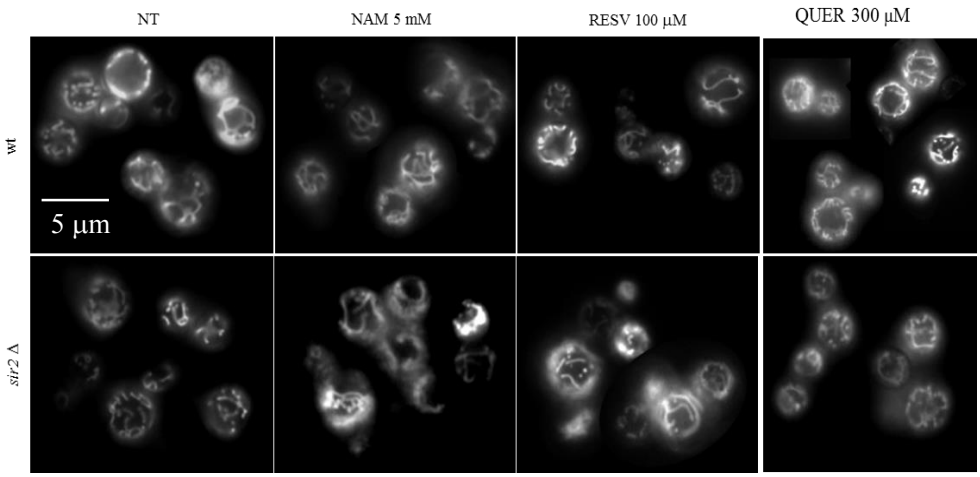
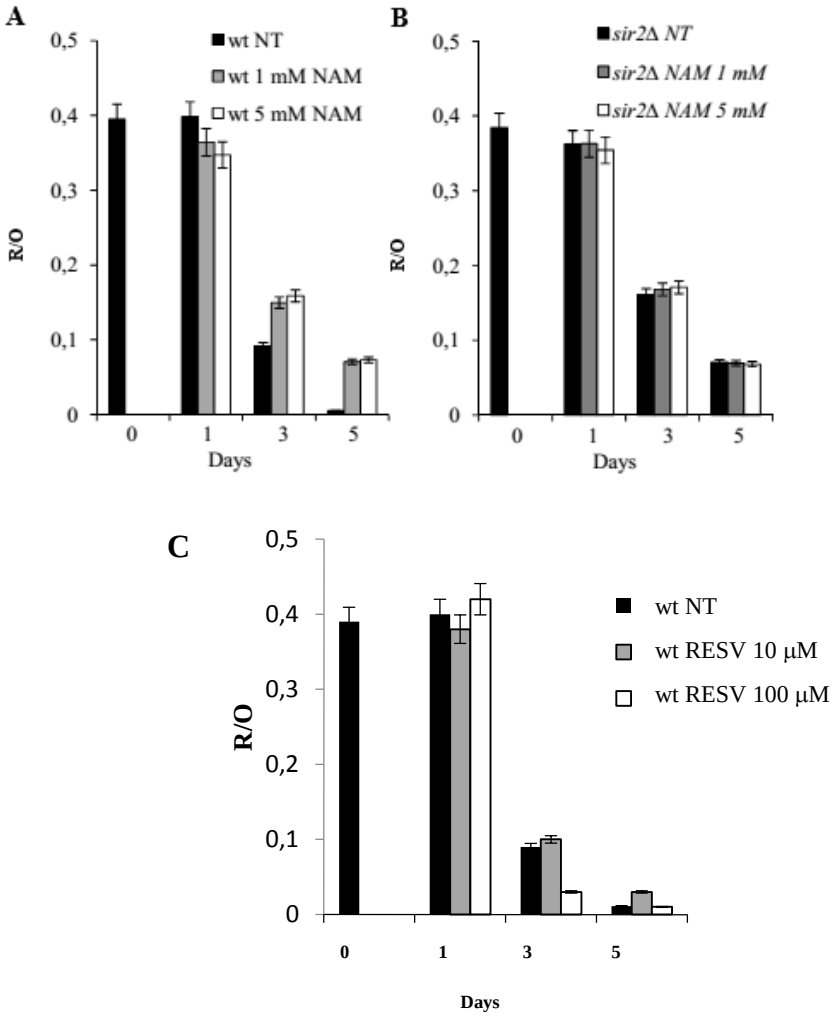


Fig. 3.6 The same cells of Fig. 3.6 at Day 7 stained with DiOC6 to visualize mitochondrial membranes. Representative images are shown.

Thus, we measured the mitochondrial redox state using a fluorescent probe, consisting in a redox-sensitive GFP that is targeted to the mitochondrial matrix (mito-roGFP) [42]. Mito-roGFP is a GFP variant where surface-exposed cysteines are added and has 2 excitation peaks at 400 nm and 480 nm and 1 emission peak

(510nm). Oxidation of the cysteines increases excitation at 400 nm while reducing that at 480 nm. Upon excitation of mito-roGFP at 480 and 400 nm, the ratio of 510 nm emission (R/O) reflects the redox state of mitochondria. In NAM- and QUER-treated and *sir2Δ* cells, in mitochondria prevailed the reducing potential over time indicative of mitochondrial functionality (Fig. 3.7 A-B-D). On the contrary, upon RESV treatment the oxidation state prevails (Fig. 3.8 C), in line with the punctuate morphology observed.



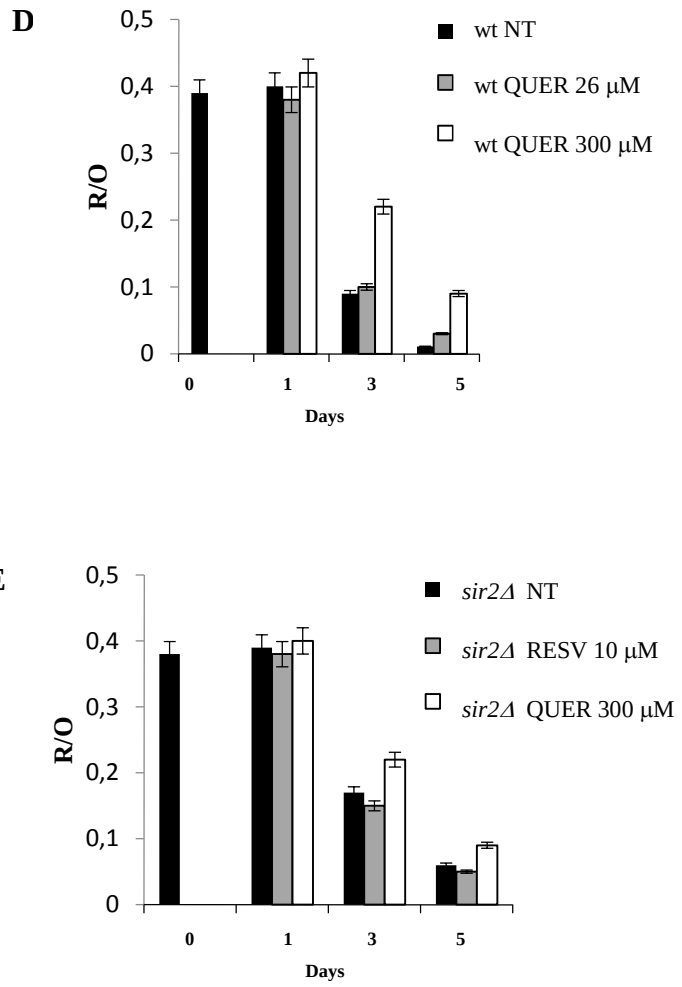
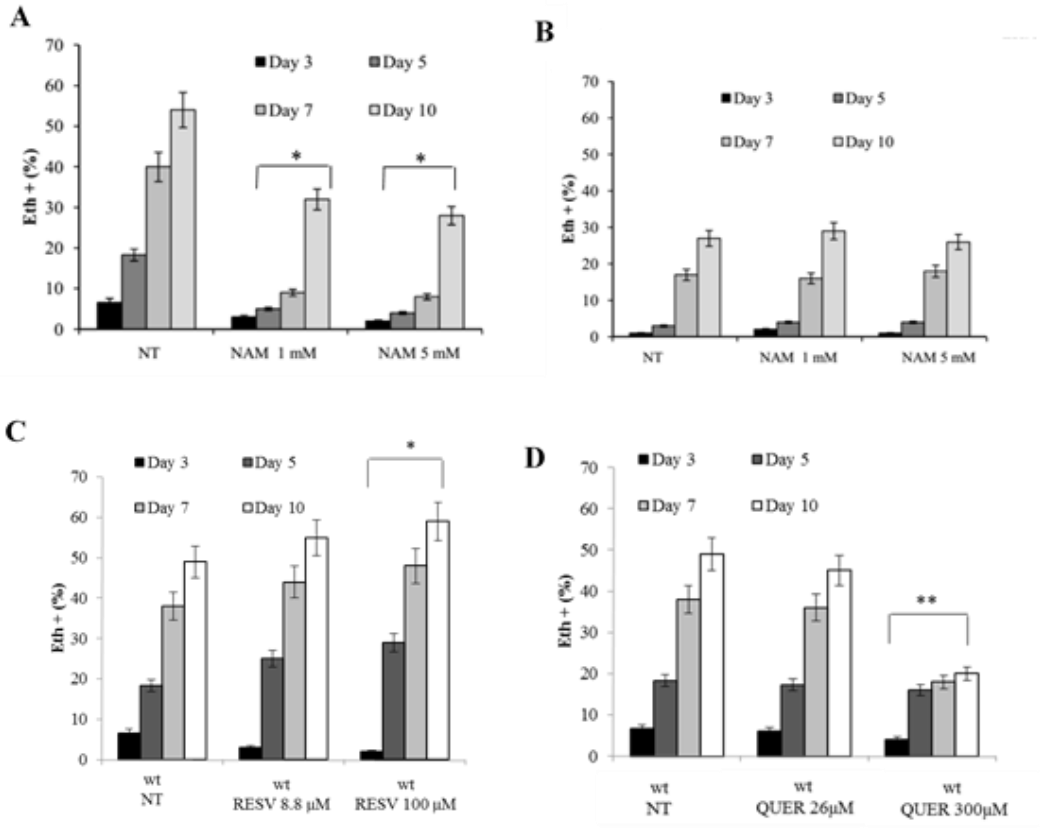


Fig. 3.7 Bar charts of the mitochondrial R/O ratio of wt (A-C-D) and *sir2Δ* (B-E) cells. Day 0, diauxic shift. NAM, nicotinamide, RESV, resveratrol, QUER, quercetin, NT, untreated cultures. Data refer to mean values of three independent experiments. SD is indicated. *P ≤ 0.05 and **P ≤ 0.01.

Besides producing energy, mitochondria are known to be the main source of ROS, as a result of the electron leakage from the ETC complexes during normal respiration. ROS formation occurs by the production of superoxide from complexes I and III, although other sites also contribute [62]. This superoxide production leads to the formation of hydrogen peroxide, and together with other

downstream products, these ROS cause oxidative damage that has been linked with aging [63]. ROS can impair mitochondrial functionality, leading to further production of ROS and thus establishing a vicious circle of oxidative damage [64]. So we decided to quantify ROS accumulation by using the DHE staining, which is specific for superoxide anion detection. All cultures accumulate ROS in a time-dependent manner (Fig. 3.8), but in NAM-treated and *sir2Δ* cells ROS content was significantly lower than wt at all the time points considered (Fig. 3.8 A-B). RESV treatment, instead, increased ROS accumulation in wt cells (Fig. 3.8 C). QUER-treated cells ROS content resulted to be the lowest at all time points considered (Fig. 3.8 D-E).



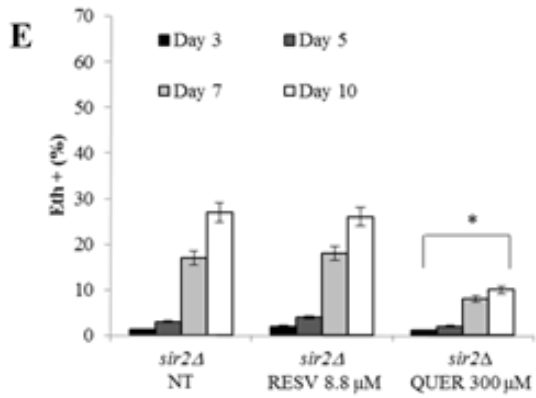


Fig. 3.8 Summary graphs of the percentage of fluorescent/superoxide positive wt (A-C-D) and sir2□□(B-E) cells (% Eth) are reported. Day 0, diauxic shift. NAM, nicotinamide, RESV, resveratrol, QUER, quercetin, NT, untreated cultures. Data refer to mean values of three independent experiments. SD is indicated. *P ≤ 0.05 and **P ≤ 0.01.

Conclusions

Our results provide evidence that NAM treatment positively affected yeast CLS and imposed a metabolic re-arrangement similar to that occurring when *SIR2* is deleted. Indeed, upon NAM treatment, the levels of both ethanol and acetate detected were lower than wt. In addition, in NAM-treated cells, likewise *sir2Δ* mutant cells, the levels of Icl1 and Pck1 enzymatic activities detected were higher than wt cells throughout chronological aging. An increased accumulation of trehalose, known to be favorable to chronological survival and to protect cells from oxidative damage, was detected, too. Thus, NAM beneficial effect on CLS correlates with an enhanced glyoxylate/gluconeogenic flux, likewise lack of Sir2 [39]. It is known that Pck1 enzymatic activity is controlled by its acetylation state and, in particular, is inhibited by the Sir2-mediated deacetylation [61]. NAM failed to extend the CLS of the *pck1Δ* mutant, whose short-lived phenotype is in line with the pivotal role played by gluconeogenesis in chronological survival. This observation pointed to a stimulatory effect exerted by NAM on gluconeogenesis, which may be due to the inhibition of Sir2 and, in particular, to the inhibition of Pck1 deacetylation by Sir2.

Both NAM treatment and lack of Sir2 favoured respiration coupling. In fact, in NAM- treated and *sir2Δ* cells, the quote of coupled respiration increased and results in lower ROS concentration. This modulation of the respiratory activity exerted by both NAM and *SIR2* inactivation resulted in a protective role on mitochondria, which retained their functionality and membrane potential over time, in contrast to wt cells, where a physiological time-dependent loss of mitochondrial functionality was observed.

On the contrary, RESV exerted a negative role on CLS. The influence of RESV on chronological survival can be positively ascribed to a modulation of the respiratory activity. In particular, the quote of coupled respiration decreased and, as a result, RESV-treated cells were more prone to generate ROS, which in turn impaired mitochondrial functionality.

QUER treatment plays a positive effect on CLS of wt and, in contrast to NAM, also in the *sir2Δ* and *pck1Δ* mutants. Also in this case, QUER-treatment cells exhibited a metabolic re-assessment in which the glyoxylate/gluconeogenic flux is favoured and respiratory activity is more efficient, avoiding the formation of ROS. These beneficial effects resulted additive to those elicited by lack of Sir2.

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Riassunto

Tutti gli organismi viventi col passare del tempo invecchiano, ossia vanno incontro ad un progressivo ed irreversibile declino funzionale/fisiologico, accompagnato da un aumentato rischio di contrarre malattie. Tra i diversi fattori coinvolti nell'invecchiamento, i nutrient-sensing pathway di TORC1/Sch9 e Ras/PKA e le Sirtuine, una famiglia di deacetilasi NAD+-dipendenti, svolgono un ruolo prioritario. Essi sono evolutivamente conservati dal lievito all'uomo, e risultano, inoltre, mediare alcuni degli effetti della Calorie Restriction (CR), un intervento che consiste nel limitare l'apporto di nutrienti senza incorrere in malnutrizione, e che è noto estendere la longevità di molti organismi. Nell'ambito della ricerca sull'invecchiamento, il lievito Saccharomyces cerevisiae è un utile sistema sperimentale. In particolare, la Chronological LifeSpan (CLS), definita come il tempo che una popolazione di cellule quiescenti può sopravvivere durante la fase stazionaria, rappresenta un modello per lo studio dell'invecchiamento di cellule post-mitotiche di mammifero, quali i neuroni e i miociti. Infatti, le cellule in fase stazionaria, pur non proliferando, rimangono metabolicamente attive e responsive agli stimoli.

Nella prima parte di questo lavoro di tesi, abbiamo indagato la relazione tra metabolismo e invecchiamento cronologico. In particolare, abbiamo dimostrato come etanolo e acido acetico, prodotti durante la crescita, possano condizionare attraverso il loro metabolismo la CLS. Infatti, gli effetti pro-aging di questi due composti possono essere prevenuti impedendo il metabolismo dell'etanolo attraverso l'inibizione specifica della sua riduzione ad acetaldeide oppure interferendo con l'uptake dell'acido acetico agendo sulle condizioni di pH extracellulare o sul trasporto mediato dall'acquagliceroporina Fps1. Abbiamo inoltre dimostrato come il destino di tali metaboliti condizioni la sopravvivenza a lungo termine. Infatti, un fattore favorevole per la CLS è rappresentato dal potenziamento dei pathway anabolici che consentono l'utilizzo delle fonti di carbonio C2 per la costituzione di carboidrati di riserva. Un'opportuna

rimodulazione tra metabolismo completamente ossidativo, basato sulla respirazione, e gluconeogenesi a favore di quest'ultima evita la rapida dissipazione delle riserve energetiche e limita la generazione di ROS dovuti all'intensa attività respiratoria. Ciò si traduce in un aumento della funzionalità dei mitocondri. Abbiamo inoltre dimostrato che il ricorso alla gluconeogenesi e al ciclo del gliossilato caratterizzano la condizione di estrema Calorie Restriction, realizzata attraverso il trasferimento delle cellule in acqua. In questa condizione, l'aggiunta di etanolo stimola con il suo metabolismo la respirazione che compete con la gluconeogenesi e, complessivamente, riduce l'effetto benefico associato alla CR sulla CLS.

Abbiamo quindi considerato alcuni composti nutraceutici in grado di influenzare, con diversi effetti, la RLS, quali NAM, RESV e QUER.

Abbiamo indagato se essi potessero condizionare anche la CLS, valutando se Sir2 fosse coinvolto. Il trattamento con NAM mima perfettamente il fenotipo sir2Δ in chronological aging come era stato osservato nel caso della RLS.

Il NAM infatti estende la CLS in egual misura all'inattivazione di SIR2 e produce gli stessi effetti metabolici, ossia un incremento della gluconeogenesi mediato dall'aumento dell'attività della Pck1 e un aumento dell'efficienza respiratoria, che preserva la funzionalità mitocondriale e riduce l'accumulo di ROS. Il RESV che induce un'estensione della RLS, ha invece un effetto opposto sulla CLS che correla non tanto con alterazioni a carico della gluconeogenesi, ma piuttosto con un incremento dell'attività respiratoria, prevalentemente inefficiente e tendente all'accumulo di ROS. I dati a disposizione non ci permettono al momento di escludere che il RESV influenzi la CLS agendo da attivatore di Sir2.

Nel caso della QUER abbiamo osservato un effetto migliorativo sulla CLS sia in cellule wt sia quando Sir2 è assente. Il trattamento con QUER potenzia la gluconeogenesi e migliora l'efficienza respiratoria in modo additivo rispetto alla mancanza di Sir2. Inoltre, la QUER ha un effetto benefico sulla CLS anche in cellule incapaci di effettuare gluconeogenesi perché prive di Pck1, indicando il coinvolgimento di altri meccanismi protettivi per la sopravvivenza cellulare. In

prospettiva, rimangono da definire quali siano questi elementi pro-longevità modulati dalla QUER e come essi si collocano nel network dei modulatori della CLS.

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Research Article

Ethanol and Acetate Acting as Carbon/Energy Sources Negatively Affect Yeast Chronological Aging

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In *Saccharomyces cerevisiae*, the chronological lifespan (CLS) is defined as the length of time that a population of nondividing cells can survive in stationary phase. In this phase, cells remain metabolically active, albeit at reduced levels, and responsive to environmental signals, thus simulating the postmitotic quiescent state of mammalian cells. Many studies on the main nutrient signaling pathways have uncovered the strong influence of growth conditions, including the composition of culture media, on CLS. In this context, two byproducts of yeast glucose fermentation, ethanol and acetic acid, have been proposed as extrinsic proaging factors. Here, we report that ethanol and acetic acid, at physiological levels released in the exhausted medium, both contribute to chronological aging. Moreover, this combined proaging effect is not due to a toxic environment created by their presence but is mainly mediated by the metabolic pathways required for their utilization as carbon/energy sources. In addition, measurements of key enzymatic activities of the glyoxylate cycle and gluconeogenesis, together with respiration assays performed in extreme calorie restriction, point to a long-term quiescent program favoured by glyoxylate/gluconeogenesis flux contrary to a proaging one based on the oxidative metabolism of ethanol/acetate via TCA and mitochondrial respiration.

1. Introduction

Human aging is associated with a host of time-dependent changes which are the clear manifestation of the progressive decline in cognitive and physical functions of an organism. Albeit extremely complex, aging has turned out to be influenced by mechanisms and nutrient/energy sensing signaling pathways that show strong evolutionary conservation. In this context, the single-celled yeast *Saccharomyces cerevisiae*, exploited as a model system, has provided valuable insight by making it possible to adopt experimental approaches that are not always feasible in higher eukaryotic systems. For example, the nutritional and metabolic status of yeast cells can be diversely coordinated by the simple choice of cultural conditions. Glucose is the preferred carbon and energy source, but in its absence other substrates such as glycerol, ethanol, acetate, or even fatty acids can be used [1]. Thus, the yeast life cycle can integrate metabolic characteristics that are typical for rapid growing cells, storage cells, or highly metabolizing cells depending on nutrient supply.

In the field of aging-related research, replicative and chronological lifespan models have been described which offer the opportunity to study the aging process of both proliferating and postmitotic quiescent mammalian cells, respectively [2–4]. The chronological lifespan (CLS) is defined as the length of time that a population of nondividing cells survives in stationary phase. Viability over time is measured as the ability to resume mitotic growth upon return to rich fresh medium [5]. In a standard CLS experiment, yeast cells are usually grown in synthetic defined media containing 2% glucose [6] where the metabolism is characterized by a high glycolytic flux, glucose fermentation, and a negligible aerobic respiration. Upon glucose depletion, the diauxic shift occurs which results in a shift from fermentation to respiration of the C2 compounds previously produced. This shift involves a massive reprogramming of gene expression including genes which encode enzymes involved in gluconeogenesis, the glyoxylate and TCA cycles. Moreover, overall growth rate is dramatically reduced. Finally, when nutrients are fully exhausted, cell division stops, and the yeast culture enters

a quiescent stationary phase [7, 8]. In the stationary phase, yeast cells display a survival-based metabolism characterized by low metabolic rates and upregulation of stress resistance resulting from the integrated responses of different signaling pathways [9].

CLS can be increased by calorie restriction (CR) which, in yeast, is generally imposed by reducing the glucose concentration in the initial growth medium [10–12] or by transferring postdiauxic cells to water (extreme CR) [5]. Moreover, inhibition/reduction activity of two pathways which sense nutrient availability, namely, TORCl-Sch9 and Ras-PKA ones, also extends CLS [13–16]. These signaling pathways lead in part to common downstream targets which include the protein kinase Rim15 and the transcriptional factors Msn2/4 and Gis1 [17–19]. These factors, besides regulating directly or indirectly stress defence mechanisms, control the accumulation/utilization of intracellular and extracellular carbon sources [20–23]. In particular, Gis1 regulates the accumulation of acetate, a metabolite involved in chronological aging [24]. Interestingly, lack of the NAD^+-dependent deacetylase Sir2, the founding member of Sirtuins, further extends the CLS of long-lived mutants such as *sch9*Δ, as well as the CLS in water indicating that the sole presence of Sir2 can serve as a "blocker" of extreme longevity extension [25]. In addition, *SIR2* inactivation induces stress resistance and affects positively the metabolism of extracellular carbon sources such as ethanol and acetate [25, 26]. These two by-products of glucose fermentation which are metabolised by yeast cells during the post-diauxic phase have been proposed as extrinsic factors promoting chronological aging [25, 27]. In fact, in some long-lived mutants, as well as in short-lived ones, an inverse correlation between the amount of extracellular ethanol or acidic acid and their CLS has been found [25, 26, 28–30]. In line with this, genetic or metabolic (CR) interventions which drive yeast metabolism away from acetic acid production increase CLS [27, 31]. Furthermore, although some connections have been established between nutrient-sensing pathways and the proaging effect of acetic acid involving superoxide anion accumulation which inhibits quiescence [32], the mechanisms by which this compound (and also ethanol) reduces the CLS are still controversial [33].

Here we present results showing that both ethanol and acetic acid contribute to chronological aging. In this context, these compounds are not simply extrinsic toxic factors, but it is their metabolic utilization as carbon/energy sources which results in proaging effects. In particular, in extreme CR, their oxidative metabolism increasing respiration impairs mitochondrial functionality and negatively affects long-term cell survival.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions. All yeast strains with null mutations were generated by PCR-based methods in a W303-1A background (*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*): *fps1*Δ (*fps1*Δ::*KlLEU2*), *sir2*Δ (*sir2*Δ::*URA3*) [34], *icl1*Δ (*icl1*Δ::*KlLEU2*), and *pck1*Δ (*pck1*Δ::*KlLEU2*) [26]. Yeast cells were grown in batches at 30°C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/L), supplemented with 2% w/v glucose. Auxotrophies were compensated for with a fourfold excess of supplements [25]. 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 Analyser, as described [35]. Doubling time (Td) was obtained by linear regression of the cell number increase over time on a semilogarithmic plot. For pH measurements, small aliquots of expired media were removed from the culture, and pH was determined using a pH meter.

2.2. Metabolite Measurements and Enzymatic Assays. 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).

Immediately after preparation of cell-free extracts, Pck1 and Icl1 activities were determined as previously described [26]. 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.

2.3. CLS Determination. Survival experiments in expired medium were performed on cells grown in minimal medium (with a fourfold excess of supplements) of 2% glucose as described by [25]. During growth, cell number and extracellular glucose, ethanol, and acetic acid were measured in order to define the growth profile (exponential phase, diauxic shift, post-diauxic phase, and stationary phase) of the culture. Cell survival was monitored by harvesting aliquots of cells starting 72 h (Day 3, first age-point) after the diauxic shift (Day 0). CLS was measured according to [25] by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 3 was considered the initial survival (100%). Survival was also monitored in the presence of 50 mM pyrazole (Sigma) which was added in the expired medium at Day 1 after the diauxic shift.

For survival experiments in water, at Day 1 cells were harvested, washed with sterile distilled water, and resuspended in a volume of water equal to the initial culture volume. Every 48 h, cells were washed with water and resuspended in fresh water to remove nutrients released by dead cells [5]. The pH of the water was adjusted to 3.2 since it was the pH value measured in the expired medium or to 5.6. Survival experiments in water containing ethanol, acetic acid, or both were performed essentially as described [25, 26, 29]. Treatments are outlined in the text.

For CLS determination in media-swap experiments, cells were grown in minimal medium of 2% glucose (with a fourfold excess of supplements) and at Day 1 after the diauxic shift, harvested by centrifugation. Cell pellets were washed

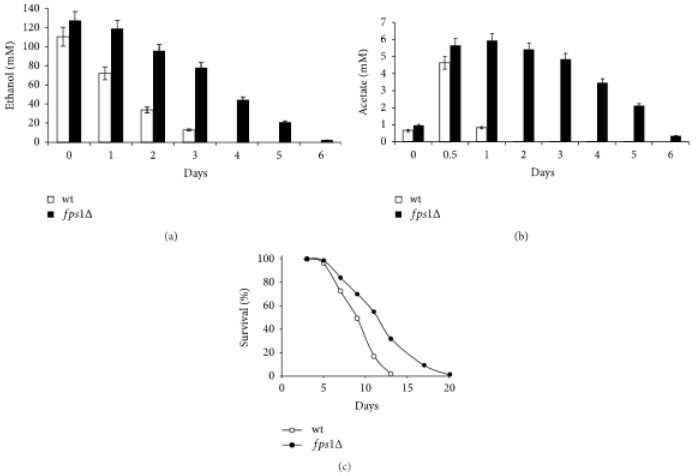

FIGURE 1: *FPS1* inactivation increases CLS in concert with a decreased uptake of ethanol and acetate. Bar charts of extracellular ethanol (a) and acetate (b) concentrations measured at the indicated time-points in wild type (wt) and *fps1Δ* mutant cultures during chronological aging. Day 0, diauxic shift. Data were obtained from mean values determined in three independent experiments. Standard deviations are indicated. (c) CLS of wt and *fps1Δ* mutant cells. At every time-point, viability was determined by counting CFUs on YEPD plates. 72 h after the diauxic shift (Day 3) was considered the first age-point (see Section 2). One representative experiment is shown.

and then resuspended in the filtered original medium or equivalently conditioned one of the indicated strain. Resuspension in media collected at Day 1 was also performed in the presence of 50 mM pyrazole. Viability was measured as previously described.

2.4. Respiration Assays. The oxygen consumption of intact cells was measured at 30°C using a "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System, Hansatech Instruments, Norfolk, UK). For all respiration assays, 2 mL of cell suspension at a concentration of 5×10^6/mL were quickly transferred from the flask to the oxygraph chamber, and routine respiration was recorded. Data were recorded at sampling intervals of 1 s (Oxygraph Plus software, Hansatech Instruments, Norfolk, UK). Respiratory rates were determined from the slope of a plot of O_2 concentration against time, divided by the cellular concentration. All assays were conducted in biological triplicate.

Index of respiratory competence (IRC) was also measured according to [36] by plating identical samples on YEPD plates and on rich medium of 3% glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.

3. Results and Discussion

3.1. Lack of Fps1 Channel Increases CLS. Ethanol and acetic acid are two normal by-products of glucose fermentation, transiently accumulated in the yeast culture medium, which restrict CLS [25, 27]. Moreover, given the low concentration reached by acetic acid in the medium of chronologically aging cells and its faster exhaustion compared to that of ethanol, its physiological relevance as an extracellular factor promoting chronological aging is a matter of debate [33]. In this context, as a first step, we examined the effects on CLS of abolishing the major route of entry into the cell of the undissociated acetic acid such as the Fps1 channel. Uptake of acetate is linked to an active transport for the dissociated form of the acid through the Jen1 and Ady2 transporters accompanied by passive/facilitated diffusion of the undissociated acid through the Fps1 aquaglyceroporin [37, 38]. The former is inducible and subjected to glucose repression [39, 40] while the passive transmembrane flux is strongly influenced by the pH of the medium. In fact, the acetic/acetate couple forms a buffer system in a dynamic equilibrium: at low pH the equilibrium increasingly favours the protonated form while at pH above the pKa of acetic acid (4.75) charged acetate anions prevail. As shown in Figures 1(a)

Table 1: pH values of exhausted media.

Days	wt	*fps1*Δ	*icl1*Δ	wt + pyrazole	*icl1*Δ + pyrazole
0	3.21 ± 0.07	3.20 ± 0.06	3.11 ± 0.06		
1	3.18 ± 0.04	3.16 ± 0.03	3.08 ± 0.05	3.18 ± 0.05	3.08 ± 0.07
2	3.13 ± 0.06	3.08 ± 0.04	2.98 ± 0.05	3.11 ± 0.04	2.89 ± 0.04
3	2.97 ± 0.05	2.90 ± 0.04	2.86 ± 0.06	2.93 ± 0.06	2.75 ± 0.07
4	2.81 ± 0.03	2.68 ± 0.06	2.68 ± 0.04	2.76 ± 0.07	2.63 ± 0.06
5	2.72 ± 0.06	2.56 ± 0.06	2.53 ± 0.06	2.65 ± 0.04	2.48 ± 0.07
6	2.70 ± 0.06	2.55 ± 0.05	2.49 ± 0.07	2.63 ± 0.07	2.43 ± 0.07

pH of the exhausted media was measured starting from diauxic shift, Day 0. Data presented are the mean values of three biological replicates. Standard deviations are indicated.

and 1(b), measurements of extracellular ethanol and acetate revealed that, at the diauxic shift, in the *fps1*Δ culture the amount of these C2 compounds was slightly higher than that in the wild type (wt) culture, in line with exometabolome data obtained during glucose fermentation [41]. However, after the diauxic shift (respiratory metabolism) a significant effect was observed on the depletion of both ethanol and acetic acid which was reduced in the mutant. In particular, as opposed to the expected fast exhaustion of acetic acid in the wt medium (Figure 1(b) and [29]), in the *fps1*Δ mutant this compound decreased very slowly, and it was still present 6 days following the entry in the post-diauxic phase (Figure 1(b)), which is in agreement with the role for Fps1 in facilitating the diffusion of the undissociated acid. In fact, during this phase in which the pH of the medium dropped to values of 2.70 for the wt and 2.55 for the *fps1*Δ mutant at Day 6 (Table 1), acetic acid is substantially undissociated, and the diffusional entry into the cells is elevated. Upon *FPS1* deletion, mutant cells can only rely on the uptake of the low fraction of acetate anions by the active transporters. Interestingly, chronologically aging *fps1*Δ cells lived longer than wt (Figure 1(c)) despite a prolonged exposure to acetic acid and ethanol.

3.2. Inhibition of Ethanol Metabolism Increases CLS. During chronological aging, after the diauxic shift, ethanol which is the main by-product of glucose fermentation, is metabolised by virtually the same pathway as acetate. In fact, after its oxidation to acetaldehyde by alcohol dehydrogenase 2 (Adh2), it is converted to acetate. Subsequently, acetate is activated into acetyl-CoA which is used to fuel the glyoxylate and TCA cycles (Figure 2(a)) [42, 43]. Consequently, we wondered whether blocking the main pathway for acetate production might influence the chronological survival of wt cells in their exhausted medium. To this end, after the diauxic shift when cells began to utilize the excreted ethanol, pyrazole which is an irreversible inhibitor of Adh2 [44] was added to the culture medium and CLS monitored. As depicted in Figure 2(b), pyrazole treatment led to CLS extension. A similar salutary effect took place also when pyrazole was added to the culture medium of postdiauxic *icl1*Δ cells (Figure 2(c)). *ICL1* encodes isocitrate lyase (Icl1), which is one of the unique enzymes of the glyoxylate cycle. During growth on C2 compounds, this cycle plays an essential role for anaplerosis of oxaloacetate which is the substrate of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Pck1) (Figure 2(a)) [43]. In the context of a CLS standard experiment, *ICL1* deletion results in a short-lived phenotype and impairment in acetate utilization [26]. Furthermore, pyrazole treatments led to a very slight acidification in the expired media of the wt and *icl1*Δ cultures (Table 1) indicating that the extracellular acidic pH alone is not sufficient to chronologically age yeast cells. Since we had already observed that pyrazole was able to abrogate the shortening effect of ethanol on CLS extension following extreme CR such as incubation in water [26], this confirms that some aspects of ethanol metabolism and not its mere presence (it enters the cells by passive diffusion) negatively affect CLS. We next performed some media-swap experiments between wt and *icl1*Δ cultures. Both strains were grown in minimal medium, and, at Day 1 after the diauxic shift, cultures were centrifuged and media were exchanged. The *icl1*Δ preconditioned medium, which contained more ethanol and acetic acid compared with the wt preconditioned one (Figures 2(d) and 2(e)) shortened the CLS of wt cells (Figure 2(b)). This detrimental effect on wt viability was abolished upon pyrazole addition, and CLS increased to the same extent as that of chronologically aging wt cells in their original medium in the presence of pyrazole (Figure 2(b)). Moreover, the wt preconditioned medium extended the CLS of the short-lived *icl1*Δ mutant (Figure 2(c)). Inhibition of ethanol oxidation by pyrazole further extended the CLS of the mutant which resulted, also in this case, similar to that of the chronologically aging mutant in its original medium supplemented with the Adh2 inhibitor (Figure 2(c)). Together these findings may point to proaging signaling effects of the metabolic pathways involved in the utilization of ethanol/acetate as carbon and energy source(s) by chronologically aging cells. This is consistent with the proposed role for acetic acid as a physiological trigger of growth signaling pathways which by promoting entry into S phase in unfavorable conditions would lead, among other effects, to replication stress in chronologically aging cells [45]. A DNA replication stress would negatively affect CLS [32, 46], and in this context experimental manipulations inducing such a stress have been recently shown to determine the loss of the reproductive capacity of chronologically aging cells [47]. Moreover, replication stress promotes apoptosis [48, 49]: a highly regulated cellular "suicide" program which is also activated during chronological aging [50]. In addition, acetic acid represents a compound which triggers apoptosis in the presence of glucose [51–53] and in glucose-derepressed *ach1*Δ

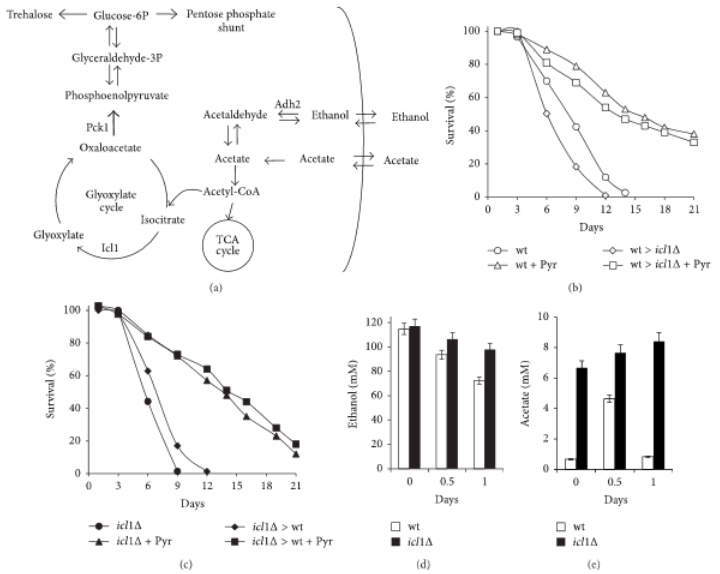

FIGURE 2: Pyrazole prevents the CLS shortening effect of ethanol. (a) Scheme of metabolic pathways allowing ethanol and acetate utilization. Only relevant reactions are shown. Adh2: alcohol dehydrogenase 2, Icl1: isocitrate lyase 1, Pck1: phosphoenolpyruvate carboxykinase 1. At Day 1 after the diauxic shift, pyrazole (50 mM) was added to the expired media of wt (b) and *icl1*Δ mutant (c) cells. In parallel, aliquots of cells were harvested and subjected to cell-free media swap with or without pyrazole. At every time-point, viability was measured as in Figure 1(c). One representative experiment is shown. Extracellular ethanol (d) and acetate (e) concentrations determined in the wt and *icl1*Δ cultures at Day 1. Day 0, diauxic shift. Standard deviations are indicated.

cells [29]. Ach1 is an enzyme involved in acetate metabolism, and its lack decreases CLS [29, 54]. Consequently, stimulation of growth induced by acetic acid after the diauxic shift in the lack of favorable conditions required for cell cycle progression would ultimately cause apoptosis.

3.3. Physiological Amount of Acetic Acid Reduces CLS. Next, we evaluated whether the physiological amount of acetic acid accumulated as a by-product of glucose fermentation could influence the chronological survival of yeast cells associated with their transfer to water, which is the extreme condition of CR known to extend CLS [25]. Therefore, we monitored the CLS of wt cells that, after the diauxic shift, were switched from expired medium to water supplemented with the amount of acetic acid (5 mM) we had detected in the expired medium (Figure 1(b) and [26, 29]). Treatments were performed in water whose pH was adjusted to 3.2 (the pH of the expired medium we measured) and in water buffered to pH 5.6. In the former condition the uptake of acetate is facilitated compared with that at pH 5.6 where the amount of the acetate anion considerably increases. As shown in Figure 3(a), the addition of 5 mM acetic acid to low pH water reduced CLS, but to a lesser extent than that elicited by ethanol [25, 26] which also in this case was supplied in amount comparable with that found in the expired medium. It is noteworthy that the addition of these C2 compounds together prevented CLS extension associated with transfer to low pH water resulting in a CLS similar to that of chronologically aging cells in their exhausted medium (Figure 3(a)). This suggests that it is a combined proaging effect of both metabolites which influences the CLS. Buffering water to pH 5.6 did not result in a CLS substantially different from that observed at pH 3.2 while the negative effect on chronological survival linked to the presence of acetic acid, ethanol, or both

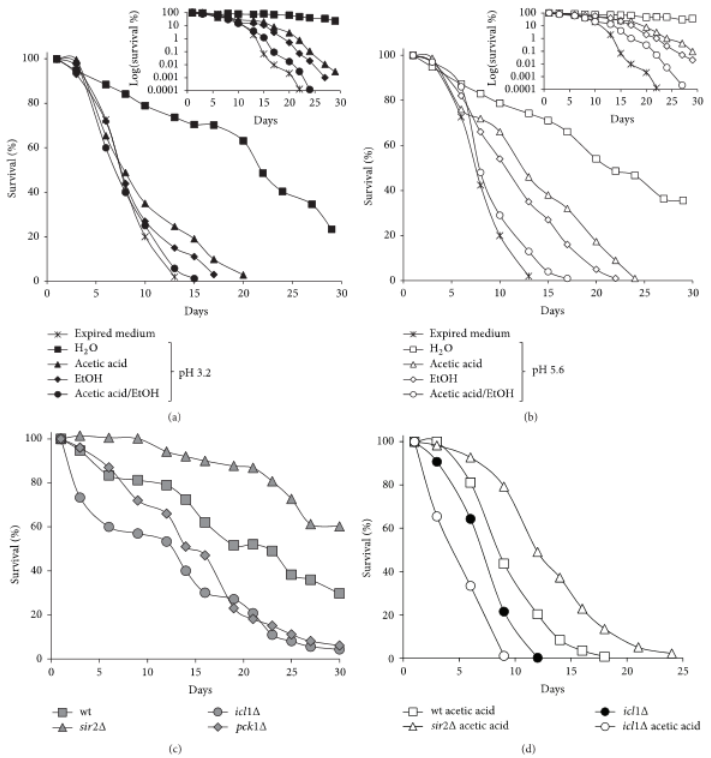

FIGURE 3: The glyoxylate-requiring gluconeogenesis plays a positive role in extreme life-span extension. At Day 1 after the diauxic shift, wt cells were switched to water adjusted to pH 3.2 (a) and to pH 5.6 (b) and challenged with ethanol (6 g/L), acetic acid (5 mM), or both. Every 48 h, cultures were resuspended in fresh water, and each time ethanol and acetic acid were added when indicated. At every time-point, viability was measured. Survival of cells in their expired medium was also monitored as control. Insets: CLS plotted on a log scale. One representative experiment is shown. (c) CLS of wt, *icl1*Δ, *pck1*Δ, and *sir2*Δ cells switched to pH 3.2 water at Day 1 after the diauxic shift. (d) In parallel, the indicated cultures were challenged with 5 mM acetic acid as in (a). Survival of *icl1*Δ cells in their exhausted medium was also monitored. One representative experiment is shown.

these compounds together was reduced (Figure 3(b)). Thus, buffering the extracellular medium alone is not sufficient to induce the fully extension of CLS observed in water, in line with data showing that an acidic environment alone is not sufficient to suppress the CLS extension associated with a CR regimen of growth which reduces acetic acid production [27, 29]. This further confirms that acidification accelerates chronological aging by influencing acetic/acetate equilibrium and consequently acetate uptake.

In the chronological aging paradigm, a proaging role is played by Sir2 which has as nonchromatin substrate the Pck1 enzyme. *SIR2* inactivation increases acetylated Pck1 in

concert with increased enzymatic activity [26, 28]. Since this correlates with an enhanced glyoxylate/gluconeogenic flux and with a more efficient acetate utilization [26], we analyzed whether the addition of 5 mM acetic acid could influence the CLS of *sir2*Δ cells that, after the diauxic shift, were incubated in low pH water. In parallel, the same analysis was performed for the *icl1*Δ mutant. As shown in Figure 3(c), the effect produced by the single *SIR2* and *ICL1* deletions on the CLS in water was the opposite. In fact, lack of Sir2 significantly extended the CLS compared with that of wt cells in agreement with [25–28] while lack of Icl1 reduced it. Interestingly, a similar decrease in cell survival has been observed following *PCK1* deletion ([28] and Figure 3(c)). Moreover, acetic acid-back *sir2*Δ cultures lived longer than acetic acid-back wt ones (Figure 3(d)). On the contrary, chronological survival of *icl1*Δ cells was affected dramatically by the same amount of acetic acid (Figure 3(d)), indicating that acetic acid, at this concentration, becomes extremely toxic for cells with an impaired glyoxylate cycle activity. Taken together these data suggest that the glyoxylate-requiring gluconeogenesis and the cell ability to metabolize acetate play positive roles in the CLS extension linked to extreme CR.

3.4. Ethanol Reduces Glyoxylate/Gluconeogenesis and Enhances Respiration of Cells in Extreme CR. Starting from the aforementioned results, for the purpose of investigating the connection between the glyoxylate-requiring gluconeogenesis and chronological longevity we measured the enzymatic activity of Pck1 and Icl1 in chronologically aging wt cells in their expired medium or transferred to water. In parallel, we also examined cellular respiration. In fact, it is well known that in the former experimental condition, when glucose is depleted, cells consume the earlier produced ethanol/acetate via gluconeogenesis (Figures 4(a) and 4(b)), and concomitantly they increase their respiration (Figure 4(c)). In the extreme condition of CR, once cells were switched to water, the levels of Icl1 and Pck1 enzymatic activities increased and remained higher than those detected during aging in the expired medium (Figures 4(a) and 4(b)). In addition, they barely respired (Figure 4(c)). It is noteworthy that when these cells were challenged with ethanol, Icl1 and Pck1 enzymatic activities were reduced (Figures 4(a) and 4(b)), and the cellular respiration increased (Figure 4(c)). Similar results (with reduction and increase to a lesser extent) were obtained when acetate substituted for ethanol (data not shown) indicating that both C2 compounds are metabolised by the CR cells. Since the ability to respire relies on functional mitochondria and a direct correlation between reduced CLS and dysfunctional mitochondria has been reported [6, 55], we decided to analyze the index of respiratory competence (IRC). This index measures the percentage of viable cells which are competent to respire [36]. At Day 1, the IRC was about 100% for chronologically aging cells in the exhausted medium, in water, and in water/ethanol (Figure 4(d)) indicating that all the cells are respiration competent. Starting from Day 12, this value began to decrease progressively for the cells in the exhausted medium and for those in water/ethanol reaching about 20% and 50%, respectively by Day 21 which is indicative of a time-dependent loss of mitochondrial functionality. On the contrary, in the extreme CR condition the IRC was still about 80% (Figure 4(d)) indicating, on the one hand, that the low level of respiration is not due to impairment in mitochondrial functionality and, on the other hand, that resuspension in water exerts a protective role on mitochondria which become more prone to damage following ethanol addition.

To this effect, a causative role in inducing mitochondrial dysfunction is played by reactive oxygen species (ROS), and, at the same time, mitochondrial dysfunction leads to increased ROS formation [56]. Moreover, mitochondria are the major intracellular source of potentially harmful ROS such as the superoxide anion. This radical can directly induce oxidative damage or can be converted to other ROS which, in turn, induce aging-associated damage [57]. Chronological aging in the absence of any extracellular nutrient, namely, water, which correlates with an increased CLS, implies that cells have to establish a survival-based metabolism where energy is conserved by shutting down expensive growth-promoting pathways and concomitantly stress resistance and access to alternate energy stores are provided. In addition, cells have to limit damage to cellular components. In this context, reducing respiration may be beneficial since, although highly efficient in producing ATP, the oxidative metabolism produces the superoxide anion which is generated in the electron transport chain.

The other feature of cells in extreme CR discovered was an increase in the enzymatic activities of Pck1 (the main flux-controlling step of gluconeogenesis) and Icl1. This feature, combined with the fact that loss of their function blocks CLS extension, further supports the notion of a positive crucial role of glyoxylate/gluconeogenesis in the control of this form of longevity [28]. Increasing glyoxylate/gluconeogenesis may be advantageous to improve survivability during chronological aging in water since gluconeogenesis switches the direction of metabolite flow towards the biosynthetic precursor, glucose-6-phosphate, which is also needed for glucose stores (Figure 2(a)). In particular, trehalose has been proposed as the carbohydrate of choice for surviving starvation and upon cell cycle reentry from quiescence [58]. Moreover, hexoses generated from gluconeogenesis can be used via the pentose phosphate pathway generating additional NADPH which is essential for the activity of antioxidant defenses [59]. On the other hand, with regard to the glyoxylate pathway, it is important to recall that it does not only have the function of fueling gluconeogenesis but can contribute to NADH production [60].

This metabolic scenario may give some explanation why the CLS extension in water is intensified following *SIR2* inactivation [25]. In fact, the increase in the acetylated active form of Pck1 due to the lack of the Sir2-targeted deacetylation enhancing the glyoxylate/gluconeogenic flux [26] might further favour the establishment of a long-term quiescent program. On the contrary, the oxidative metabolism of ethanol/acetate via the TCA and mitochondrial electron transport chain increasing respiration may generate harmful ROS which impair mitochondrial functionality. This, in

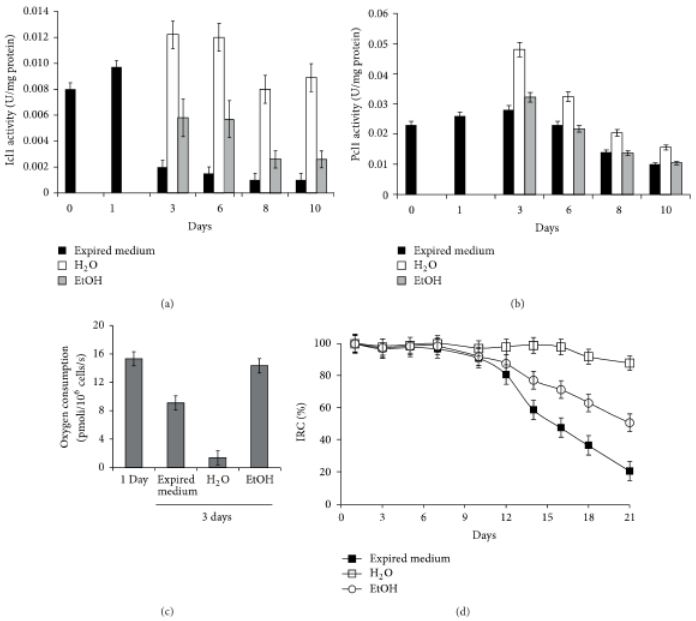

FIGURE 4: Ethanol affects the glyoxylate-requiring gluconeogenesis and the respiration of cells in extreme CR. At the indicated time-points, Icl1 (a) and Pckl (b) enzymatic activities were measured in wt cells during chronological aging in their expired medium and after the switch to water or water/ethanol as in Figure 3. Day 0, diauxic shift. (c) Cellular respiration of the same cells in the indicated experimental conditions. Error bars are the standard deviation of three replicates. (d) Chronologically aging wt cultures at the indicated time-points were serially diluted, plated onto YEPD and YEPG plates, and the index of respiratory competence (IRC) was determined. Standard deviations of three independent experiments are indicated.

concert with induced growth signals in the lack of favorable conditions required for cell cycle progression [32], most likely negatively affects cell survival. Bearing in mind that the relationship between respiration, ROS, and CLS is very complex, how can the proaging effect induced by ethanol in nutrient starvation conditions fit with the ability of pregrowth on the same respiratory carbon/energy source to extend CLS [61, 62]? In fact, in addition to the role played by a mitochondrial respiratory threshold in regulating CLS [63], mitochondrial respiration affects chronological survival through ROS generation. They can be either deleterious or beneficial depending on the biological context/phases of the yeast cell cycle in which they are produced [57]. Although mitochondrial ROS have been associated with damaging effects which promote and/or accelerate chronological aging [64], they also function as signaling molecules with hormetic effects on longevity [65, 66]. In particular, elevating mitochondrial ROS during yeast exponential growth elicits an adaptive response which promotes CLS extension [67]. Similarly, the effects on CLS observed following growth on ethanol [61, 62] are also in line with an adaptive mitochondrial longevity signal generated during active growth which contributes to establishment of a better quiescent program.

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