UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze

Corso di Dottorato di Ricerca
in Biotecnologie Industriali, XXV ciclo

Exploring the metabolism beyond cell aging in yeast

Tesi di: Nadia Casatta

Tutor: Prof.ssa Marina Vai

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Anno Accademico 2011-2012

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Nadia Casatta

Maricola 062639

Tutor: Prof.ssa Marina Vai

Coordinatore: Prof. Marco Vanoni

Università degli Studi di Milano-Bicocca
Piazza dell'Ateneo Nuovo 1, 2126 Milano

Dipartimento di Biotecnologie e Bioscienze
Piazza della Scienza 2, 20126 Milano

"Aging and death do seem to be
What Nature has planned for us.

But what if we have other plans?"

Bernard Streheler

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Abstract

All living organisms undergo a progressive physiological decline with age that results in an increased risk of the development of many diseases. Up to now, many factors have been shown to be involved in aging, like oxidative damage, telomere erosion, mitochondrial dysfunction, genomic instability or epigenetic changes, but, although many efforts made, none of them is commonly recognized as the primary molecular cause of this phenomenon. The budding yeast *Saccharomyces cerevisiae* has proven to be an experimental useful model for studying the aging process leading to the identification of pathways whose counterparts can be found in higher eukaryotes and in particular in humans. In yeast, the two paradigms of aging are described: the replicative aging and the chronological aging. The latter refers to the aging of cells in a quiescent-like state, with chronological lifespan (CLS) defined as the length of time that non-dividing yeast cells can maintain replicative potential. CLS is influenced by several factors, both intrinsic and extrinsic. The former group includes the signal transduction pathways involved in stress and nutrient responses, such as the TOR/Sch9 and the Ras/PKA pathways. Among the latter, in yeast two byproducts of the cell metabolism seem to play a determinant role: acetate and ethanol. In particular, their presence in the exhausted growth medium has a pro-aging effect, even though the exact role they play is still a matter of debate.

Moreover, a key role in lifespan regulation is widely recognized for Sir2, the founder of Sirtuins, a family of highly conserved NAD-dependent histone deacetylases. Unlike the other families of deacetylases, Sirtuins couple protein deacetylation with the cleavage of NAD^+, a feature that makes them key elements for the interconnection between cell homeostasis and aging. In addition, Sirtuins also influence the activity of many metabolic enzymes in humans by modulating their acetylation state, strengthening the linkage between the metabolic *status* of the cell and Sirtuin function.

In this context, this work aims to deepen the knowledge on some extrinsic and intrinsic regulators of chronological aging. Among intrinsic factors, we

analyzed Sir2, which plays a pro-aging role in CLS. We found that the lack of this deacetylase influences some aspects of the cell metabolism, with a particular regard to ethanol and acetate. Among the extrinsic factors, and particularly dealing with acetate, we focused on Ach1, a mitochondrial enzyme, whose function has not been well characterized yet but that may play a key role in the metabolism of acetic acid at mitochondrial level.

To analyze the interconnections among Sir2 activity, lifespan and the cell metabolism, we performed experiments in batch with fermentative and non-fermentative carbon sources, during chronological aging and in chemostat (glucose-limited cultures pulsed with ethanol and acetate) where growth parameters together with metabolite contents were analyzed. During chronological aging, CLS was also determined. Since in cells lacking Sir2 we determined an increase in acetate and ethanol catabolism, we focused on gluconeogenesis (in particular on Pck1, the rate limiting enzyme) and on the glyoxylate cycle (in particular on one of the two unique enzymes of this cycle, Icl1). In fact, both processes are fundamental when cells are growing on acetate and ethanol. By measuring the activity of Pck1 and Icl1, we found that in *sir2*Δ cells both these enzymatic activities were enhanced. In particular, the increased activity of Pck1 correlated with a higher acetylation level of this protein, giving also experimental evidence of a model where it has been proposed that Pck1, acetylated by Esa1, could be the target of Sir2-mediated deacetylation. Moreover, to check whether the activity of these enzymes was related with the CLS phenotype of *sir2*Δ cells, we inactivated *ICL1* and *PCK1* in *sir2*Δ cells to analyze the longevity and the metabolite level of the double mutant strains during chronological aging: both *icl1*Δ and *pck1*Δ mutants accumulated high level of extracellular acetate and ethanol and were short-lived mutants. In addition, *ICL1* and *PCK1* inactivation had epistatic effects on *sir2*Δ cells. Moreover, consistent with an increased gluconeogenetic flux, trehalose levels in *sir2*Δ stationary phase cells were higher compared with wild type cells. On the whole, we provided evidence that *SIR2* inactivation positively affects acetate metabolism by enhancing the glyoxylate-requiring gluconeogenesis. In the aging context, this implies lower levels of negative extracellular factors and a major accumulation of

protective trehalose which create a beneficial environment for long-term survival of non-dividing cells.

Then, we focused on acetate, and in particular on Ach1, a mitochondrial enzyme whose exact function has not been well characterized. In the '90, Ach1 was identified as mitochondrial hydrolase, even though the physiological role of acetyl-CoA hydrolysis was not clear. Recently, in *Aspergillus nidulans* an enzyme with high sequence identity with Ach1 was identified which is involved in the process of propionate detoxification. On the basis of this, the hypothesis arose that in *S. cerevisiae* as well this enzyme could catalyze a transferase reaction by activating, rather than hydrolyzing, acetyl-CoA from acetate. In this work of thesis, we characterized the phenotype of *ach1*Δ cells, with the aim of better understanding the role of this protein in acetate metabolism and any potential implications on mitochondrial functionality and on CLS modulation. We found that chronologically aging *ach1*Δ cells accumulated a high amount of extracellular acetate which correlated with a short-lived phenotype. This phenotype was strictly dependent on extracellular acetate since, when the acid stress was abrogated either by a calorie restricted regimen (no acetic acid production) or by transferring chronologically aging *ach1*Δ cells to water, cell survival was restored. Moreover, the short-lived phenotype of chronological aging *ach1*Δ cells is accompanied by ROS accumulation, a compromised mitochondrial activity and a precocious activation of the Yca1-dependent apoptotic pathway. In agreement with this compromised mitochondrial activity, we also saw that *ach1*Δ cells had severe problems to grow in media containing acetate as carbon source, underlining a primary role of Ach1 enzymatic activity in acetic acid detoxification which is important for mitochondrial functionality. Mitochondrial functionality which also plays an essential role for chronological cell survival.

Since we found an inverse correlation between extracellular ethanol and acetic acid level and CLS, further experiments were performed to clarify the role played by these two pro-aging factors. Data obtained support the hypothesis that at physiological levels is not the mere presence of ethanol

and acetic acid to influence the CLS but it is their metabolism. Thus, both these C2 compounds act as carbon sources that prevent entry of cells into a calorie restriction-like state, the only one in which cells are able to maintain a long term survival.

1

Introduction

One of the first biotechnological tools even known is the budding yeast *Saccharomyces cerevisiae,* exploited for wine making, brewing or baking. It is listed as a GRAS (Generally Regarded as Safe) organism; its genome is completely sequenced since 1996 [1] and a complete library of gene deletion mutants has been constructed and widely analyzed [2,3]. This, together with the diverse and efficient genetic tools available, the easy transformation and the fast cultivation made *S. cerevisiae* a useful tool also for studying many processes.

Moreover, the basic cellular machinery and molecular processes appears to be highly conserved in other eukaryotes, including humans [4]. As a consequence, since it was recognized that many yeast genes have functional orthologues in mammalian organisms, yeast has become an effective model system for the study of diverse cellular processes, including mechanisms involved in lipid metabolism [5], apoptosis [6] or cancer [7]. Furthermore, yeast cells can efficiently express heterologous proteins: this aspect has been widely exploited for studies of protein that did not have their counterpart in yeast. As a consequence, the so-called "humanized" yeast systems have been constructed to study specific disease-related proteins to unravel disease-related processes or to identify novel medicinal compounds [8]. Examples of this are common in the field of neurodegenerative disorders, in particular for protein-misfolding disorders such as Alzheimer's, Parkinson's or Huntington's disease [9,10].

One of the most complex processes to understand is aging since this phenomenon involves many different aspects at the same time, such as the cell metabolism, the transcriptional control or cell death induction. To unravel the role and the interconnections among all the processes leading to aging, the unicellular eukaryote *S. cerevisiae* was proven to be a powerful simpler model organism. The foundation of yeast aging was laid in 1959, when Mortimer and Johnston first discovered that yeast cells can divide a finite number of times that can be measured [11].

This chapter details some advances in the aging research, firstly giving an overview on the conserved pathways that modulate aging; afterwards, it will deepen more specifically into the known mechanisms and processes in yeast that control chronological aging.

1.1 Aging

1.1.1. Definition and model systems

The first evidence underlining the complexity of this process comes from the definition of the term 'aging'. It could be generally referred to as 'the passage of time', but it is mostly considered as the 'biological process of growing older', often in a negative sense. Many possible different definitions of aging can be given, but they all more or less comprise the idea of a deleterious, progressive and intrinsic process, that is also conserved among the species. Aging studies are increasing in biomedical research. The demographics of the world are rapidly changing, leaving a population with an increasing number of elders and a declining number of working-age individuals to support them. Aging itself is the leading risk factor for a variety of widespread diseases, among which most of neurodegenerative diseases. If it was possible to understand aging and modify its rate, the consequences would hopefully be a reduced incidence or progression of diseases and an increased health span. The potential of interventional approaches targeted at aging has yet to be realized because aging is a complicated multisystem process. However, research over the last decades has led to significant improvements in the knowledge. One of the most striking and recent findings is that it is possible to administer a clinically approved drug, rapamycin, to mice at 20 months of age and extend both their lifespan and health span [12].

Over the years, many theories have emerged to explain what process or mechanism drives aging and which are the causes, since almost every important discovery in molecular or cellular biology has led to a new family of theories of aging. Most of them have old origins, but the inherent difficulties of studying human aging make testing these theories a long and expensive process. Moreover, interpreting the results from longevity studies is frequently controversial and discriminating between causes and effects is often impossible. Nevertheless, some theories have gathered more empirical support than others. The aging process may derive from changes occurring

in parallel in different tissues due to intrinsic cellular mechanisms or changes in one tissue may be predominant. Some authors argue that aging is located within one tissue, while others defend that aging originates in all tissues. Neurons and the brain might play a regulatory role in aging [13]. Furthermore, intrinsic changes may occur in human cells as we age and it appears that intrinsic cellular mechanisms play a key role, though these can be modulated by extracellular factors. Of the many theories of aging that exist, the family of 'damage-based theories' defends that aging results from a continuous process of damage accumulation originating in by-products of metabolism; thus, a certain form of damage accumulates throughout the entire lifespan and causes most aspects of aging. Typically, this damage is a by-product of normal cellular processes, or a consequence of inefficient repair systems. On the other hand, 'programmed theories of aging' argue that aging is not a result of random or stochastic process but is rather driven by genetically regulated processes. In fact, it cannot be denied that aging has a strong genetic component. Of importance, even damage-based theories of aging recognize that certain genetic factors, such as defensive or protective genes, play a role in aging. Likewise, programmed theories of aging recognize that some forms of damage contribute to aging and that environmental factors influence the outcome of aging to some degree [14,15]. So the difference lies in the underlying mechanism: damage-based theories of aging argue that aging is predominantly a result of interactions with the environment or damage from chemical reactions, while programmed theories argue that aging is predetermined and occurs on a fixed schedule triggered by genetic programs.

From an evolutionary point of view, the advantage of a system that includes aging would be that the population size would be limited, for example to secure food stocks, and that there would be an acceleration of turnover of generations for adaptation to changing environments. However, it remains hard to see how this could have been developed at the level of single individuals. If one member had a dysfunctional 'internal clock' gene, for instance because of a mutation, it would have the advantage of a longer life and quickly overcome the others. This reasoning does not consider the idea of the selection at the level of the group, according to which certain altruistic behaviors beneficial at group level can be selected during evolution (even though detrimental at individual level). These theories seem to have been

applied mostly to mammalian species; however, recently, several reports claim to observe this altruistic behavior in unicellular organisms, for instance the apoptosis-like features in *S. cerevisiae* [6]. The explanation of these observations could reinforce group selection theory also in unicellular organisms.

As said before, yeast has proven to be a qualified model for different kinds of research. Nevertheless more than a doubt arouse at the beginning whether it was also a good model to study the aging process. It took also some time before it was found that yeast cells as well can age. No more than 50 years ago, Mortimer and Johnston made the observation that was the basis for the application of genetic analysis to the study of aging in yeast. Observing the repeated divisions of individual yeast mother cells, these scientists noted that in a genetically identical population of yeast, each cell ceased to divide after giving birth to a finite number of daughter cells [11]. For a given strain of yeast, the likelihood of mortality increased exponentially with increasing numbers of cell divisions. When Mortimer and Johnston plotted their data, they made the striking observation that the mortality curve for a population of yeast closely resembled the mortality curves for many other organisms, including humans [11]. In the years that followed, radical advances characterized the study of yeast biology: also on the forefront of aging research, yeast provided an invaluable model system.Before the crucial experiments by Mortimer and Johnston, it was commonly believed that yeast could divide forever. However, *S. cerevisiae* is an asymmetrically budding yeast, providing an easily recognizable difference between the mother cell (soma) and her offspring (germ line). Besides the difference in size, the mother cell is clearly distinguishable by the bud-scars left after separation of mother and daughter.

Actually, there are several advantages for choosing *S. cerevisiae* for doing aging research:

- it ages rather fast;
- it is relative easy to determine the replicative age of a cell (by counting the bud scars);
- at least several homologies have been found between aging yeast and higher eukaryotes concerning aging, for example the insulin/glucose

signaling and calorie restriction features [16], as described in detail below;
- the aging of yeast can be controlled by external factors and can be easily manipulated.

One of the tasks that remain is to determine at which extent, these pathways are specific for yeast or the other models of aging and which are conserved among more complex organisms.

Aging in humans

Contrary to mortality rates that increase exponentially with age, the human functional decline tends to be linear, declining slowly over time. Briefly, aging is characterized by changes in appearance, such as a gradual reduction in height and weight loss due to loss of muscle and bone mass, a lower metabolic rate, longer reaction times, declines in certain memory functions, declines in sexual activity, menopause in women, a functional decline in audition, olfaction and vision, declines in kidney, pulmonary and immune functions, declines in exercise performance and multiple endocrine changes [17,18].

While evidences for models systems clearly demonstrates that single gene mutations can extend lifespan in these organisms, it has yet to be conclusively proven that this is the case for humans. The first direct evidence for the existence of a gene that confers exceptional longevity on humans was provided by Puca and colleagues in 2001 [19]: a genome-wide scan of 308 long-lived individuals identified a *locus* on chromosome 4 that is associated with long lifespan. Although no specific mutations are known that increase human lifespan, several genetic diseases appear to accelerate many features of the normal aging process. This class of disease is collectively known as segmental progeroid syndromes (among which Hutchinson-Guilford Syndrome and Werner Syndrome) [20].

Aging in humans has been studied at cellular level as well. Hayflick first noted in 1965 that human cells have a finite replicative capacity *in vitro* [21]. After him, this phenomenon of growth arrest after a period of apparently normal cell proliferation is known as the 'Hayflick limit'. Afterwards, it has then been demonstrated that the replicative lifespan of human cells is determined by telomere length. In parallel, as described before, several

theories of human aging propose that many of the degenerative processes that occur with age are the result of accumulated cellular damage; this cellular damage could be the result of exposure to environmental insults or could be a byproduct of normal cellular function [22].

Aging in model systems

The long lifespan, difficulties obtaining genetically pure isolates and obvious ethical considerations makes it too difficult to directly study many aspects of aging in humans. Fortunately well characterized systems for aging research can be done with the same purpose in mice, rats, flies, worm and yeast.

Invertebrate model organisms have acted as engines of discovery for genes and mechanisms involved in extension of life span, but the mouse is the most practical mammal for establishing if homologous genes and processes can extend healthy lifespan, and hence lead to human clinical trials [12,23,24]. Much of the research on rodents has been concentrated on the effects of calorie restriction (CR) on lifespan. Already in 1935, McCay and colleagues first showed that CR could extend both mean and maximum lifespan of rodents [25]. Since then, CR has been found to extend lifespan in a wide diversity of organisms (see Chapter 1.2.3). in rodents it was also demonstrated the importance of hormonal signaling on lifespan.

Caenorhabditis elegans was one of the first organisms in which mutations that extend lifespan were identified. Unlike yeast, it allows studies of different cell types and organs, such as the nervous or digestive systems. It is also more closely related to mammals [22]. In *C. elegans*, more than 50 longevity genes have been identified, most of which in well defined pathways. Similar to rodents, hormonal signaling and metabolism appear to be critical for lifespan determination. Resistance to stress, another common theme among model organisms, also was found to correlate with longevity. Finally, in *C. elegans*, it was shown for the first time that the orthologue of the yeast longevity gene *SIR2*, Sir-2.1, functions to extend lifespan in this higher eukaryote as well [26], even though the function of Sir-2.1 in worms remains still unclear.

The fruit fly *Drosophila melanogaster* is another important model for establishing evolutionary conserved mechanisms, for studying events in

different tissues, which are more numerous and differentiated than in *C. elegans* and for examining sex differences [22]. Even though only few mutations extending lifespan have been discovered, still, as for other organisms, insulin signaling, stress response and CR are important determinants of fly lifespan.

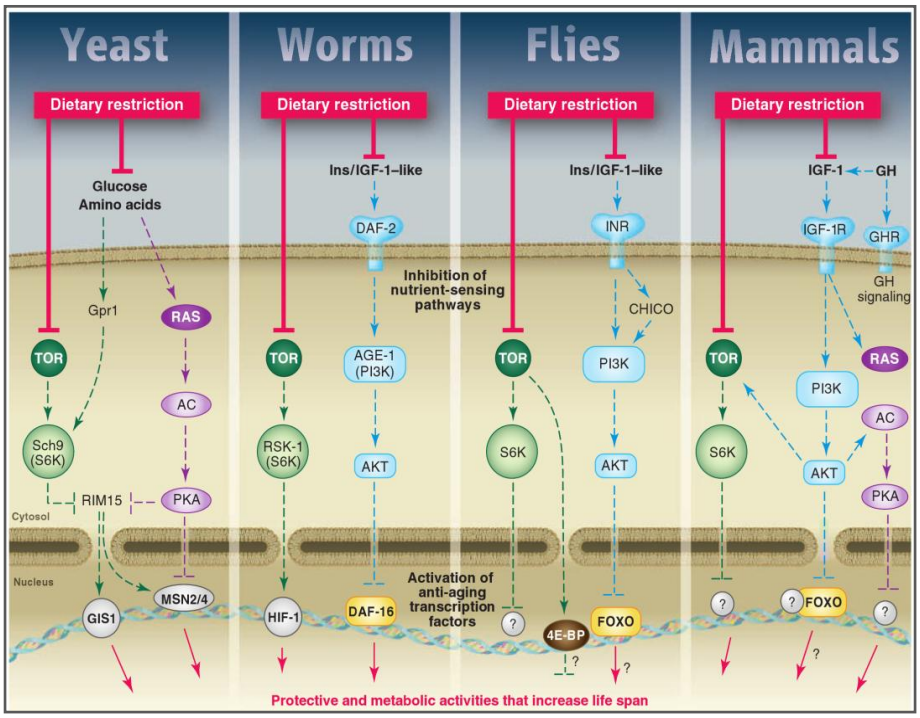

Fig. 1.1 A model for the conserved pathways that regulate longevity in various model organisms and mammals. Nutrient signaling seems to be the common element that dramatically influence lifespan extension in every organism studied (see also next paragraph for further information). CR reduces the activity of various signal transduction pathways either directly (yeast) or indirectly through the reduced levels of growth factors such as IGF-1 (worms, flies, mammals). The role of AKT and S6K in aging appears to be conserved in yeast, worms, flies and mice. Similar transcription factors inactivated by either the PKA/AKT or TOR/S6K pathways affect cellular protection and/or aging in all the major model organisms. Notably, in the multicellular worms, flies and mice, these genes may promote aging within the cells in which they are expressed but also in other cells through the regulation of circulating factors (Adapted from [22]).

Findings from model organisms have recently begun to be tested in monkeys but also in human volunteers, including subjects with mutations analogous to those that extend the healthy lifespan of laboratory animals [27–29], further underlining the importance of the research on model organisms.

1.1.2. Chronological and replicative aging

In yeast, two aging models have been defined: replicative and chronological aging (Fig. 1.2). Replicative lifespan (RLS) is defined as the number of daughter cells an asymmetrically dividing mother generates in the presence of nutrients before senescence [30]. With replicative aging, cells bud off a limited amount of daughter cells. In this case, lifespan is measured by number of divisions instead of using a chronological time-scale. On the other hand, chronological lifespan (CLS) is the mean and maximum survival period of a population of non-dividing cells in stationary phase. In this context, viability over time is defined as the ability to restart growing upon reefing [31]. These non-dividing yeast cells are used as good models for differentiated non-dividing mammalian cells, in spite of the differences in metabolic activity rate [32,33], as said before.

Although being a powerful tool in longevity studies, the two ways in which yeast lifespan analysis can be done reveals discrepancies and similarities between the approaches, with a striking example being Ras2. Deletion of this protein (amongst other things involved in nutrient signaling through the protein kinase A pathway) leads to an extension in lifespan for stationary phase cells and to a decrease in lifespan for dividing cells [34]. However, when the adenylate cyclase Cyr1 (also involved in the PKA signaling pathway) is mutated, both the replicated and the chronological lifespan are extended [32]. These observations and the observations that passage through stationary phase advances replicative aging [35,36] shows that the two aging modes are strictly related.

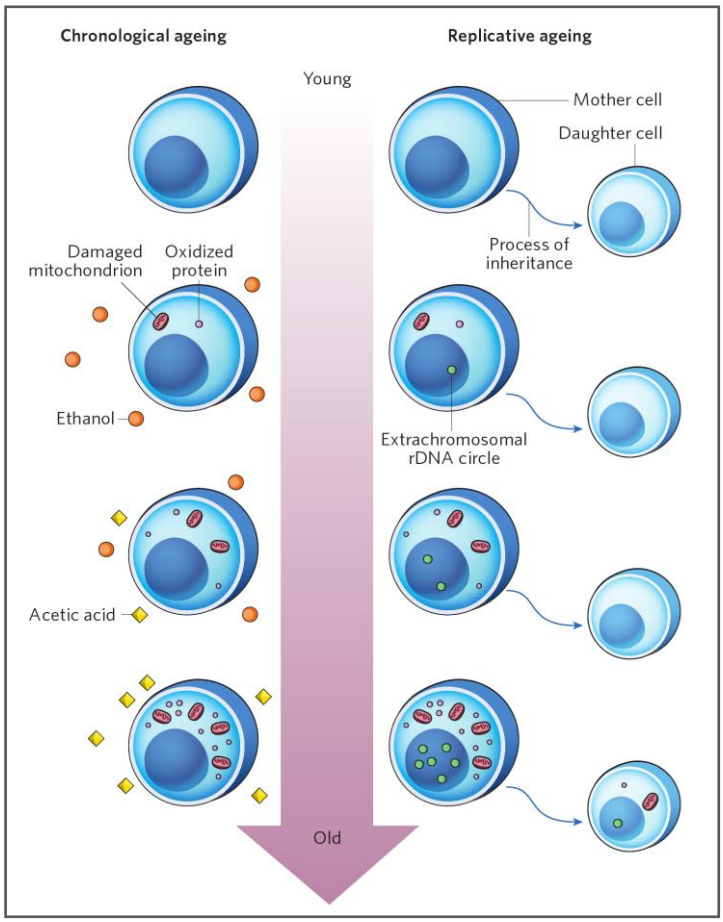

Fig. 1.2. The aging processes in yeast. In a chronologically aging cell (left), damages accumulate in these non-dividing cells: damaged mitochondria and also oxidized proteins accumulate and probably contribute to chronological senescence. In replicative aging yeast cells (right), damage is asymmetrically inherited by the mothers. Here, nuclear extra-chromosomal ribosomal DNA circles, cytoplasmic oxidized proteins and damaged mitochondria contribute to replicative senescence. In very old mother cells, asymmetry breaks down and the daughter cell can inherit sufficient damage to become prematurely aged (from [37]).

As previously said, RLS studies date back more than 50 years [11]. Since then, nearly 100 yeast replicative aging genes have been identified whose deletion enhances longevity; it has been estimated that up to 2% of the

nonessential genes are likely to fall into this category; moreover, approximately 20% of the nonessential genes result in short lifespan when deleted [38,39]. In Fig. 1.3 are reported the main known pathways regulating RLS. One of the most famous yeast aging genes is *SIR2*, which encodes the founding member of the Sirtuin family of NAD-dependent protein deacetylases (see Chapter 1.3) [40]. One mechanism by which Sir2 activity promotes replicative longevity is by suppressing homologous recombination in the ribosomal DNA (rDNA) that can cause the formation of extrachromosomal rDNA circles (ERCs) [30]. Recent studies have suggested other roles for *SIR2* in yeast aging, not directly involving the regulation of the chromatinic structure but pointing to modulate the function of extra-nuclear substrates. For example, loss of Sir2 results in a defect in asymmetric retention of oxidatively damaged cytoplasmic proteins in the mother cell [41,42]. Furthermore, it was hypothesized that Sir2 can control RLS also in an ERC-independent manner, by controlling cytoskeleton function and polarity [43,44]. Importantly, this has been one of the first evidences that Sir2 could act directly on non-histone substrate. Mitochondrial function as well plays a critical, but poorly understood, role in RLS determination. This was clearly shown for the first time by studies from the Jazwinski lab demonstrating that induction of the retrograde response pathway, which transmits signals of mitochondrial stress to the nucleus, can increase RLS in certain genetic backgrounds [45]. Recently, it was also reported that enhanced proteasome activity is able to increase RLS by a mechanism that is genetically distinct from both CR and Sir2 [46]. This finding is consistent with a growing body of researches linking aging with loss of normal homeostasis of proteins in a variety of organisms, thus strengthening the idea that multiple yeast longevity pathways are conserved [47]. Like ERCs, damaged proteins are asymmetrically segregated to the mother cell during cell division. So it might be that elevated proteasome activity extends lifespan by reducing the age-related increase of this type of damage.

In order for an organism to be evolutionarily successful, maintaining a functional germline is essential. In part, aging yeast do this by maintaining damaged molecules in mother cells, in an attempt to protect their daughters from aging. Meiosis, however, is likely the principal manner by which yeast recombine their genome in the wild. A recent study suggests that

mechanisms exist to preserve the integrity of spores of aging diploid cells induced to sporulate [48]: by mechanisms still unknown, aging diploid cells induced to sporulate are able to remove age-associated damage.

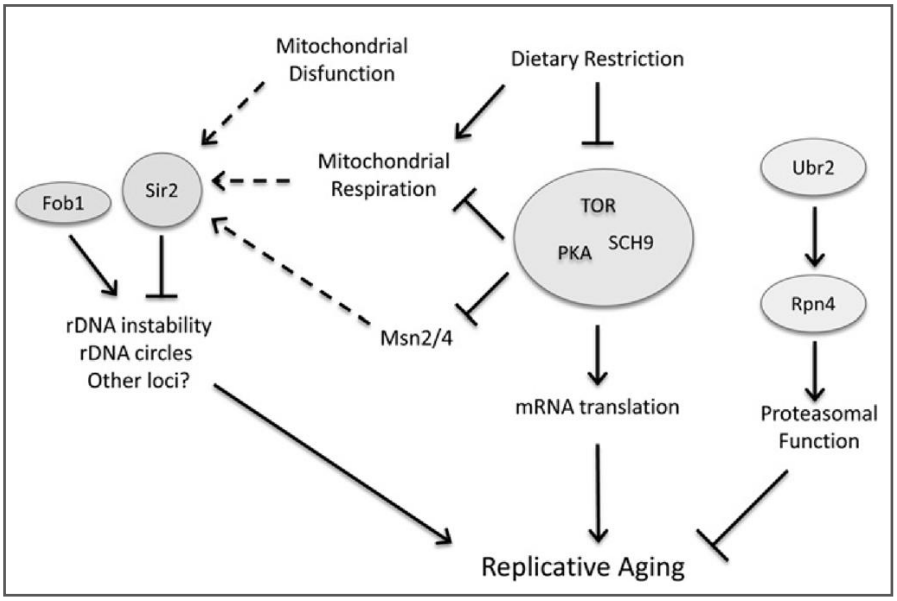

Fig. 1.3. Genetically distinct pathways modulating RLS. Sir2 and Fob1 influence RLS largely through their role in the rDNA, although there is evidence for aging-related functions for Sir2 at other *loci*, including telomeres, as well as by modulating asymmetric inheritance of cytoplasmic damage. CR is mediated through reduced signaling of the protein kinase A (PKA) and Target of Rapamycin (TOR) pathways. Ubr2 and Rpn4 influence RLS by modulating proteasome activity. The relationship of the retrograde response and mitochondrial dysfunction to these pathways remains unclear. Dotted lines represent potential points of crosstalk among proposed pathways (from [39]).

If replicative aging may be linked to aging of mitotic or stem cell populations in a complex organism, chronological aging may more closely resemble aging of non-dividing cells [39]. CLS is typically measured by growing a culture of yeast cells up to the post-diauxic and stationary phase [31]. The major genes and pathways regulating CLS in yeast show

remarkable similarities to those in worms, flies and mammals. As reported in Fig. 1.4, there are two major pro-chronological aging pathways in *S. cerevisiae*, both of which sense nutrient availability and control their utilization: the TOR/Sch9 pathway, activated by amino acids and other nutrients [32]; and the Ras/PKA pathway, mainly activated by glucose, but affected by other nutrients as well [49,50]. These two signaling pathways have partially overlapping, yet distinct, pro-aging effects [51]. Their regulatory role inside the cell will be further discussed in Chapter 1.2. A primary mode of action of both of them is their convergence on the stress-resistance regulator Rim15 and on the transcription factors Msn2/Msn4 and Gis1, as will be described more in detail in Chapter 1.1.6. In addition to stress, these factors regulate metabolism and the accumulation and utilization of intracellular and extracellular carbon sources [51,52]. Interestingly, Sir2 seems also connected to Msn2/4 activity and regulation of both RLS and CLS [53]. Results from many studies highlight the multi-factorial nature of chronological aging and point to several damage and stress pathways with pro-senescence effects, including:

- oxidative stress [54];
- mitochondrial dysfunction and reactive oxygen species (ROS) [52,55,56];
- reduced autophagy [57];
- nuclear DNA damage, mutagenesis and replication stress [58];
- metabolic alterations [51];
- extrinsic stress [59,60];
- as well as many other factors [61].

The most likely interpretation is that coordinated regulation of a variety of protective systems by stress, CR or mutations in nutrient signaling pathways promotes longevity extension. Importantly, repair and replacement systems, as well as epigenetic changes, are also likely to play a key role in yeast age-dependent cellular damage and mortality (as reviewed in [39]).

In the next paragraphs, some aspects related to yeast aging will be described more in detail, such as the apoptotic process, calorie restriction, pH homeostasis, as well as some key modulations of nutrient sensing pathways.

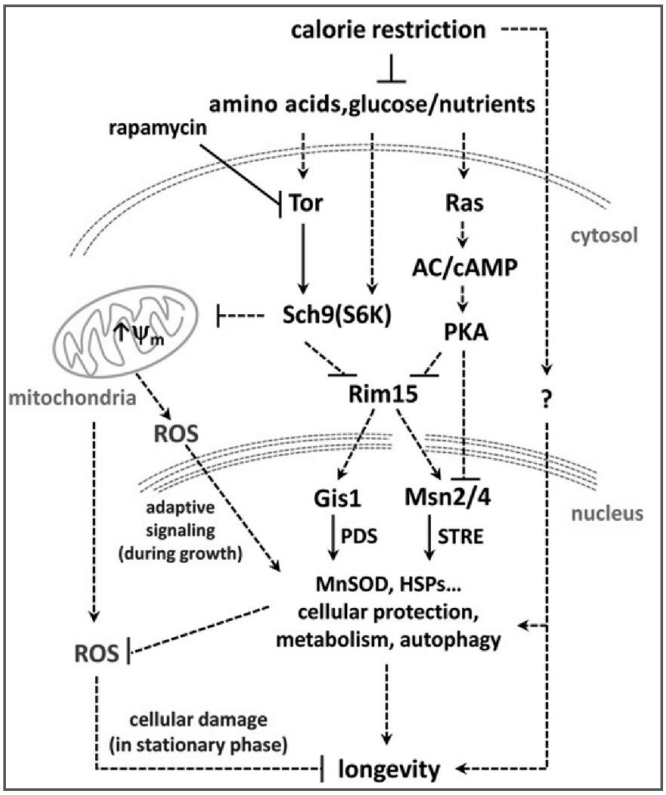

Fig. 1.4. CLS major regulatory metabolic pathways. The nutrient-sensing pathways controlled by Sch9, TOR and Ras converge on the protein kinase Rim15. A major portion of the effect of CR on longevity appears to be mediated by the downregulation of the Ras/PKA and TOR/Sch9 pathways. Reduced TOR/Sch9 signaling also increases coupled mitochondrial respiration and membrane potential during growth, leading to an adaptive mitochondrial ROS signal (from [39]).

1.1.3. Apoptosis

Chronologically aging yeast cells show features of apoptotic death such as nuclear condensation/fragmentation, phosphatidylserine exposure and caspase activation [62]; ROS formation is enhanced in agreement with a

central role for superoxide in the activation of yeast apoptosis during aging. Some genetic interventions have also been described that delay chronological aging and the appearance of the associated apoptotic features, such as the inactivation of the *YCA1* (coding for the metacaspase Yca1), *AIF1* or *NDE1* genes (coding for the yeast orthologue of the AIF-homologous mitochondrion associated inducer of death) or the overexpression of the stress-dependent transcription factor Yap1 [63,64]; under normal growth conditions, Yap1 localizes in the cytoplasm due to the interaction with Crm1, a nuclear export receptor. During oxidative damage, Yap1 accumulates in the nucleus where it drives the transcription of many genes, among which especially those involved in ROS scavenging such as *SOD*, catalases as well as some genes involved in DNA repear or cell cycle check point control [65–67]. However, the most effective interventions so far identified on CLS that delay also the appearance of apoptotic markers are again the deletion of either *RAS2*, *TOR1* or *SCH9* [32]. When wild type cells incubated in medium are compared to long-lived *sch9*Δ mutants, a difference is observed in apoptotic markers appearance, but also important differences can be observed in terms of activation of the stress response: for example, *sch9*Δ mutants are less susceptible to oxidative stress, mainly due to a high expression of *SOD2*, the gene coding for the mitochondrial superoxide dismutase [68]. A similar phenotype has been observed in calorie restricted cells: normally, the activation of an "aging program" blocks cell protection and accelerates cell death.

The existence of such a program for a unicellular organism has to be considered in the context of a population of millions of yeast, where cellular "suicide" represents a survival strategy for the group [69]. Studies conducted in the last years have shown that longevity is genetically determined and depends on evolutionary conserved pathways. In simple organisms such as yeast and worms the downregulation of these pathways is induced by starvation and promotes the activation of somatic maintenance to guarantee survival until nutrients become available. The discovery of these aging pathways has led to the "programmed longevity theory" according to which aging is caused by the genetically programmed decline of a repair and maintenance system that can control cellular damage. The "programmed and altruistic aging theory" hypothesizes instead the existence of a genetic program activated in order to "sacrifice" the majority of the population to

allow the survival of a few adapted organisms. According to this theory natural selection functions at the level of the group rather than at individual level [69,70].

Since the discovery of yeast apoptosis in 1997 [71], it has become apparent that, among other cellular processes, the apoptotic core machinery is conserved in yeast to a degree that makes it a suitable model organism to approach pending questions on human apoptosis and its deregulation in the context of neoplasia, neurodegenerative diseases and aging. Assays for apoptotic and/or necrotic cell death, such as clonogenic determination of viability, accumulation of ROS, DNA fragmentation, exposition of phosphatidylserine or cell integrity assays are routinely used in the field of yeast. It is important to underline that it is only the combined application of these assays that accurately determines apoptotic death: the evaluation of just one of these methods cannot alone be sufficient to define an observed death phenotype as 'apoptosis' [72].

Numerous stimuli can induce yeast apoptosis, provided for example externally in the form of chemical or physical stress, via heterologous expression of human apoptotic proteins (like Bax or Bcl2 [73]) or by the yeast cells themselves, as part of lethal signal transduction pathways. Actually, the first proof of ROS being key regulators of yeast apoptosis arose from experiments involving treatment with low doses of hydrogen peroxide (H_2O_2) [74]. Still now, ROS are considered key players in this process [55,56,75]. Oxygen radicals are defined as molecular derivatives of oxygen with one or more unpaired electrons (Fig. 1.5).

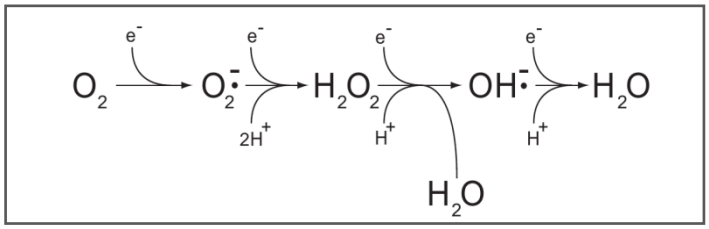

Fig. 1.5. Stepwise reduction of oxygen to subsequently superoxide, hydrogen peroxide, hydroxyl radicals and finally water.

Because of these unpaired electrons they are 'eager' to react with other molecules. In biological systems, all kinds of constituents ranging from DNA, to phospholipids to proteins and metabolites, can be altered in their structure. These changes can be amongst other things, DNA double strand breaks, lipid modifications and dysfunctional proteins. Actually, the term "Reactive Oxygen Species" is a broader definition also covering for instance hydrogen peroxide. Hydrogen peroxide is not an oxygen radical in the strict sense because it has no unpaired electrons. However it is an important intermediate; so, to include it, the term ROS was coined. Because the chemical properties of the ROS are different, their preferred cellular targets differ. 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 (reviewed in [55]). On the whole, ROS-induced damage is complex and frequently irreversible; moreover, it further impairs mitochondrial function, rendering them in turn prone to further ROS generation. However, being able to use oxidative phosphorylation to obtain energy comes together with the adverse side effects of the damaging oxygen radicals. Cells adapted to these threats and devised several protection mechanisms, such as antioxidant enzymes (glutathione reductases, superoxide dismutases or catalases), small antioxidant molecules (glutathione, vitamin E or vitamin C), trehalose or repair and turnover systems (ubiquitin system, DNA repair) [76].

Several molecular factors are involved in apoptosis, including the yeast metacaspase Yca1 (see below) and the apoptosis-inducing factor-1 (Aif1) [64]. Acetic acid is a commonly used compound to induce yeast apoptosis [77]. Treatment of yeast cells with acetic acid leads to temporary activation of the proteasome system and release of cytochrome *c* from mitochondria [78,79]. Cytochrome *c* is a mitochondrial protein with a function in the respiratory chain and, in mammals, additionally a well-characterized lethal factor implicated in the activation of caspase-9 in the intrinsic pathway of apoptosis. However in yeast its role has not been completely clarified upon release into the cytosol. It has also been hypothesized that it could represent an ancestral pro-death factor, whose lethal role might only rely on the indirect consequence of respiratory dysfunction and subsequent ROS

accumulation, with no direct intervention in the apoptotic machinery itself [72]. Moreover, the signaling of acetic acid-induced apoptosis is linked to amino acid metabolism as well as to the TOR pathway, which both connect the nutritional condition of the cell to the aging process (reviewed in [72]). A variety of additional agents have been reported to induce an apoptotic phenotype in yeast. These include high ethanol concentration, hypochlorous acid, high salt, UV irradiation or heat stress, but also several compounds that normally constitute nutrients, such as supra-physiological concentrations of glucose itself, ornitric oxide (NO), produced by cells themselves in certain conditions as side product of the ammonium metabolism [80,81].

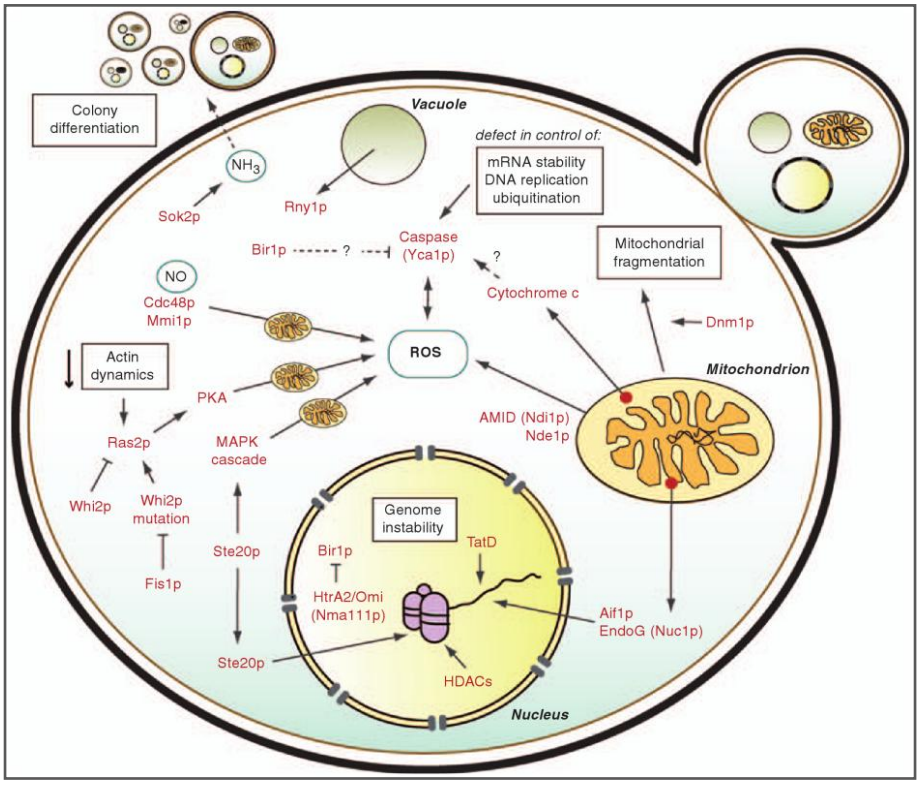

Fig. 1.6. The network of factors involved in yeast apoptosis. Important roles are played by several small molecules as lethal mediators, as well as by an increasing number of orthologues of crucial mammalian apoptotic proteins involved in different conserved proteasomal, mitochondrial, vacuolar, and epigenetically

regulated cell death pathways. Between brackets, the names of the yeast orthologues of the human proteins described are reported (taken from [72]).

The regulation of such a complex process like apoptosis requires the interplay between small molecules, proteins and pathways involving the nucleus, mitochondria, the cytosol and lysosomes. In Fig. 1.6, the interconnection of the different players involved in yeast apoptosis are represented. The first molecular characterization of yeast apoptosis emerged from experiments on CDC48 mutants, linking cell death to proteomic alterations in mitochondria, release of cytochrome *c* to the cytosol, increased ROS production and development of apoptotic markers [74]. Yeast bears at least one orthologue of mammalian caspase: the metacaspase Yca1. Though different cleavage specificity – mammalian caspases cleave their substrates after aspartate (an acidic residue), metacaspases after arginine or lysine (basic residues), both metacaspases and caspases play a pivotal role during cell death execution. This, in addition to the common natural, death-related target molecules, argues for a functional conservation between caspases and metacaspases [82]. So far, in addition to Yca1, three other proteases have been described in the yeast apoptotic scenarios: the separase Esp1 [83], the nuclear HtrA-like protein Nma111 (nuclear mediator of apoptosis) [64] and Kex1 [84].

1.1.4. Calorie restriction

A conserved non-genetic intervention that promotes lifespan extension is CR [85,86]. Dietary restriction (DR) is defined as a reduction of particular or total nutrient intake without causing malnutrition. Dietary restriction in this broad sense includes CR, in which total food intake is reduced. Understanding the molecular mechanisms of how DR slows aging and age-associated diseases has gained importance in the last years. Anyway, in humans, DR is already known to provide major and sustained beneficial effects against obesity, insulin resistance, inflammation or oxidative stress, in agreement with the metabolic and functional changes observed in dietary-restricted rodents [87]. Moreover, it induces some hormonal adaptations

(like increased adiponectin and reduction in testosterone or insulin) together with reduced cholesterol or blood pressure, all risk factors for cardiovascular disease [87]. However, extreme DR can lead to several detrimental health effects such as amenorrhea, infertility, osteoporosis or immune deficiencies. Thus, it will be important to examine these negative side-effects in dietary-restricted subjects that are not malnourished. As reviewed in [88], in certain species, restriction of individual components of the diet, like proteins or amino acids, is sufficient for lifespan extension; an important challenge in this field includes examining the role of individual nutrients on aging and to further find whether these effects are conserved among species. Anyway, the utilization of the powerful genetic tools available in the simpler and short-lived model organisms like *S. cerevisiae*, *C. elegans* and *D. melanogaster* is giving fundamental insights on the basic mechanisms of the protective effects of CR. One of the proposed mechanisms is that under poor nutrient conditions the cell reallocates resources to survive harsh environmental conditions [88].

Of relevance, well-studied key players in CR and lifespan modulation, such as NAD^+ levels, amino acid metabolism and generation of ROS, are all dependent on mitochondria and, more in detail, mainly on mitochondrial respiratory activity [94]. Having the powerful opportunity to diversely coordinate the nutritional and metabolic status of yeast cells by a simple change of growth media, the response to various stresses has been used to study distinct cell death pathways under these conditions. For instance, the special ability of *S. cerevisiae* to switch on and off respiration in response to changes in the carbon source, together with the possibility to obtain strains lacking mitochondrial DNA (defined as *petite* or *rho* strains), allowed many genetic evaluations of the mitochondrial role during cell death [95]. In Fig. 1.7, the main changes induced in the cell by different type of carbon sources on the metabolism are represented.

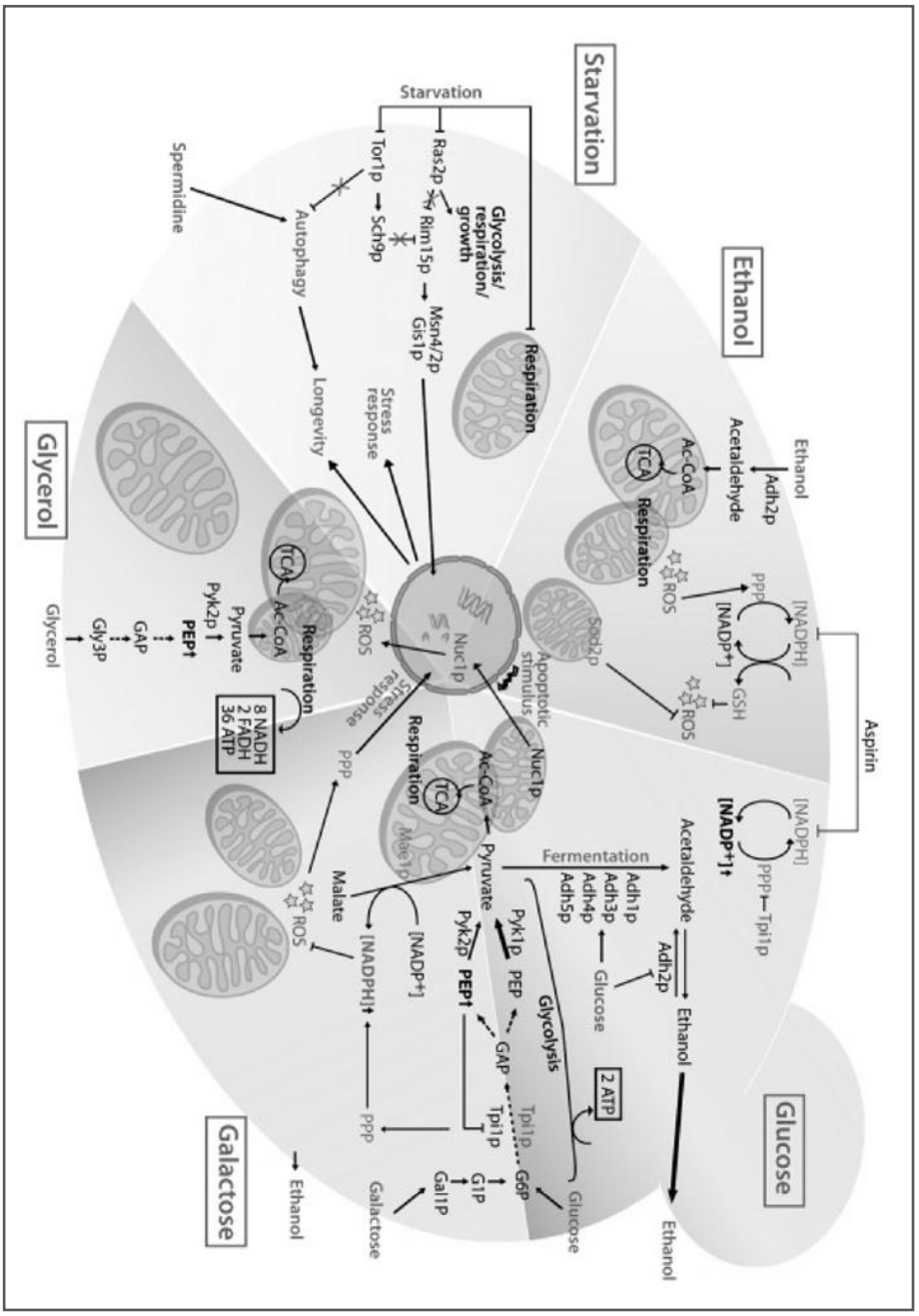
Starvation
Ethanol
Glucose
Glycerol
Galactose
Starvation
Tor1p
Sch9p
Ras2p
Rim15p
Msn4/2p
Gis1p
Glycolysis/
respiration/
growth
Autophagy
Spermidine
Longevity
Stress
response
Respiration
Ethanol
Adh2p
Acetaldehyde
Ac-CoA
TCA
Respiration
ROS
PPP
[NADPH]
[NADP+]
GSH
Sod2p
Aspirin
[NADPH]
[NADP+]↑
PPP
Tpi1p
Acetaldehyde
Adh2p
Ethanol
Fermentation
Adh1p
Adh3p
Adh4p
Adh5p
Glucose
Pyruvate
Pyk1p
Pyk2p
PEP↑
PEP
Glycolysis
GAP
Tpi1p
G6P
G1P
Gal1P
2 ATP
Glucose
Galactose
Ethanol
Nuc1p
Apoptotic
stimulus
Stress response
Mae1p
Malate
[NADP+]
[NADPH]↑
PPP
Glycerol
Gly3P
GAP
PEP↑
Pyk2p↑
Pyruvate
Ac-CoA
Respiration
8 NADH
2 FADH
36 ATP

Fig. 1.7. Changes in growth media and their effects on cell death. Glucose presence activates those alcohol dehydrogenases (Adh1,3,4,5) that are responsible for ethanol formation during fermentation. Additionally, glucose represses Adh2, inhibiting the vice-versa production of acetaldehyde from ethanol. Ethanol and glycerol stimulate expression of the less active Pyk2, which furthermore enhances intracellular levels of PEP. Enhanced PEP leads to an activation of the PPP, increases respiratory metabolism via pyruvate and acetyl-CoA intermediates. Upon strict carbon starvation metabolic fluxes are restricted and the stress response mechanisms and autophagy are induced, which ultimately results in longevity. Abbreviations: Ac-CoA-acetyl-coenzyme A; GAP-glyceraldehyde 3-phosphate; Gal1P-galactose 1-phosphate; G1P-glucose 1-phosphate-G6P, glucose 6-phosphate; Gly3P-glycerol 3-phosphate-GSH, glutathione; NADP-nicotinamide adenine dinucleotide phosphate; PEP-phosphoenolpyruvate; PPP-pentose phosphate pathway.

In *S. cerevisiae,* CR was proposed to extend both RLS and CLS by targeting many age-associated processes rather than a single limiting factor [89]. CR can increase intracellular stores of trehalose and protect against oxidative stress, responses which have been attributed to maintenance of mitochondrial health [90,91]; moreover, CR inhibits TOR activity, an intervention that should increase CLS in part by an adaptive mitochondrial longevity signal involving increased ROS levels [92,93]: the idea is that these effects are mediated by a metabolic shift toward mitochondrial respiration induced by CR. Recently, it was also reported that CR cells exhibit a lower respiratory rate in stationary phase with respect to wild type cell and that lower overall mitochondrial effort could be the reason why CLS is extended [90]. In addition, CR also induces autophagy: enhanced degradation of damaged mitochondria, proteins or other macro-molecules could reduce the overall burden of cell damage.

In Table 1.1 some of the effects that mitochondrial metabolism can exert on lifespan are listed.

Table 1.1. Physiological conditions linked to mitochondrial function that affect yeast aging (adapted from [94]).

Physiological conditions that affect yeast aging	**Mitochondrial effect**	**Lifespan effect**
Defective mother/daughter segregation	Organelle misdistribution, accumulation of senescence factors.	Decreased RLS.
Activation of retrograde signaling	Higher α-ketoglutarate and amino-acid production, glutamate accumulation.	Increased RLS. Dependent on *RTG2*.
Inhibition of autophagy	Accumulation of protein aggregates.	Decreased CLS.
Histone deacetylase activation	NAD^+ regeneration in mitochondria are substrates for nuclear Sirtuins.	Increased RLS, but not CLS. Some results are strain-specific.
NAD^+ levels	Normal respiratory activity regenerates NAD^+, a substrate for Sirtuins.	Increased RLS and CLS.
Increased respiration	Higher NAD^+, decreased ROS production and oxidized products.	Increased RLS and CLS.
Glucose restriction	Dependent on respiratory enhancement and changes in NAD redox state.	Increased RLS and CLS.
Amino acid restriction	Activates retrograde signaling.	Increased RLS.

Studied for more than 70 years [25], CR can be mimicked in yeast by reducing the concentration of glucose or other nutrients in the growth media to varying degrees, resulting in enhanced RLS and CLS [96]. Of interest, no effect of CR has never be seen in Crabtree-negative yeasts, as well as when galactose, raffinose, glycerol or ethanol are the carbon source in Crabtree-positive yeasts [97]. Glucose signaling in Crabtree-positive yeasts leads to changes in a complex regulatory network involving many aspects of the energy metabolism, including modulating enzyme activities by altering mRNA, translation, amounts of the protein and/or promoting post-translational modifications (reviewed in [94]). As anticipated before, in yeast

as well, limiting amino acid content was proven to have an effect similar to CR: CLS is modulated by changes in their content in the medium, mainly regarding those amino acids that are involved in branched chain amino acid biosynthesis: leucine, isoleucine, valine and threonine [98]. Furthermore, the lack of glutamate or mitochondrial dysfunction triggers the retrograde (RTG) signaling pathway, which is in turn able to extends RLS, but may lead to accumulation of ERCs [45]. On the other hand, RTG is well known to crosstalk with the TOR pathway, which impacts lifespan, as will be detailed below. Also as a consequence of RTG inhibition by TOR, gene deletions of the TOR pathway or reduced TOR signaling extend both RLS and CLS [38,52,99].

1.1.5. Medium acidification

Another feature that is able to modulate (by shortening) CLS is media acidification [53,59], a phenomenon that is accompanied by ROS production and by those mitochondrial changes commonly associated with aging in yeast and other organisms [86]. Longo and colleagues showed that ethanol and medium acidification reduce the CLS [53]. In contrast, the Kaeberlein laboratory proposed that it is acetic acid, rather than ethanol, that increases mortality [37,59]. In addition, they proposed that the effects of CR can be explained by the reduced acidification of the medium: with alternative carbon sources, in stationary phase culture pH shows a significant inverse correlation with CLS [97,100]. It currently remains unresolved whether the positive effect of pH buffering on CLS arise primarily due to protection against acetic acid toxicity, altered sensitivity to ethanol in the culture, reduced intracellular oxidative stress or other additional mechanisms [36]. Dealing more specifically with acetic acid, it was recently shown that acetic acid accumulation can be prevented by deletion of *FPS1* [101,102], a gene coding for an aquaglyceroporin membrane channel that facilitates an energy-independent transmembrane transport into the cell of small molecules, such as water or glycerol, as well as of the undissociated acetic acid [103]. When grown at low pH, *S. cerevisiae* acquires enhanced resistance to acetic acid through loss of Fps1 function, mediated by transient activation of the mitogen-activated protein kinase Hog1. Hog1 directly phosphorylates Fps1,

targeting this channel for endocytosis and degradation in the vacuole. At least two other acetate carriers in derepressed cells have been identified: Jen1 and Ady2 [104,105]. Both these proteins are repressed by the presence of glucose, while the transcription of the corresponding genes is activated in the post diauxic phase [106]. Jen1 was identified as necessary for the uptake of lactate but it was also shown that it can transport other monocarboxylates, including acetate [104]; the protein Ady2 was found later to be essential for acetate transport activity in cells grown in acetic acid [105].

What is sure is that one of the crucial and fundamental tasks of a living cell is to maintain the homeostasis of intracellular pH concentrations and that this becomes a big effort for the cell especially when the extracellular pH becomes lower. In this context, it was also observed that the H^+-pumping ATPases and other salt channels are reported to be highly conserved in fungi and plants [108]. In yeast, the cytosol is maintained typically more alkaline and negatively charged than the extracellular environment in order to generate an electrochemical proton gradient that serves as a source of free energy for important cellular processes, among which organic acid export across the plasma membrane. To be noted, as reported in Fig.1.8, in eukaryotic cells, most organelles have their own specific pH value, associated with the different processes that take place in those compartments [107]: for example, H^+ gradients over various membranes are involved in the transport of nutrients by either symport or antiport [107], and the ΔpH over the mitochondrial inner membrane, created by activity of the electron transport chain, is used for ATP synthesis [109].

Since cell membranes are not freely permeable to hydrogen ions, transmembrane proton gradients are established by active proton-pumping mechanisms like the H^+-ATPase. The master enzyme that controls cell pH, nutrient and ion transport and the overall cell growth is the plasma membrane H^+-ATPase; besides, *S. cerevisiae* possess an F-type H^+-ATPase in the mitochondrial inner membrane and a V-type H^+-ATPase in the vacuolar membrane, responsible for acidification of other organelles in the secretory pathway [110]: pumping excess protons to the vacuole was proven to be a very efficient way of maintaining cytosolic pH (pH_c). Still, the master regulator of pH_c is the P-type H^+-ATPase Pma1 [108,111,112]. P-type ATPases are membrane proteins that couple transport of cations to ATP

hydrolysis. Pma1 is one of the most abundant proteins in the plasma membrane, comprising as much as the 50% of total membrane proteins [113] and pumps protons out of the cell at a stoichiometry of H^+ translocated per ATP hydrolyzed, as found also from studies in *Neurospora crassa* (reviewed in [107]). More, Pma1 is able to generating a 10,000-fold difference between the concentration of protons on either side of the membrane and therefore is inextricably linked with yeast growth. However, the magnitude of the gradient between in and outside the cell depends on the presence of other cations as well, notably K^+, which is exchanged for H^+ in a 1:1 stoichiometry, being H^+-ATPase closely linked to ion homeostasis [107].

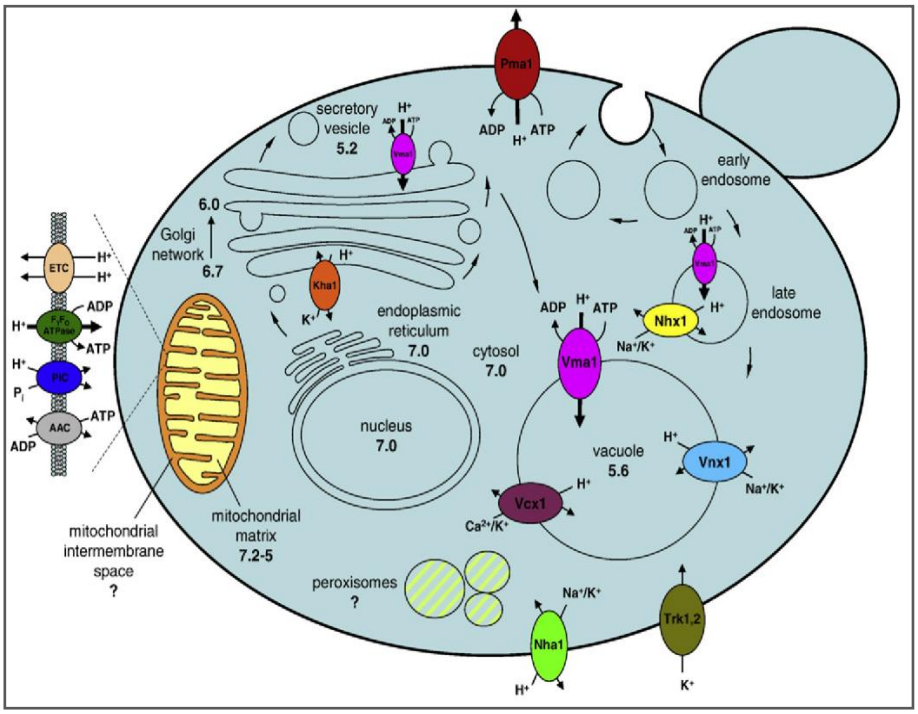

Fig. 1.8. Contributors to pH regulation. pH of the cell compartments are indicated. The two main contributors to the building of difference in potential across the membranes by export of protons against the gradient at the cost of ATP are the P-type ATPase Pma1 in the plasma membrane and the V-type ATPase (Vma1) in various organellar membranes; other organellar transporters use the energy stored in the difference in membrane potential to transport ions and to contribute to pH_i

homeostasis. In the mitochondrial inner membrane protons are pumped out of the mitochondrial matrix by activity of the electron transport chain (from [107]).

1.1.6. Regulation of metabolism

The fact that metabolism is involved in aging was already clear from the correlations between the 'rate of living' and lifespan and from experiments with CR. As mentioned above, in higher eukaryotes such as *C. elegans*, *D. melanogaster* and also mice, the insulin pathway is connected to longevity since a long time. Noteworthy, in *S. cerevisiae* as well, glucose metabolism is regulated with such an enormous precision as in higher eukaryotes.

Reduced activity of two major nutrient sensing pathways can extend both types of yeast lifespan. The first is centered on an amino acid sensing pathway, including TOR and the serine-threonine kinase Sch9. Deletion of *SCH9*, which has sequence and functional similarity to the mammalian ribosomal protein S6 kinase (S6K), causes an increase of several folds in both CLS and RLS [32,38], as does the deletion of *TOR1* or the inhibition of TOR activity [51,99]. Moreover, alterations to protein synthesis are strongly implicated in extension of replicative lifespan by reducing TOR/Sch9 activity and may play a key role in CLS as well [114]. Extension of CLS by reduced TOR activity depends on the transcription factor Gis1, which in turn activates many other protective systems [51]. The second pathway includes three proteins: Ras, adenylate cyclase Cyr1 and PKA [32,115]. This pathway seems to be of crucial importance in sensing the environmental conditions. Both the availability of the different essential nutrients but also the lack of any stressful conditions can positively influence PKA activity. However it is still not completely clear how different stressful environmental conditions are sensed by PKA [50]. In Fig. 1.9, some mechanisms of activation of the PKA pathway in high nutrient condition are resumed.

Anyway, the activation of PKA has several consequences in the cell [49,116]:

- trehalose and glycogen content decreases;
- stress response is downregulated;

- gluconeogenesis is downregulated;
- glycolysis is upregulated;
- growth is induced.

It is remarkable that mutation or overexpression of several components of this system were all found to affect lifespan.

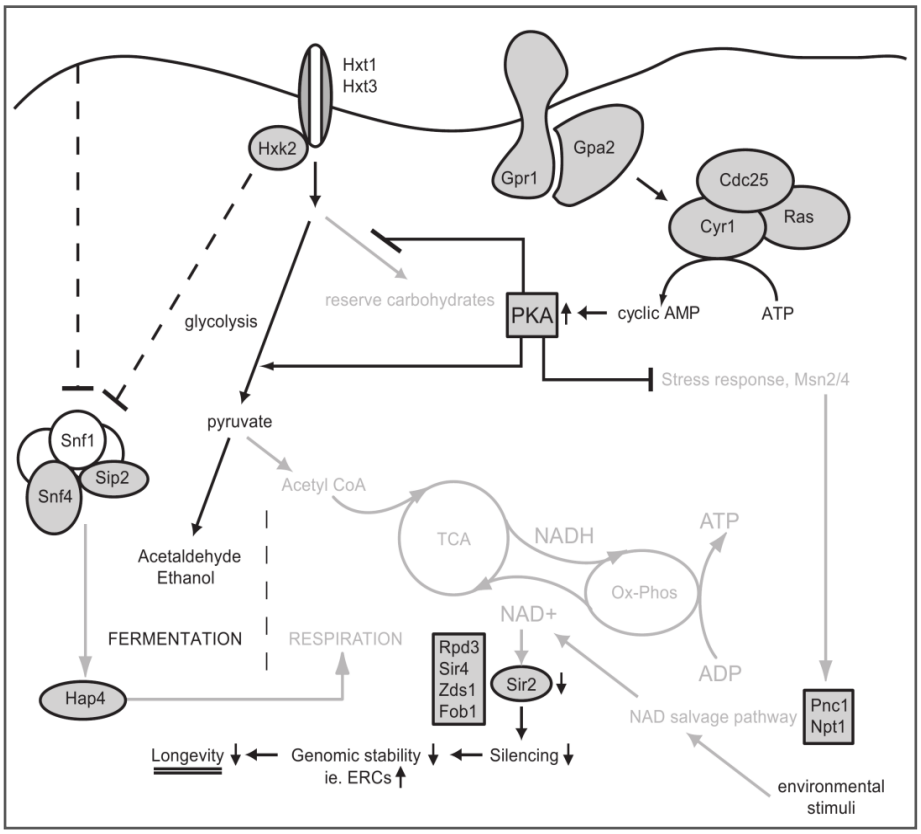

Fig. 1.9. Proposed mechanism of PKA pathway involvement in longevity during high growth rates. Light grey arrows denote inactive pathways (which, in turn, are activated in conditions of low growth rate). The availability of nutrients leads to the formation of cAMP and subsequent activation of PKA. This leads to a whole range of events, from induction of glycolysis to the repression of stress response including reserve carbohydrate formation. Of course, the Ras/PKA pathway is not the only one that is involved. To note, during high growth rate, NAD^+ flux will decrease, possibly leading to less active Sir2. This protein can also stimulated by the NAD^+ salvage pathway, which is induced by environmental stimuli as low glucose, oxidative stress, heat stress and other low dose stress.

As downstream effector on which both PKA and TOR converge, Rim15 regulation is fundamental and also highly regulated. Rim15 in turn is thought to activate the transcription factors Gis1, Msn2 and Msn4, which turn on genes that are needed for long term survival [117]. It is commonly assumed that the early events before entering into stationary phase can influence the final destiny of a cell lifespan. In this context, the role of Rim15 in determining many aspect of cellular adaptation to starvation condition results of a great importance: in fact, a tightly controlled regulation occurs to Rim15 itself, as described in Fig. 1.10.

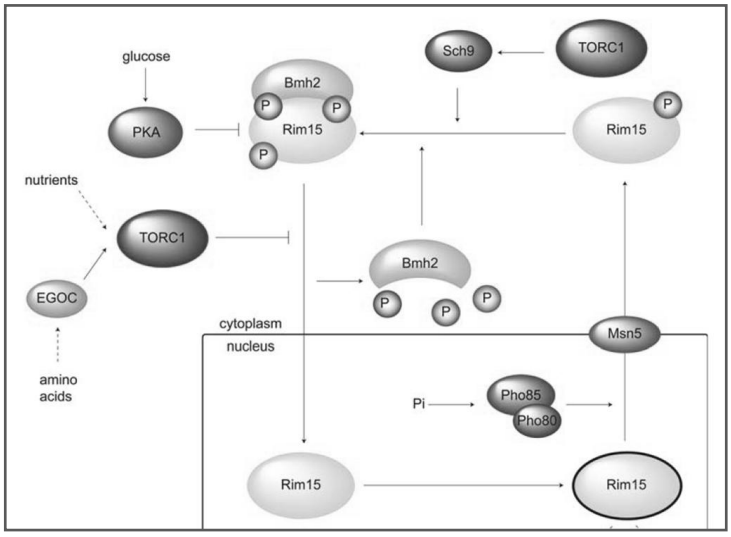

Fig. 1.10. The Rim15 protein kinase is regulated by different nutrient signaling protein kinases. In response to glucose, PKA directly phosphorylates and thereby inactivates the kinase activity of Rim15. Active TOR and Sch9 promote cytoplasmic retention of Rim15. Notably, TOR favours phosphorylation of Rim15 via activation of Sch9 and via inhibiting a yet unidentified protein phosphatase to promote the association of Rim15 with a 14-3-3 protein for its optimal sequestration in the cytoplasm. Rim15 is subject to auto-phosphorylation, which apparently stimulates nuclear export. Also the complex protein kinase Pho85 coupled with its cyclin Pho80 can control the nuclear export of Rim15 (taken from [50]).

Msn2 and Msn4 are two closely related zinc finger proteins; the transcriptional activation of genes depending on them is very complex. Briefly, Msn2 is regulated by nuclear translocation or by increased binding of Msn2 to the STRE elements in the promoters of stress-responsive genes. PKA can negatively regulate the transcription of Msn2-dependent genes by directly phosphorylating Msn2 and keeping it in the cytosol (reviewed in [50,118]). Subcellular localization of Msn2 is also regulated by the TOR pathway, which promotes interaction of Msn2 with a 14-3-3 protein anchoring Msn2 in the cytosol.

Gis1 acts in the PKA pathway downstream of Rim15. Msn2/Msn4 and Gis1 are not functionally equivalent since they are able to differentiate between similar STRE and postdiauxic shift (PDS) promoter elements. Expression of Gis1-dependent genes is regulated by both the PKA and the TOR pathway and depends on Rim15 [117,119]. Gis1 is also a zinc finger protein, found to regulate gene expression after glucose depletion, when yeast cells shift their metabolism from fermentation of glucose to oxidation of ethanol [120]. In fact, induction of several genes at the diauxic shift is dependent on Gis1, which acts through a PDS motif, present in the promoters of these genes [119,121]. 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].

Of interest, several genes involved in glycerol and acetate metabolism are regulated by Gis1 and Rph1, among which also Ach1; in addition, several genes involved in acetyl-CoA metabolism are downregulated by Gis1 [122], as reported in Fig. 1.11. due to the emerging role of acetate, glycerol as well as acetyl-CoA itself in the modulation of lifespan extension, these findings provided an further interesting links between nutrient signaling, metabolic regulation and the control of aging in yeast, even though the phenotype of *gis1*Δ and *rph1*Δ cells (short CLS and low acetate accumulation) remain to be explained.

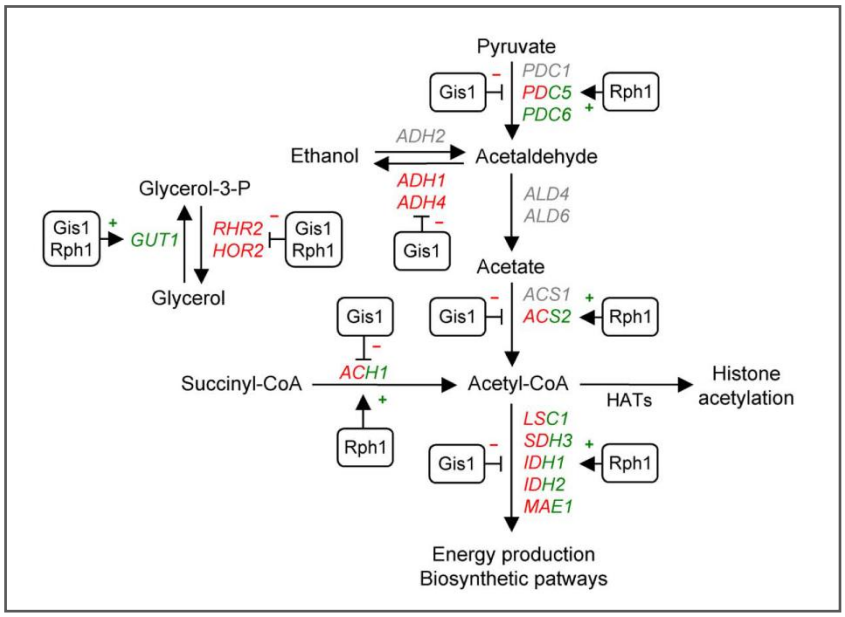

Fig. 1.11. Glycerol and acetate metabolic pathways in yeast. The effects of Gis1 and Rph1 on genes encoding some key enzymes are shown. It should be noted that regulation in several cases is likely to be indirect, being mediated by the repression or activation of some other transcription factor (from [122]).

Most of the genes upregulated during the diauxic shift, but not all of them, are targets of Msn2/Msn4 [123]. Interestingly, a correlation was found between the genes upregulated at the diauxic shift with those upregulated in a strain with mutated form of histone H4: the derepression of many of the genes at the diauxic shift resulted to be associated with a decreased level of histone H4 acetylation [124]. Availability of nucleo-cytosolic acetyl-CoA regulates histone acetylation and the level of nucleo-cytosolic acetyl-CoA may reflect metabolic changes during the diauxic shift [125]. This is in agreement with the finding that the level of acetylated histone H3 and H4 decreases as cells enter the diauxic shift and stationary phase [125]. Histone acetylation could thus provide an additional fine tuning of the transcriptional reprogramming during the diauxic shift and connect the metabolic state of the cell with a chromatin structure and transcription and with lifespan determination [125].

1.2 Yeast stationary phase

1.2.1. Stationary phase

The signal transduction pathways responsible for organizing the transition to the diauxic shift and stationary phase also affect aging of yeast cells. In fact, as previously mentioned, downregulating the PKA or the TOR pathways can for example extend lifespan [32,34,99]: as discussed before, this is partially due to an increased cellular protection against oxidative stress through the regulation by the transcription factors Msn2/Msn4 and Gis1 [31]. This indicates that the longevity of yeast cells is tightly linked to the signaling and transcription mechanisms that are important for transition to quiescence.

Like all microorganisms, *S. cerevisiae* spends most of its natural lifetime in a reversible, quiescent state primarily induced by limitation for one or more essential nutrients. When sources of carbon, nitrogen, phosphate or sulfur become limiting, cells cease growing, arrest cell division and acquire a distinct set of physiological, biochemical and morphological features that allow cells both to survive extended periods of starvation and to transit back to the proliferating state upon refeeding [126,127]. While some aspects of the quiescence program are nutrient specific [50,128], yeast cells still establish a core quiescence program regardless of which nutrient is limiting.

Although the many shared features, an important difference between quiescent yeast and metazoan cells has to be considered: unlike many types of quiescent metazoan cells (such as neurons), quiescent yeast cells display a significantly reduced (even though still active) metabolism. However, despite of this, stationary phase non-proliferating yeast cells still display a respiratory metabolism, which is similar to the kind of metabolism of many non-proliferating human cells. Thus, yeast still represents an excellent model for studying quiescence pathways common to higher eukaryotes.

Quiescent yeast cells are mainly studied by harvesting cells to saturation (stationary phase) in glucose-containing media: cells enter into quiescence after distinct adaptive phases, which critically affect the cell lifespan and the ability to cope with environmental stresses in their future [49,129]. When

glucose in the medium is consumed, *S. cerevisiae* starts using respiratory substrates (such as ethanol and acetate) as carbon sources for aerobic growth, leading to a post diauxic phase. It has been assessed that at the diauxic shift many transcriptional changes occur in the cell: the mRNA level of around 700 genes increases, while the mRNA level of approximately 1000 genes declines [123]. The massive reprogramming of gene expression includes, among others, genes involved in the gluconeogenesis, the TCA cycle and the glyoxylate cycle [130,131]. In addition, mitochondrial mass and respiration are increased, both causing substantial changes in metabolites crucial for cellular function and survival [95] and, likely as a consequence of the on-setting respiratory activity, of genes encoding antioxidant defenses that allow scavenging and/or the destruction of reactive oxygen species (ROS) [76,132]. When also ethanol and acetate are depleted from the medium and no other carbon source is available, cells enter quiescent or stationary phase (G_0). Anyway, nutrient deprivation of diploid cells does not necessarily lead to quiescence: when growing on nonfermentable carbon source such as acetate and starved for nitrogen, they normally enter meiosis and sporulate [118].

The final characteristics of stationary phase cells reflect their integrated responses and adaptations triggered by progression through distinct, sequential physiological phases:

- they are unbudded and contain unreplicated nuclear DNA;
- their metabolism is mainly respiratory;
- the overall transcription rate is lower and translation is reduced [133];
- they have a thickened cell wall [134];
- they contain more abundant mitochondria and morphologically distinct condensed chromosomes [135];
- they are more resistant to heat and osmotic shocks [136], as well as to treatment with toxic drugs [134];
- they accumulate reserve carbohydrate such as trehalose;
- unlike exponential cells, they are able to survive for extended periods of time without nutrients [126].

Recently it has been observed that stationary phase cultures exhibit a complex, heterogeneous community structure mainly composed by two

distinct sub-populations [137], as shown in Fig. 1.12. An extensive characterization of the two fractions revealed that:

- the high-density cells (made up mainly by daughters and young mothers) were mostly unbudded and showed several features of quiescent cells while low-density cells were both budded and unbudded, and morphologically more similar to dividing cells (non-quiescent);
- the low-density cells lost viability more rapidly than the others over time, they were more sensitive to heat-shock and they produced more ROS than quiescent cells;
- the non-quiescent cells showed higher levels of apoptotic markers (DNA fragmentation and phosphatidylserine exposure) than quiescent cells [138,139].

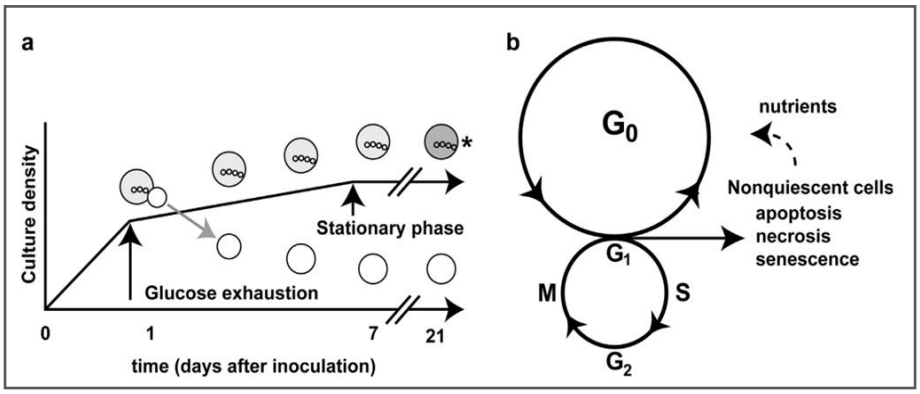

Fig. 1.12. Models for the formation of quiescent and non-quiescent cells in chronologically aged yeast cultures and the relationship with the mitotic cell cycle. (A) After glucose exhaustion, during the diauxic shift, quiescent daughter cells are formed and can be isolated from non-quiescent cells by density. The asterisk indicates a non-quiescent fraction that is heterogeneous and contains both budded and unbudded cells. (B) Daughter cells produced during the diauxic shift enter G_0 and reenter the mitotic cell cycle when nutrients become available. Non-quiescent cells are heterogeneous; may continue in the mitotic cell cycle; and can become senescent, apoptotic or necrotic. As dead cells break down, they release nutrients that can be used by G_0 cells (broken arrow). (from [137]).

It will be relevant to understand the dynamics in the generation of these two types of cell sub-populations to establish whether their ratio changes over time and whether quiescent cells can become non-quiescent once the apoptotic program is activated (Fig.1.12). These physiological differences in cell populations contributes to the temporal plasticity of the mortality rate within an aging stationary-phase culture [140]. Nevertheless, studies of aging factors have identified distinct properties of quiescent cells that can collectively define the essence of the quiescence program in yeast [31,37].

Hereafter, some of the major characteristics of stationary phase cells will be briefly described.

Trehalose

Trehalose is a disaccharide formed by two glucose units linked in a α,α-1,1-glycosidic linkage, apparently absent from vertebrates but present in yeasts, fungi and plants. It has a great importance since it plays a variety of roles, from being an energy source to a stress protectant [141]. The synthesis of trehalose is represented in Fig. 1.13 and is catalyzed by a trehalose-synthesis protein complex composed of four proteins. Trehalose 6-phosphate synthase Tps1 synthesizes trehalose 6-phosphate by the condensation of glucose 6-phosphate and UDP-glucose. Trehalose is then generated by dephosphorylation of trehalose 6-phosphate by Tps2. Tps3 and Tsl1, the residual components of the complex, are considered to be regulatory subunits [141,142]. As to trehalose hydrolysis, two enzymes are involved: a neutral cytosolic trehalase, Nth1, and an acidic trehalase, Ath1 [143]. The cellular localization and functions of Ath1 are still controversial.

In *S. cerevisiae*, it has been pointed out that there is a strong correlation between trehalose content and stress tolerance and the cellular levels of trehalose are tightly controlled by an enzymatic balance between its synthesis and degradation [144,145]. In *S. cerevisiae*, trehalose is known as major stress protectant, whose synthesis is induced by many environmental stress conditions at the transcriptional level [126,146,147]. Because of the peculiar characteristics of this disaccharide, it is believed that it may contribute to the stress tolerance of cells by preserving membrane integrity, by stabilizing proteins and by suppressing the aggregation of denatured proteins during heat shock [148].

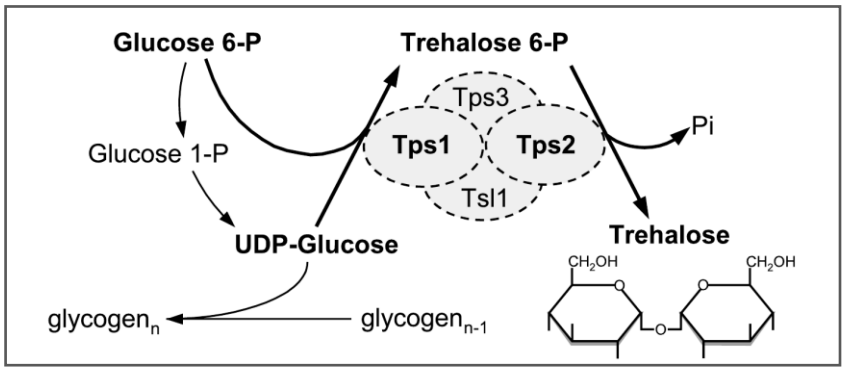

Fig. 1.13. The pathway of trehalose synthesis. Trehalose is synthesized in two steps (thicker arrows) starting from uridine-diphosphoglucose (UDP-glucose) and glucose 6-P. The enzymes catalyzing the synthesis, trehalose 6-P synthase (Tps1) and trehalose 6-P phosphatase (Tps2), are part of a complex in which Tsl1 and Tps3 participate to maintain the stability of the complex. The figure also shows the relationship between trehalose and glycogen biosynthesis through the use of UDP-glucose as starting block in both pathways (from [141]).

During the post-diauxic phase and growth on ethanol, yeast cells accumulate trehalose, which is then degraded as starvation proceeds [126]: in addition to its general protective role, trehalose may also contribute to energy homeostasis as potential source of glucose. In fact, it has recently been proposed that, upon refeeding of stationary phase cells, trehalose can rapidly be mobilized, which may at least in part contribute to a fast cell cycle progression upon return to growth [149]. In fact, as cells enter prolonged stationary phase after growth, trehalose levels tend to be maintained, whereas glycogen stores decrease. Some of these glycogen and trehalose stores may be used to support basal metabolic activities as cells persist in stationary phase; however, it is clear that trehalose is preferentially maintained over glycogen as the quiescent state progresses. On exit from quiescence, trehalose stores are metabolized before glycogen. Thus, the ability of a cell to accumulate sufficient trehalose stores is a key determinant of whether it can enter and exit quiescence successfully. Trehalose has been widely reported to function as a stress protectant by acting as a chemical chaperone to help minimize protein aggregation or as an osmolyte to minimize water loss [150]; nevertheless, although trehalose may directly function as a chaperone or as an osmolyte, it may simultaneously serve as a

critical energy reserve. Shi and coworkers speculate that there are several significant reasons why trehalose might be a preferred energy source for survival of a variety of adverse conditions [149]; it is a nonreducing disaccharide formed from two molecules of glucose. Because of its glycosidic linkage, trehalose is more resistant than most carbohydrates to heat and acid-induced decomposition. Thus, trehalose represents a source of energy that itself would endure a variety of harsh conditions. Moreover, cleavage of one glycosidic bond in trehalose would release two glucose equivalents that can be directly used for energy. On the base of these considerations and on their results, the authors also stated that the perturbation of pathways that negatively impact trehalose accumulation or metabolism might compromise the ability of cells to rapidly exit the quiescent state and thus compromise their survivability. Conversely, those that enhance trehalose storage or reduce trehalose metabolism while persisting in the quiescent state might elongate cell survival [149].

Autophagy and protein degradation

Autophagy is a vacuolar degradative pathway for many proteins and damaged and/or unnecessary organelles. It is most potently stimulated by nitrogen starvation and, to a lesser extent, also by starvation for other essential nutrients including carbon [151,152]. Autophagy begins with the formation of double-membrane vesicles, termed autophagosomes, which sequester cytoplasmic material to be fused with the vacuole. The inner vesicle, released into the vacuolar lumen, is then degraded by a series of vacuolar hydrolases and stationary phase-induced proteases [153]. Following efflux from the vacuole, the corresponding degradation products can then be metabolically recycled, a process that contributes significantly to the survival of cells during starvation [154].

Signaling networks and metabolism

As discussed more in detail in Chapter 1.1.6, both PKA and TOR are key regulators of nutrient sensing during cell growth that critically participate in the cell decision to enter into quiescence [155,156]. An additional signaling network with the Snf1 protein kinase at its core regulates the entry into quiescence. Lastly, recent evidence suggests that the Pho85-signaling

pathway is another important modulator of the setup of the quiescence program [50].

Some of the physiological changes that occur as cells enter the quiescent phase are subject to post-transcriptional control by PKA: PKA antagonizes both the metabolic transition from glycolysis to gluconeogenesis and the induction of trehalose and glycogen synthesis by different means, including:

- the stimulation of the glycolytic 6-phosphofructo-2-kinase Pfk2 and pyruvate kinases Pyk1/2 [157];
- the inhibition of the gluconeogenic fructose 1,6-bisphosphatase Fpb1 [158],
- the activation of the neutral trehalase Nth1 [159];
- the activation of the glycogen phosphorylase Gph1 [160];
- the inhibition of the glycogen synthase Gsy2 [161].

1.2.2. Carbon metabolism: glycolysis, fermentation, respiration

In the microbial world, an unbelievable diversity exists in sugar metabolism, but a more surprising unity exists among yeasts. Of the hundreds of yeast species currently recognized, all the investigated one seem to predominantly use the Embden-Meyerhof-Parnas pathway (also known as glycolysis) for the conversion of hexose phosphates to pyruvate [162]. The unity in the carbohydrate metabolism of yeasts becomes most evident for their fermentative sugar metabolism: for each species exhibiting a fermentative sugar metabolism, ethanol is the predominant fermentation products [162]. Glycerol is another frequently found product since its formation enables the reoxidation of NADH generated during the conversion of sugar into biomass, in the cases in which NADH cannot be reoxidized by respiration [163]. In addition to ethanol and glycerol, fermenting yeast cultures often generate other fermentation products, in particular organic acids (such as acetate) or higher alcohols, esters and aldehydes [59,164]. Notably, acetate accumulation in the medium is due to an imbalance between its production rate from acetaldehyde and its conversion to acetyl-CoA.

Like other microorganisms, *S. cerevisiae* has developed efficient regulatory systems to allow rapid changes in metabolic pathways. When glucose is available it is preferentially used over other carbon sources. This phenomenon, defined as catabolite repression, is achieved in part by inhibition of the synthesis of enzymes required for the utilization of alternative carbon sources, such as those involved in the gluconeogenesis, the glyoxylate cycle, the TCA cycle or the respiratory chain [165,166], as well as of proteins required for the active transport system across the plasma membrane of acetate, lactate and pyruvate [167].

The first glycolytic enzyme of yeast was isolated more than 80 years ago [168] and the complete pathway had already been elucidated by 1940. By now, most enzymes in central carbon metabolism have been extensively characterized and the genes encoding them have been identified [169]. The first stage in glycolysis is a series of preparatory rearrangements, reactions that do not involve oxidation-reduction and do not release energy but lead to the production from glucose of two molecules of the key intermediate, glyceraldehyde 3-phosphate. In a second stage, oxidation-reduction occurs being produced both ATP and two molecules of pyruvate. In a third stage pyruvate is subjected to a series of oxidation-reduction reactions and may originate by fermentation products such as ethanol, lactic acid and CO_2 or be channeled into the TCA cycle for respiration (as described in Fig.1.14). In glycolysis itself, the net gain to the organisms is two molecules of ATP per glucose fermented. Glycolysis leads also to the production of two NADH which will be re-oxidized in the reduction of pyruvate, the organic fermentation product.

The intracellular glucose enters the glycolytic pathway after a phosphorylation step, leading to glucose-6-phosphate. This molecule is a central key element for different aspects of the cell metabolism, since it is the precursor both of the pentose phosphate pathway and of synthesis of oligo- and poly-saccharides [170]. The irreversible phosphofructokinase and the irreversible pyruvate kinase are considered key regulatory enzymes in glycolysis and their activity is influenced by numerous effectors, including AMP/ATP ratios, internal phosphate availability, ammonium ions and fructose 2,6-biphosphate [116,171]. Another very important step in glycolysis is the conversion of glyceraldehyde-3-phosphate into 1,3-

bisphophoglycerate catalyzed by glyceraldehyde-3-phosphate dehydrogenase. Its activity is believed to be of importance especially in stress conditions [172], but also for being a 'switching station', directing carbon flow appropriately, not only in yeast but also in higher eukaryotes. In fact, for example, in humans it plays a key role in the regulation of carbon metabolism in different tissues [173].

On the whole, the glycolytic enzymes are regulated not only by mechanisms of transcriptional regulation. Still, the majority of glycolytic transcripts are increased upon glucose addition. Although the signaling mechanism has not been completely elucidate, the induction is led by an increase of the intracellular concentration of some of the glycolytic intermediates [162,174]. Glucose itself has a strong effect on the presence of pathways surrounding glycolysis: in fact, its presence causes the transcriptional repression of genes involved in gluconeogenesis, respiration and alternative carbon source utilization; this repression is mainly mediated by the main glucose repressor Mig1, the non-phosphorylated form of which binds to upstream repression sequences found in numerous target genes [175]. Anyway, the transcriptional induction of all these pathways is not caused by one single mechanism, even though genes involved in respiration are mainly induced through the Hap2-5 complex [176], while gluconeogenic genes are induced through Adr1 and Cat8/Sip4 [171]. In parallel, central metabolism is strongly regulated by other fast regulatory mechanisms: for example, rapid degradation of proteins plays an important role in regulation of the glycolytic enolase Eno2 under osmotic and temperature stress [177] or high affinity hexose transporters Hxt6-Hxt7 after an increase in glucose concentration [178]. Moreover, activities of glycolytic enzymes are directly regulated through phosphorylation. The fastest regulation of sugar metabolism is implemented through changes in low molecular weight molecules such as substrates, products and allosteric effectors. Not surprisingly, abundantly present glycolytic enzymes are mainly regulated by the changing metabolites to quickly adapt to changes in sugar availability. As an example, an intermediate of the upper part of glycolysis, fructose-1,6-biphosphate, activates an enzyme of the lower part of glycolysis, pyruvate kinase [179].

Glycolysis can take place under aerobic as well as anaerobic conditions and the fate of pyruvate will determine the type of energy metabolism that is

being used. The two major fates of the pyruvate produced in glycolysis are either its oxidation to CO_2 or its fermentation to ethanol or lactate through oxidoreductive metabolism [162]. Transformation to ethanol can take place in anaerobic conditions or at high glucose concentrations even in aerobic conditions in those yeasts that present a Crabtree effect such as *S. cerevisiae*. The Crabtree effects can be defined as the occurrence of alcoholic fermentation under aerobic conditions [180,181]. The most important fermentation pathway occurring in yeasts is the alcoholic fermentation. Pyruvate obtained through this process is decarboxylated to acetaldehyde via pyruvate decarboxylase, encoded by *PDC1-PDC5* genes in *S. cerevisiae*, some of which are essential to grow on glucose [182]. Acetaldehyde is then reduced to ethanol via alcohol dehydrogenases. The kinetic and regulatory characteristics of alcohol dehydrogenases, the regulation of the transcription of the genes that encode them and their subcellular localization determine the direction of the reaction catalyzed under physiological conditions [183]. On the whole, alcohol dehydrogenases are implicated at least in three cellular events [184,185]:

- ethanol utilization as carbon source;
- production of ethanol as a end-product of fermentation;
- in the acetaldehyde-ethanol redox-shuttle.

Acetic acid is also a by-product of the metabolism of certain yeasts including *S. cerevisiae*, being synthesized from acetaldehyde in the presence of aldehyde dehydrogenase [116,171]. Simultaneous production of ethanol and acetate is frequent and is regulated by the expression and affinity for acetaldehyde of each enzyme [186]. Acetate production is also increased at alkaline pH values and this is explained by the pH optimum of the aldehyde dehydrogenases [187].

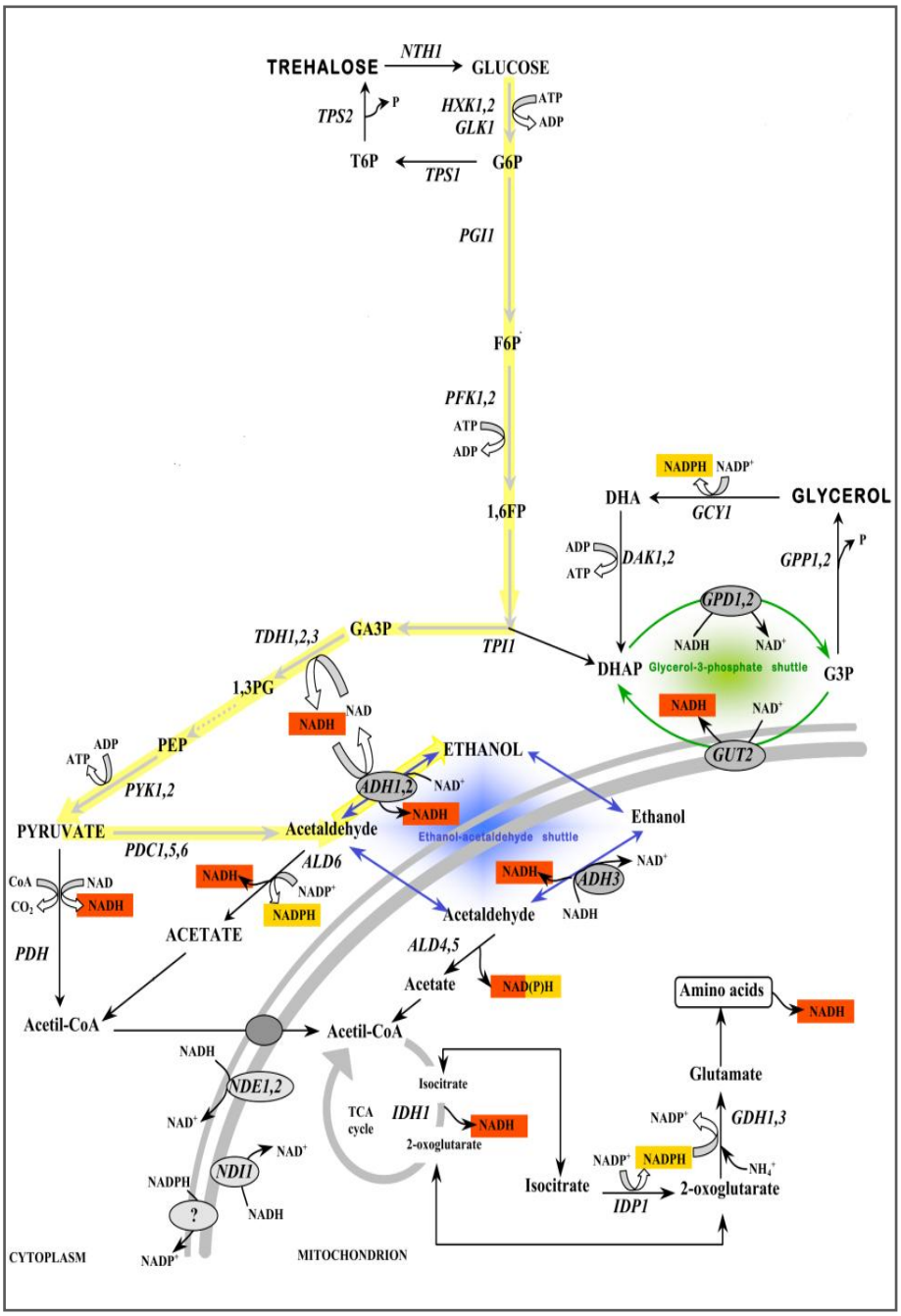
TREHALOSE
NTH1
GLUCOSE
TPS2
P
HXK1,2
GLK1
ATP
ADP
T6P
TPS1
G6P
PGI1
F6P
PFK1,2
ATP
ADP
1,6FP
NADPH
NADP+
DHA
GCY1
GLYCEROL
ADP
ATP
DAK1,2
GPP1,2
P
GPD1,2
NADH
NAD+
TDH1,2,3
GA3P
TPI1
DHAP
Glycerol-3-phosphate shuttle
G3P
1,3PG
NADH
NAD
NADH
NAD+
GUT2
ADP
ATP
PEP
ETHANOL
PYK1,2
ADH1,2
NAD+
NADH
Ethanol
PYRUVATE
Acetaldehyde
Ethanol-acetaldehyde shuttle
PDC1,5,6
ALD6
NADH
NADP+
NADPH
NADH
ADH3
NAD+
CoA
NAD
CO2
NADH
Acetaldehyde
NADH
ACETATE
ALD4,5
PDH
Acetate
NAD(P)H
Amino acids
NADH
Acetil-CoA
Acetil-CoA
NADH
NDE1,2
NAD+
Isocitrate
Glutamate
TCA
cycle
IDH1
NADH
2-oxoglutarate
NADP+
GDH1,3
NAD+
NDI1
NADPH
NADP+
NADPH
NH4+
?
NADH
Isocitrate
2-oxoglutarate
IDP1
CYTOPLASM
NADP+
MITOCHONDRION

Fig. 1.14. Scheme of the central carbon metabolism in yeast. Mechanisms at the interface between the NADPH and NADH-yielding reactions in assimilatory and dissimilatory sugar metabolism and the mitochondria respiratory chain in *S. cerevisiae*. Ethanol-acetaldehyde shuttle is represented in blue. Glycerol-3-phosphate shuttle is represented in green. Glycolysis is represented in yellow. Yellow and red boxes highlight reactions where NADPH and NADH are produced respectively. Abbreviations of metabolites: G6P-glucose-6-phosphate; T6P-trehalose-6-phosphate; F6P-fructose-6-phosphate; 1,6FP-fructose-1,6-biphosphate; GA3P-glyceraldehyde-3-phosphate; DHAP-dihydroxyacetone phosphate; G3P-glycerol-3-phosphate; DHA-dihydroxyacetone; 1,3PG-1,3-diphosphoglycerate; PEP-phosphoenolpyruvate; Genes: HXK-hexokinase; GLK1-glucokinase; TPS1-trehalose-6-phosphate synthetase; TPS2-trehalose-6-phosphate phosphatases; NTH1-trehalase; PGI1-phosphoglucose isomerase; PFK-phosphofructokinase; TPI1-triose-phosphate isomerase; GPD-glycerol-3-phosphate dehydrogenase; GPP-glycerol-3-phosphatase; GCY1-glyceroldehydrogenase; DAK-dihydroxyacetone kinase; GUT2-mitochondrial glycerol-3-phosphate dehydrogenase; TDH-glyceraldehyde-3-phosphate dehydrogenase; PYK-pyruvate kinase; PDC-pyruvate decarboxylase; PDH-pyruvate dehydrogenase complex; ALD6-cytoplasmic acetaldehyde dehydrogenase; ADH1,2-cytoplasmic alcohol dehydrogenases; ADH3,4,5-mitochondrial alcohol dehydrogenases; IDH1-mitochondrial isocitrate dehydrogenase; IDP1-mitochondrial isocitrate dehydrogenase; GDH-glutamate dehydrogenase; NDI-internal NADH dehydrogenase; NDE-external NADH dehydrogenases.

Frequently, oxidation of pyruvate to CO_2 occurs via the TCA cycle under aerobic conditions. Nevertheless, pyruvate may be used simultaneously to produce ethanol (fermented) and oxidized through the TCA cycle (respirated), being this very common type of metabolism termed respiro-fermentative [162]. Pyruvate enters the TCA cycle undergoing a decarboxylation catalyzed by a pyruvate dehydrogenase complex, a mitochondrial multi-enzyme complex formed by three different components [188–190]. Acetyl-CoA can also be formed in the cytosol through the so-called pyruvate bypass that implies the synthesis of acetyl-CoA through the concerted action of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. Acetyl-CoA is a central molecule in yeast metabolism as an essential building block in anabolic processes such as fatty acids, sterols and amino acids synthesis [162,191]. This is corroborated by the fact that a *S. cerevisiae* strain deleted in both genes, *ACS1* and *ACS2*,

coding for acetyl-CoA synthetase (ACS), does not grow in media containing the most simple and common carbon sources [192]. The two forms of ACS evidence differences in regulation, localization and kinetic aspects suggesting different, but not yet clear, roles in metabolism.

Of the major metabolic pathways in *S. cerevisiae* that originate from pyruvate, only the conversion of pyruvate into ethanol has an exclusively dissimilatory function: ATP production from substrate-level phosphorylation reactions in glycolysis can only occur when the NADH produced in this pathway is reoxidized [193]. The energetically more favourable, respiratory dissimilation of carbohydrates requires coupling of glycolysis to the TCA cycle. The assimilatory function of the TCA cycle as generator of biosynthetic precursors should be, in principle, incompatible with its role in dissimilation, since complete dissimilation of acetyl-CoA requires that oxaloacetate would be regenerated at each turn of the cycle. In fact, during growth on carbohydrates, the source of TCA-cycle intermediates for biosynthesis is compensated by the carboxylation of pyruvate to oxaloacetate, catalyzed by pyruvate carboxylase [194]. The TCA cycle is carefully regulated to ensure that its level of activity corresponds closely to cellular needs. In its primary role as a means of oxidizing acetyl groups to CO_2 and water, the TCA cycle is sensitive both to the availability of its substrate, acetyl-CoA, and to the accumulated levels of its principal end products, NADH and ATP. Actually, the ratio NADH/NAD^+ and the energy charge or the ATP/ADP ratio seem to be even more important than the individual concentrations [193].

TCA cycle, as said before, is an amphibolic pathway since it fulfils both catabolic and anabolic functions. In yeast, two major anaplerotic pathways are the pyruvate carboxylase, the cytoplasmic enzyme that generates oxaloacetate [195,196] and the glyoxylate cycle [197–200]. Glyoxylate cycle is essential for growth in minimal medium in 2C-carbon sources, such as ethanol or acetate: glyoxylate bypass is prominent in these conditions since this pathway provides carbon skeletons for the biosynthesis of other macromolecules. In fact, *Schizosaccahromyces pombe* lacks the enzymes of the glyoxylate cycle and cannot grow on ethanol [201].

During respiration growth, both cytosolic and mitochondrial NADH can be reoxidized by the respiratory chain. NADH generated in the mitochondrial

matrix is oxidized by an internal NADH dehydrogenase [202]. Like plant mitochondria, but unlike mammalian mitochondria, yeast mitochondria oxidize also cytosolic NADH directly, through an external NADH:ubiquinone oxidoreductase [203]. Like the internal one, this enzyme is localized in the mitochondrial inner membrane but its active site faces the mitochondrial intermembrane space [202]. Alternatively, cytosolic NADH can be oxidized by the respiratory chain via existing shuttle systems that generate NADH in the mitochondrial matrix, such as the glycerol-3-phosphate shuttle [204] or the ethanol-acetaldehyde shuttle [205]. All known pathways of respiratory NADH oxidation in *S. cerevisiae* converge then at the ubiquinone pool. Ubiquinone donates its electrons to cytochrome *c;* terminal oxidation of cytochrome *c* by molecular oxygen is catalyzed by cytochrome *c* oxidase [202].

1.3 Sirtuins

1.3.1. The Sirtuins family

Over three quarters of a century ago, Clive McCay and colleagues first noted that rats kept on a calorie-restricted diet lived longer than freely fed controls [25]. However, since that discovery, the molecular mechanism that drives this lifespan extension has remained elusive. The yeast Silencing Information Regulator 2 (Sir2) came out on top in a screen for modulators of yeast lifespan [40,115]. This discovery launched a new field in biology, the study of the histone deacetylase Sir2 and its orthologues in mammals, called Sirtuins. Histone and protein deacetylases are divided into four classes with the yeast proteins Rpd3 (class I), Hda1 (class II) and Sir2 (class III), serving as charter members of the three major classes, while human HDAC11 is the sole member of class IV, conserved in mice but not in yeast [206]. Class III deacetylases are unique in the sense that they need NAD^+ as a cofactor, driving a complex reaction that couples lysine deacetylation to NAD^+ hydrolysis [115,207]. As such, Sirtuins activity may be controlled by NAD homeostasis and be related to changes in the cell metabolic status. On the whole, having fundamental regulatory roles in transcription, metabolism and genome maintenance (see below), Sirtuins are desirable targets for therapeutic development. Of relevance, evidence has imputed mammalian Sirtuins in carcinogenesis, although the mechanisms involved seem to be diverse and complex: in fact, some Sirtuins, such as SIRT2 and SIRT6, seem to work as tumor suppressors, but others, such as SIRT1, are apparently bifunctional: operating as both tumor suppressors and oncogenic factors depending on the context and the study conditions [208–210]. The complexity of unraveling the role played by Sirtuins in the cell is also reflected in the opposite role of yeast Sir2 in shortening CLS and elongating RLS. Thus, in the study of any process involving these proteins a high accuracy has to be paid in order to report results that might not depend only on specific experimental conditions.

Due to its ability to promote silencing, Sir2 was long suspected of possessing histone deacetylase activity and Sir2 levels were known to correlate with

histone deacetylation at targeted areas of the yeast genome [211]. In yeast, this activity is essential for the maintenance of silencing. Silencing of genomic DNA was first observed by repression of genes near certain translocation breakpoints in *D. melanogaster*. This and further studies in yeast have led to the identification of factors acting in *trans* to mediate silencing. Among these are the proteins encoded by the *SIR* genes, Sir1, Sir3 and Sir4, which are differently responsible for silencing at the three major silent chromatin domains in yeast: mating type *loci*, telomeres and the ribosomal DNA (rDNA) repeats as described in Fig. 1.15 [212,213]. Gene expression patterns and cellular fate are tightly connected to the establishment and maintenance of silent chromatin or heterochromatin domains. It was observed that certain factors can modulate the recruitment of histone deacetylases to specific silent chromatin domains, even though the mechanisms of action that affect the distribution of histone deacetylases among all the major silent chromatin domains has not been fully clarified [214]. Sir2 is required for the maintenance of RLS since this process is highly dependent on the stability of the rDNA repeats, which consist of ~190 rRNA genes that are tandemly arranged on chromosome XII and reside within the nucleolus, a major nuclear compartment that is also the site of ribosomal biogenesis [215]. Hyperactive or aberrant recombination at this site can lead to chromosomal instability and to a shorter RLS, also promoted by the formation of ERCs [40]. Thus, recombination within the rDNA repeats is tightly regulated. Recombination within the repeats is promoted by an open chromatin structure. Sir2 operates at rDNA as part of a complex called RENT (Fig. 1.15), by limiting the access to RNA polymerase and to the recombination machinery. The function of telomeric Sir2 is also required for the maintenance of RLS [216], and acts as the enzymatic subunit of the SIR complex together with Sir3 and Sir4 [214]: firstly, Sir2 and Sir4 are recruited to telomeres, then Sir3 associates and the complex can spread into subtelomeric regions promoting silent chromatin assembly. Disruption of this Sir2-dependent telomeric silencing is linked with subtelomeric DNA instability and a shorter lifespan [40,216]. Sir2 also localizes to and represses gene expression at the silent mating-type *loci*, *HMR* and *HML*, even though whether this event can influence lifespan is still unclear. Recent studies aim to underline that, since Sirtuins, such as Sir2 or the mammalian counterpart SIRT6, operate each at multiple age-related *loci*, some factors exist that limit their localization to certain *loci* by promoting in parallel other lifespan-

sustaining roles of these Sirtuins elsewhere in the genome, or even more generally in the whole cell [217].

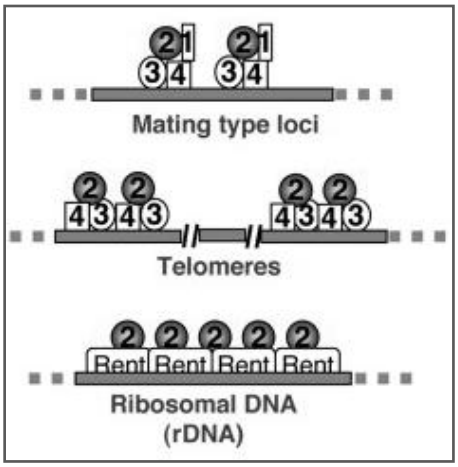

Fig. 1.15. The SIR complexes mediate silencing at the mating type loci, telomeres, and rDNA *loci*. Sir2 associates with Sir1/3/4 to repress the transcription of mating type *loci*, while Sir2/3/4 mediate telomere maintenance. In these two *loci*, Sir3 is suggested to mediate the spreading of SIR complex along the region. At the rDNA loci, Sir2 is recruited to the nucleolus to form RENT complexes, which also contains the proteins Net1/Cfi1 and Cdc14, to repress the recombination of rDNA repeats and to maintain genome integrity (adapted from [213]).

Sir2 specifically deacetylates histone H3 on lysines K4 and K56 and histone H4 on lysine K16 in *vivo* [218–220]. Moreover, recently also H3 K79 was proposed as a substrate of Sir2 deacetylation [221]. Both H3 K4 and H4 K16 are located on the flexible N-terminal tail of the histone, and H3 K56 is located in the core of histone H3 at the entry-exit points of DNA on the nucleosome [218–220]. The tail residue of H3 K4 was already known to be critical for silencing. The intimate involvement of this highly conserved histone tails in yeast silencing suggests that global repression mechanisms in more complex eukaryotes may be related at some fundamental level to the yeast system. However, the fact that bacteria have Sir2-like proteins, but lack histones and silent chromatin, suggested also that Sir2 might have a

more general, but again well conserved, function not strictly related to gene silencing.

Several unique features characterize the Sir2 enzymatic reaction. Initially, Sir2 and some of its orthologues were found to possess an ADP-ribosyltransferase activity, catalyzing the breakdown of a NAD substrate and the addition of an ADP-ribose moiety to target proteins [222]. However, this reaction was soon overshadowed by the discovery of a more robust NAD^+-dependent histone deacetylase activity present in the yeast Sir2 protein as well as in its closest mouse orthologue, SIRT1 [218–220]. Histone deacetylation by Sir2 also cannot proceed without NAD^+, nor can other nicotinamide adenine dinucleotides (such as NADH, $NADP^+$ or NADPH) substitute for NAD^+ [218]. Better, on the contrary, NADH and nicotinamide both function as effective inhibitors of Sir2 catalytic activity, providing an effective mechanism for regulating Sir2 function *in vivo* [16]. Actually, the requirement of NAD^+ for the Sir2 enzymatic reaction was unexpected for thermodynamic reasons. The deacetylation reaction catalyzed by the other classes of histone deacetylases, a simple hydrolysis reaction, is energetically favorable, begging the question of why Sir2 would couple such a reaction to the breakdown of NAD^+, a metabolically important compound. In fact, NAD^+ is not a cofactor of Sir2 reaction but is actually consumed by it [223]. Another important peculiarity of this reaction is that Sir2 catalyzes the transfer of the acetyl group to the ADP-ribose cleaved from NAD^+, resulting in the formation of O-acetyl ADP-ribose (OAADPr) [207,223]. Thus, the Sir2 reaction produces two major products in addition to deacetylated lysine: nicotinamide and OAADPr (Fig.1.16).

While Sir2 is generally believed to influence silencing through the deacetylation of histone H4, it is formally possible that any of these three products of the Sir2 reaction could be required for creation and maintenance of the silenced state. OAADPr was originally proposed to trigger some sort of signal transduction cascade. More recently, this molecule has been shown to play an integral role in the assembly of the SIR complex in yeast, promoting the association of multiple copies of Sir3 with Sir2/Sir4, inducing important structural changes in the complex [224]; OAADPr was also linked with decreased reactive oxygen species levels, gene silencing and ion-channel activation [225]. Anyway, the unique catalytic mechanism of

Sirtuins that consumes NAD^+ provides a direct connection between protein deacetylation and central metabolic pathways [226]. In particular, a direct link between cellular NAD^+ levels and the regulation of chromatin accessibility and gene expression could be established. Many metabolic enzymes, including several in glycolysis and the TCA cycle, utilize NAD^+ as an electron acceptor and reduce it to NADH. Thus, the activity of Sirtuins could be influenced by fluctuations in free NAD^+ levels deriving from changes in cellular metabolism, for example, in response to feeding or fasting [227]. Of relevance, such regulation of Sir2 by NAD^+ levels was one of the earliest hints that small molecule metabolites might be important for regulating gene expression through histone modifications [228].

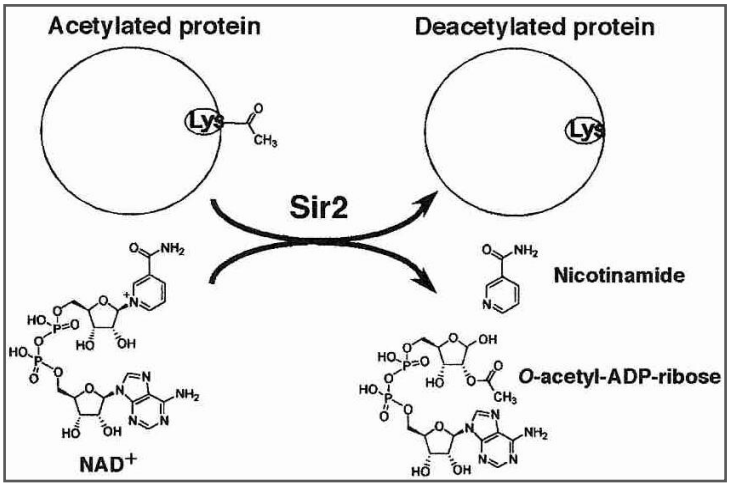

Fig. 1.16. Sir2/Sirtuins deacetylation reaction. Sir2 and Sirtuins catalyze NAD^+ dependent deacetylation of histone tails or other non-histone acetylated proteins; the reaction transfers the acetyl group from acetylated lysine residues to the ADP-ribose moiety of NAD^+, generating deacetylated histone tails nicotinamide and the novel metabolite OAADPr.

Mammals have seven Sirtuins (SIRT1-7), that possess NAD^+-dependent deacetylase, deacylase and ADP-ribosyltransferase activities [229]. They have been found in different subcellular locations, including the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2) and mitochondria (SIRT3,

SIRT4 and SIRT5) and they are also expressed differently in the various tissues of the organism (see below and Fig. 1.17). Structural analysis of the Sirtuin family members reveals a conserved catalytic core composed of two sub-domains. The two domains are connected by several loops that form a binding cleft for the nicotinamide and ribose moieties of NAD^+ and the acyl lysine substrate. Several invariant amino acids are located in the cleft and are responsible for substrate binding and catalysis [230]. The varying hydrophobicity and charge distribution of the acyl-substrate binding cleft allows for varied substrate selectivity among the different human Sirtuins [231]. In addition to acetyl-CoA, some other abundant acyl-CoAs might serve as donor molecules for the post-translational modification of lysine residues both in histone and non-histone proteins. While many of these newly described modifications were reported on histone proteins, post-translational succinylation and malonylation were identified and verified in several metabolic enzymes from mammalian cells [231–233].

Many laboratories focused on elucidating Sirtuins role in higher organisms. Most of these studies have described a key role for SIRT1 in regulating the metabolic response to CR. The strongest link between mammalian Sirtuins and the anti-aging effects of CR comes from SIRT3, which mediates the prevention of age-related hearing loss by CR [234]. Indeed SIRT3 has been shown to directly modulate ROS by deacetylating MnSOD [235,236]. This evidence suggests a broader role for SIRT3 in regulating age-related pathologies that depend in cellular levels of ROS. Evidence for a positive effect of Sirtuins on longevity also comes from the recent discovery that SIRT6 overexpression extends lifespan in mice [237], being this is the first demonstration that a mammalian Sirtuin positively regulates lifespan. Nowadays, the number of Sirtuin targets is continually increasing. SIRT1 deacetylates a number of histone and non-histone proteins including histones H3 and H4, p53, NF-κB, phosphoglycerate mutase 1 and PGC-1α [238]; SIRT3 deacetylates and modulates the activity of several metabolic enzymes including long chain acyl-CoA dehydrogenase, MnSOD, acetyl-CoA synthase 2 and isocitrate dehydrogenase 2 [238].

1.3.2. Influence on the cell metabolism

Because Sirtuin enzymatic activity is dependent upon the presence of NAD, their activity is directly linked to the metabolic state of the cell. Indeed, nearly every Sirtuin has been shown to play a role in regulating metabolism and energy homeostasis, often in roles that help the cell adapt to periods of low energy input. Indeed, Sirtuins are involved in multiple metabolic pathways (Fig. 1.17).

Because of the importance of proper metabolic function in aging, the Sirtuins are positioned to play important roles in aging based on their function in regulating some metabolic processes. In a number of species, Sirtuins are required for the increased lifespan observed in response to CR [239]. The mammalian Sirtuins regulate stress responses, cell survival and the insulin and fat metabolism [240]. In particular, a key role is played in the regulation of acetate metabolism and acetyl-CoA homeostasis. On the base of this and on the importance of Sirtuins in the regulation of aging and longevity, acetate metabolism itself is likely to play an important (and somehow underappreciated) role in aging. In fact, although the physiological consequences of acetylation on every metabolic enzyme are not yet known, the requirement of acetyl-CoA for protein acetylation suggests that acetate metabolism will contribute to the regulation not only of the chromatinic status, but also of several metabolic pathways via its impact on protein acetylation [241].

Lipid metabolism

Lipid catabolism is especially important during fasting and its regulation is tightly linked to HAT/HDACs modifications [242]. SIRT1 is induced in several tissues during CR [243] and responds to the need of the organism for energy by stimulating lipid breakdown through the transcription factor FoxO1. SIRT1 also inhibits the ability of the cell to synthesize fat by deacetylating a lipogenic activator [234]. The metabolic effect of SIRT3 appear far to be straightforward: SIRT3 is upregulated by fasting in liver and brown adipose tissue and promotes mitochondrial oxidative metabolism via deacetylation of numerous metabolic enzymes [244]. However, not all Sirtuins act in the same direction: knocking down SIRT4 increases fatty acid oxidation, suggesting that this Sirtuin opposes its mitochondrial counterpart SIRT3 [245].

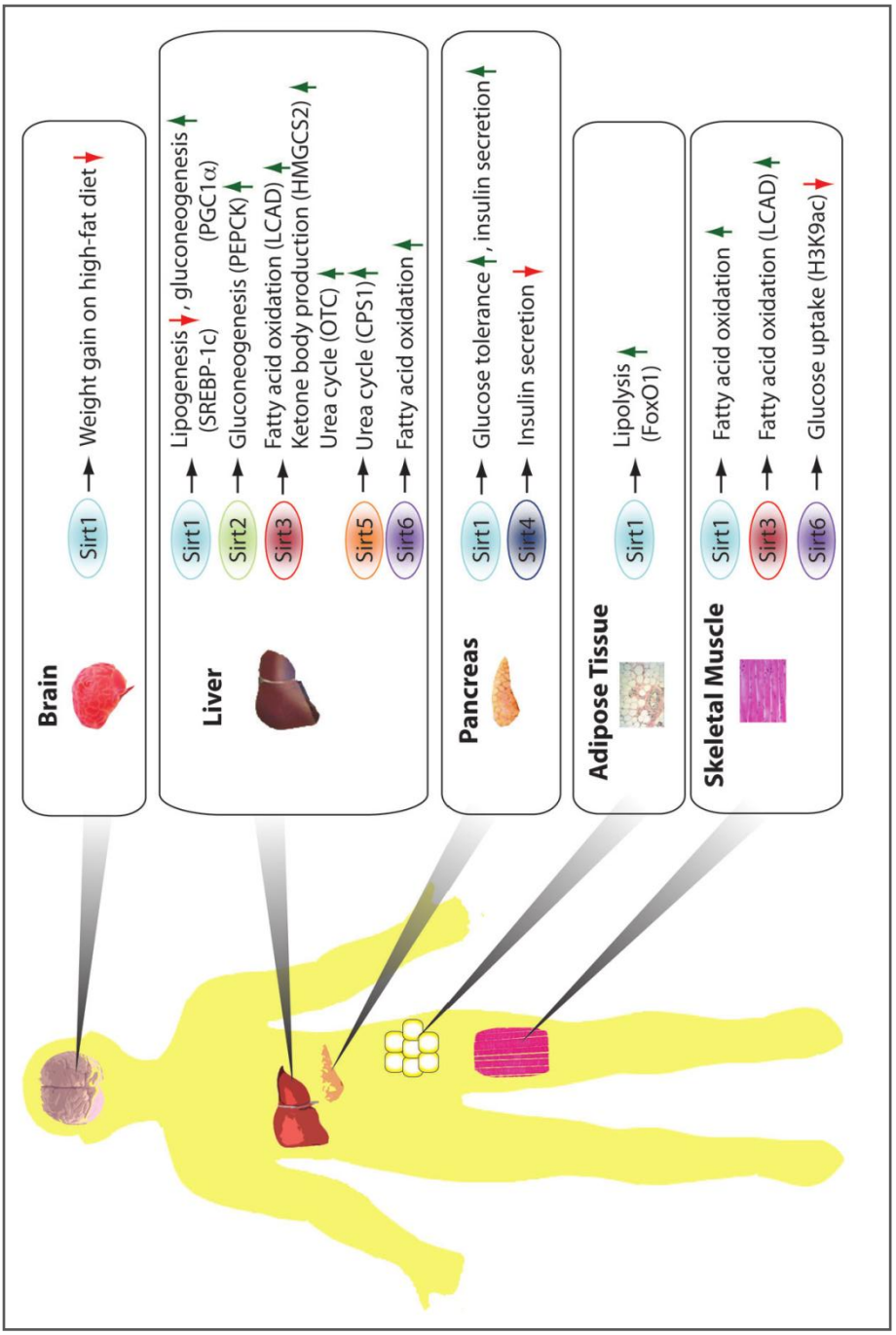

Fig. 1.17. Metabolic functions for the mammalian Sirtuins in different tissues (taken from [234]).

Glucose metabolism

Most of the wok done so far on how Sirtuins regulate glucose metabolism has focused on SIRT1. In response to fasting, SIRT1 expression is increased by an inducer of gluconeogenic genes. Increased SIRT1 protein levels result in deacetylation and, consequently, the activation of PGC-1α, a regulator of genes involved in mitochondrial biogenesis, thermogenesis, ROS detoxification and gluconeogenesis. Activation of PGC-1α by SIRT1 turns on the expression of a number of catabolic proteins in metabolism. In response to refeeding, transcription of *sirt1* gene is decreased, serving as a molecular switch to the anabolic state [246]. Other recent studies show also that the transcription of *sirt3* is induced by PGC-1α in muscle cells, brown adipose and hepatocytes [247]. Recently, other Sirtuins have appeared on the stage as key regulators of glucose homeostasis: SIRT6 is an important regulator of glucose uptake and metabolism, since it lowers the expression of some glycolytic genes through deacetylation of histone H3 K9 [248]. Moreover, SIRT2 takes part in glucose metabolism as well, by promoting gluconeogenesis through deacetylation and stabilization of the rate-limiting enzyme, PEPCK [249].

Acetyl-CoA homeostasis

Acetate is an abundant and important metabolite; however, as mentioned above, before entering central metabolism, it must be activated into acetyl-CoA by ACS; then, it can enter either the glyoxylate cycle or directly feeds into the TCA cycle. ACS are very well conserved enzymes from bacteria to humans [250]; interestingly, also some aspects of their regulation, as reported in Fig. 1.19, have been maintained across the evolution. In contrast to prokaryotes, eukaryotic cells contain two isoforms of ACS which are localized in the cytoplasm and the mitochondria. Generally in the cytosol, acetyl-CoA is used for anabolic processes, such as fatty acid biosynthesis or can regulate histone acetylation at nuclear level, whereas the mitochondrial acetyl-CoA is mainly used for energy generation (reviewed in [241]). Only more recently have researchers begun to appreciate the possibility that, apart from NAD homeostasis, also levels of acetyl-CoA itself could be rate-limiting for specific protein acetylation modifications, as reported in Fig. 1.18 [228,251,252].

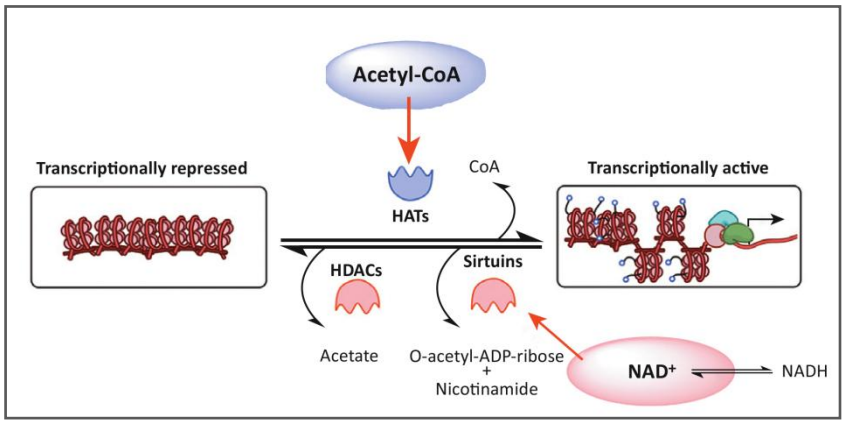

Fig. 1.18. Small-molecule metabolites in the cell are involved in the regulation of gene expression. For example, histone acetyltransferases (HATs) use acetyl groups from acetyl-CoA to acetylate lysines along the histone tails that protrude out of the octamer. This modification facilitates opening of the chromatin and allows various transcription factors to access the DNA to turn on gene transcription. By contrast, HDACs remove acetylation marks from histones and return the chromatin to its closed conformation. A subset of HDACs, Sirtuins, are regulated by NAD^+ levels in the cell (adapted from [228]).

In human, acetate can freely diffuse through the blood to peripheral tissues, where extrahepatic AceCS2 (see Appendix I) uses this free acetate and ATP to generate acetyl-CoA. The expression of AceCS2 is activated at the transcriptional level in skeletal muscle during fasting. Similar to AceCS1, AceCS2 enzymatic activity is regulated by reversible acetylation [253]. A single acetylation site is present in a region of AceCS2 that is highly conserved in all ACS of different species, including *Salmonella enterica, S. cerevisiae, C. elegans, D. melanogaster* and mice. Deacetylation of AceCS2 leads to its enzymatic activation [241,253]. This is the case of SIRT1 and SIRT3 that regulate the acetylation ad the enzymatic activity of mammalian cytosolic AceCS1 and mitochondrial AceCS2, respectively, by deacetylating a single lysine residue [253]; the same happens in bacteria. In fact, in *S. enterica*, acetylation of ACS by a protein acetyltransferase inhibits its enzymatic activity: deacetylation of the same lysine by the NAD^+-dependent protein deacetylase CobB, the bacterial Sir2 orthologue, activates ACS (Fig. 1.19) [254].

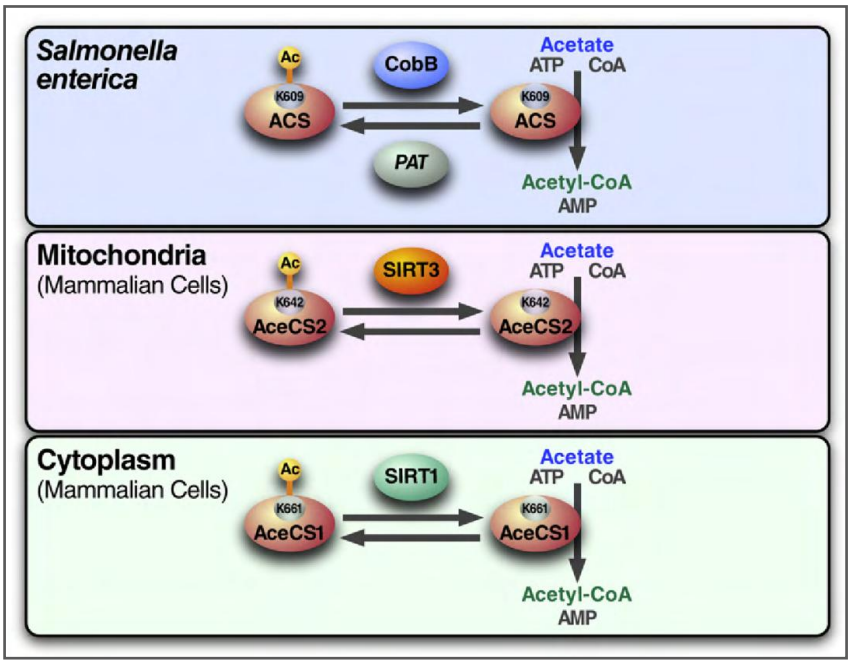

Fig. 1.19. Regulation of ACS by reversible acetylation. The ACS activity is regulated by reversible acetylation via the competing activities of a protein acetyltransferase and a protein deacetylase. ACS are enzymatically inactive when acetylated while they are activated by deacetylation. Active ACS catalyze then the conversion of acetate, ATP and coenzyme A into acetyl-CoA and AMP (taken from [241]).

In *S. cerevisiae*, two ACS isoenzymes are present and both are critical for metabolic regulation. Acs1 is a mitochondrial enzyme needed for growth under gluconeogenic conditions, and Acs2 is the cytosolic enzyme required for growth on glucose [255,256]. However, up to now, no conserved acetylated residue *in vivo* has been found in yeast ACS, that could be regulated by Sir2 and that could in turn regulate the activity of these enzymes, even though the sequence is highly conserved and the lysine that is the target of the Sirtuins is also present [257].

Other metabolic processes

Sirtuins have important functions in additional metabolic pathway that promote adaptation to periods of low energy input. They also upregulate the electron transport chain and the urea cycle (reviewed in [234]). A lot has yet to be still understood about the enzymatic functions of Sirtuins, and future studies will surely investigate the mechanisms through which Sirtuins might work together to coordinate metabolic responses.

1.4 Scope of this thesis

In these days, the biology of aging in humans is a topic of intense interest. Theories are many, but few related results have been presented and specific molecular or genetic determinants of longevity have yet to be identified. In contrast, the biology of aging in model systems is relatively well understood. One of the best characterized models for studying aging is the budding yeast *S. cerevisiae;* in yeast, a clear correlation exists between metabolism and aging, as described in the previous paragraphs. The effect of CR on longevity, as well as the fact that mutations in several genes involved in nutrient signaling influence lifespan, suggest that environmental conditions like the presence of nutrients are of major importance for determining longevity. Moreover, not only the availability of nutrients but also other stressful conditions are able to elongate lifespan. However, whether the hypothesis that presence of nutrients might be or not sensed as a stressful condition [258] remains still unclear, together with the reason why this would influence longevity.

Nowadays, the mission of this field is to continue the molecular characterization of genes encoding proteins that somehow influence longevity and to understand the mechanisms lying behind them, in order to be able, one day, to apply this knowledge to mammalian systems. With this long-term goal in mind, experiments in *S. cerevisiae* to unravel the interconnection between the cell metabolism and the aging machinery have been conducted and the results described in this thesis.

Chapter 1 gives an overview on the current knowledge on the aging process in yeast as well as in higher eukaryotes and the importance of the regulation of the cell metabolism during aging. A topic received special attention: the particular influence of Sir2 and of the Sirtuin family on the cell metabolism and lifespan extension.

In Chapter 2, the attention will be focused on the founding member of the Sirtuin family, the deacetylase Sir2. We observed that this protein plays an important role in the regulation of carbon metabolism, since *SIR2*

inactivation correlates with higher ethanol and acetate catabolism, as well as with an enhanced activity of gluconeogenesis and of the glyoxylate cycle. Thus, we observed that a general reassessment of the carbon metabolism toward gluconeogenesis correlates with CLS extension.

Chapter 3 is focused on a mutant strain lacking the Ach1 enzyme, where to some extent we found the opposite phenotype to *sir2*Δ strain; the long-lived *sir2*Δ cells do not accumulate acetic acid in the culture medium, while the short-lived *ach1*Δ cells accumulate acetic acid at higher extent than wild type cells, inversely correlating the amount of acid release and CLS extension. Furthermore, in this Chapter, the correlation of this pro-aging phenotype in cells lacking Ach1 with an early insurgence of a Yca1-dependent apoptotic markers is presented.

In Chapter 4, the role of extracellular ethanol and acetic acid on CLS is discussed, pointing to distinguish whether the influence of these metabolites on chronological longevity is mainly due to their intrinsic toxicity or to the effects of their metabolism.

1.5 References

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2

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

2.1 Abstract

Yeast chronological aging is regarded as a model for aging of mammalian post-mitotic cells. It refers to changes occurring in stationary phase cells over a relatively long period of time. How long these cells can survive in such a non-dividing state defines the chronological lifespan. Several factors influence cell survival including two well known normal by-products of yeast glucose fermentation such as ethanol and acetic acid. In fact, the presence in the growth medium of these C2 compounds has been shown to limit the chronological lifespan. In the chronological aging paradigm, a pro-aging role has also emerged for the deacetylase Sir2, the founding member of the Sirtuin family, whose loss of function increases the depletion of extracellular ethanol by an unknown mechanism. Here, we show that lack of Sir2 strongly influences carbon metabolism. In particular, we point out a more efficient acetate utilization which in turn may have a stimulatory effect on ethanol catabolism. This correlates with an enhanced glyoxylate/ gluconeogenic flux which is fuelled by the acetyl-CoA produced from the acetate activation. Thus, when growth relies on a respiratory metabolism such as that on ethanol or acetate, *SIR2* inactivation favors growth. Moreover, in the chronological aging paradigm, the increase in the acetate metabolism implies that *sir2*Δ cells avoid acetic acid accumulation in the medium and deplete ethanol faster; consequently pro-aging extracellular signals are reduced. In addition, an enhanced gluconeogenesis allows replenishment of intracellular glucose stores which may be useful for better long-term cell survival.

2.2 Introduction

Aging is a time-dependent progressive and irreversible physiological /functional decline of an organism that is accompanied by an increased vulnerability to both environmental stress and diseases and increased risk of mortality. On the whole, aging is a complex multi-factorial process modulated by interplay between genetic and environmental factors. No single variable can adequately capture the full extent of this complexity since several processes interact simultaneously and operate at different levels of functional organization [1, 2]. The budding yeast *Saccharomyces cerevisiae* is one of the most established model systems used for aging-related research which provides, among others, the opportunity to study and compare the aging processes of both proliferating and non-proliferating cells in a simple single-celled organism. In fact, in yeast two aging paradigms have been described: replicative and chronological. In the former, replicative lifespan (RLS) is defined as the number of daughter cells an asymmetrically dividing mother generates in the presence of nutrients before senescence [3]. In the latter, chronological lifespan (CLS) is the mean and maximum survival period of a population of non-dividing cells in stationary phase. Viability over time, in this case, is defined as the ability to resume mitotic growth upon return to rich fresh medium [4].

In the stationary phase, yeast cells display a survival-based metabolism and acquire physiological and morphological features, including a thickened cell wall, accumulation of storage carbohydrates and increased stress resistance, which result from the integrated responses of different signalling pathways. The establishment of a quiescent program allows the cells to survive starvation and resume growth when nutrient conditions become favorable again [5]. CLS can be extended by either inhibition/reduced activity of two major nutrient-sensing pathways such as TORC1-Sch9 and Ras-PKA ones, or by calorie restriction (CR), the practice of limiting nutrient intake which in yeast is generally imposed by reducing the glucose concentration in the growth medium [6-8]. Defects in TORC1-Sch9 or Ras-PKA signaling as well as CR lead to in part common downstream targets (Rim15, Msn2/4 and

Gis1) which ultimately by increasing endogenous stress defence mechanisms contribute to enhance cell survival [6, 9].

Moreover, during chronological aging, in addition to the well known intrinsic factors such as hydrogen peroxide and superoxide [10], cellular stresses also include extrinsic factors such as ethanol and acidic acid. In fact, in some settings, the presence in the growth medium of these two by-products of the yeast metabolism restricts CLS [11, 12].

In the chronological aging paradigm, a pro-aging role is also played by Sir2 [12]. Sir2 is the founding member of Sirtuins, a family which comprises the unique class III of NAD^+-dependent deacetylases known to be evolutionary conserved regulators of aging [1, 7, 13]. Much work in yeast has focused on histone deacetylation, but a wide range of non-chromatin substrates for the other Sirtuins have been identified which are involved in different metabolic processes including energy production, the urea cycle, fatty acid and acetate metabolism [14, 15]. Concerning the acetate metabolism, in mammals, acetate derived from both exogenous and endogenous sources, is activated to acetyl-CoA either in the cytoplasm by acetyl-CoA synthetase 1 (AceCS1) or in the mitochondria by AceCS2 [16, 17]. The Sirtuins, SIRT1 and SIRT3 deacetylate AceCS1 and AceCS2, respectively, promoting their activity [18]. Both SIRT1 and SIRT3 play an important role during energy-poor diets [19]. In particular, under long-term fasting or CR, acetate is released from the liver and utilized for energy production in extraepatic tissues following AceCS2 activation [16, 20]. In this context, SIRT3 might modulate the reprogramming of mitochondria to low energy input [21]. The SIRT1/3-dependent regulation of AceCS1/2 and the implication of the two Sirtuins in aging and in the CR-mediated longevity response [13, 22, 23], have suggested an involvement of the acetate metabolism in the aging process [16].

Similarly to SIRT1/3, a Sir2 ortholog, CobB, in *Salmonella enterica* activates through deacetylation the acetyl-CoA synthetase (Acs in bacteria and yeast) allowing acetate utilization for acetyl-CoA synthesis and bacterial growth on acetate [17, 24]. In *S.cerevisiae* two Acs isoenzymes, Acs1 and Acs2, are present which differ with respect to kinetic properties and cellular localization [25, 26]. To date, there is no evidence of a reversible acetylation involved in their enzymatic activation [25]. Interestingly, Acs2 is required

for replicative longevity [25], further supporting the notion that the acetate metabolism can play an important role during aging. This is also suggested by data showing that genetic interventions which drive yeast metabolism away from acetic acid production increase CLS [11].

In this study, we provide evidence that in the budding yeast, *SIR2* inactivation actually influences positively the acetate utilization by way of an increased flux of the glyoxylate/gluconeogenic pathway. In the chronological aging paradigm, this implies low levels of toxic extracellular factors (ethanol and acetic acid) and an increase of protective intracellular factors (trehalose) in the *sir2*Δ cultures which all together may favor a better long-term survival and extension of CLS.

2.3 Materials and methods

2.3.1. Yeast strains and growth conditions

All yeast strains used in this work are listed in Table S2.1. All deletion strains were generated by PCR-based methods [27]. Genomically 3HA-tagged strains were obtained as described [28]. The tagged strains were undistinguishable from the congenic untagged ones with respect to overall morphology, cellular volumes, duplication times and CLS. The accuracy of all gene replacements and correct deletions/integrations was verified by PCR with flanking and internal primers. Primer sequences are available upon request. Standard methods were used for DNA manipulation and yeast transformation.

Table S2.1: strains used in this study.

Strain	Relevant genotype	Source
W303-1A	*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	P.P. Slominski
YVU21	W303-1A *sir2*Δ*::URA3*	[a]
YVU81	W303-1A *icl1Δ::KlLEU2*	This study
YVU82	W303-1A *sir2*Δ*::URA3 icl1Δ::KlLEU2*	This study
YVU83	W303-1A *pck1Δ::KlLEU2*	This study
YVU84	W303-1A *sir2*Δ*::URA3 pck1Δ::KlLEU2*	This study
YVU90	W303-1A *PCK1-3HA::KlURA3*	This study
YVU91	W303-1A *sir2*Δ*::HIS3 PCK1-3HA::KlURA3*	This study
GRF18c	*MAT*α	[b]
GRF18L	*MAT*α *his3-11,15*	[b]
YVU65	GRF18L *sir2*Δ*::HIS3*	This study

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Yeast cells were grown in batches at 30°C in rich medium (YEP, 1% w/v yeast extract, 2% w/v bacto peptone) with the indicated carbon source at 2%. For the acetate-YEP medium (pH 4 or 5.8) precalculated amounts of 0.2 M acetic acid and 0.2 M sodium acetate solutions were mixed and added to YEP medium to obtain the required pH and molarity (0.1 M). pH 5.8 was selected since it was the initial pH of the unbuffered YEP medium. For cells grown in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/l), auxotrophies were compensated for with a four-fold excess of supplements [12]. All strains were inoculated at the same cellular density (culture volume no more than 20% of the flask volume). Growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyzer, as described [29]. Doubling time (Td) was obtained by linear regression of the cell number increase over time on a semilogarithmic plot.

2.3.2. Ethanol and acetate pulses

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

calculated as in [33]. Each pulse experiment was carried out in triplicate. Trends reported refer to a representative experiment.

2.3.3. Metabolite measurements

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

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

2.3.4. Enzyme assays

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

2.3.5. Immunoprecipitation and Western analysis

Cellular extracts for anti-HA immunoprecipitation were prepared essentially as described [36] in the presence of protease inhibitors (1 mM

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

2.3.6. Acetic acid sensitivity

Exponentially growing cells on glucose were harvested and resuspended (10^7 cells/ml) in YEP/glucose (YEPD) pH 5.8, YEPD pH 4 or YEPD pH 3 (set with HCl) medium containing 80, 100 or 120 mM acetic acid. Cells were incubated for 200 min at 30°C with shaking [37]. After treatment, cells (serially diluted) were spotted onto YEPD plates. Plates were incubated at 30°C for 3 days.

2.3.7. CLS determination

Survival experiments in expired medium were performed on cells grown either in YEPD or in minimal medium (with a four-fold excess of supplements)/2% glucose as described by [12, 38]. 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 [12] by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 3 was considered the initial survival (100%).

Survival experiments in water and in water containing ethanol were performed on cells grown in minimal medium/glucose (with a four-fold excess of supplements) as described by [12]. Every 48 h, ethanol was added to the culture after washing at a concentration (6 g/l) equal to that found in the expired medium at Day 0. In parallel, 50 mM pyrazole (Sigma) was added [39]. Viability was measured as described above.

For CLS determination in media-swap experiments, cells were grown in minimal medium/glucose (with a four-fold 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.

2.4 Results and discussion

2.4.1 Chronological aging *sir2*Δ cells avoid extracellular acetic acid accumulation

In chronological aging, two by-products of glucose fermentation, ethanol and acetic acid, which accumulate in the yeast culture medium, have CLS-shortening effects [11, 12]. Since CLS extension in *sir2*Δ mutants is associated with an increase in the depletion of extracellular ethanol, which is re-introduced into the metabolism via its oxidation to acetate (Fig. 2.1A), we wondered whether the extracellular acetate/acetic acid (see below) was also influenced by *SIR2* inactivation. To this end, we analyzed the kinetics of ethanol and acetate accumulation in wild type (wt) and *sir2*Δ cultures during growth on YEP/2% glucose (YEPD) and the subsequent utilization of these C2 substrates during the post-diauxic phase.

During the exponential phase, when growth is sustained by a prevalent fermentative metabolism, the glucose decrease was accompanied by ethanol accumulation that in both strains followed the same kinetics (Fig. 2.1B). After the diauxic shift, when cells switched to a respiration-based metabolism, ethanol was depleted more rapidly in the *sir2*Δ culture compared with the wt one (Fig. 2.1B and C). With regard to acetate, in the wt culture, its concentration initially increased and then slowed down (Fig. 2.1D). Interestingly, in the *sir2*Δ culture very low levels of excreted acetate were detected (Fig. 2.1D). Moreover, measurements of ethanol and acetate in the culture media of cells grown in minimal medium also revealed that, after the diauxic shift, in the *sir2*Δ culture, ethanol was exhausted earlier than in the wt one (Fig. S2.1A) in line with changes observed by [12] and acetate was detected at very low levels (Fig. S2.1B). Thus, lack of Sir2 affects the extracellular abundance of two chronological aging factors, ethanol and acetic acid, but unlike the case with ethanol, the reduced accumulation of acetic acid seems to be linked to a reduced secretion of the fermentation product.

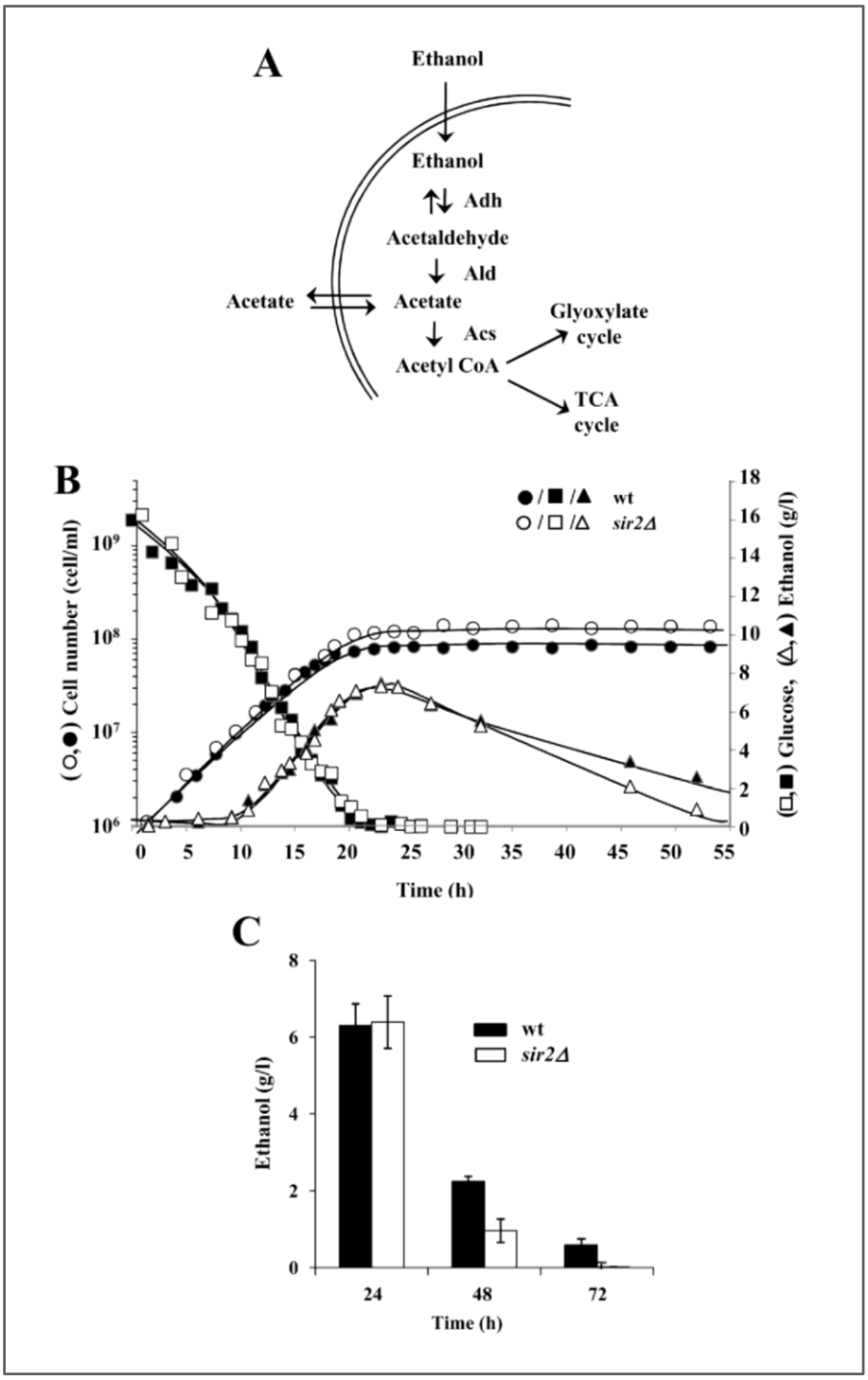
It is known that culturing cells in rich YEPD medium rather than in synthetic defined minimal medium extends CLS [40-42] and this was the case for both wt and *sir2*Δ cultures (Fig. 2.1E and F). Besides, *sir2*Δ cells aged in YEPD medium lived longer than the wt ones (Fig. 2.1E). In agreement with [12], no significant difference in CLS was observed between the two strains grown on minimal medium when CFUs were monitored until 99.9% of the population died (Fig. 2.1F).

However, CFUs monitored below this value (Fig. 2.1F) and some experiments performed in another genetic background [12] may suggest that *SIR2* inactivation also affects positively chronological survivability in minimal medium. Known interventions that increase CLS, such as CR, promote longevity by reducing also the extracellular amount of acetic acid [11, 43]. It is therefore conceivable that the low level of this compound in the *sir2*Δ medium might contribute to determine the *sir2*Δ long-lived phenotype.

Table 2.1: *sir2*Δ mutant growth is favored on ethanol

Medium	Strain	Td (h)*
Glucose	wt	1.39 ± 0.09
	sir2Δ	1.41 ± 0.03
Ethanol	wt	4.38 ± 0.36
	sir2Δ	3.30 ± 0.24
Acetate pH 5.8	wt	2.19 ± 0.2
	sir2Δ	2.21 ± 0.15

Duplication time (Td) of the indicated strains growing on different carbon sources. *Td was calculated as ln2/k, where k is the constant rate of exponential growth. Data represent the average of three independent experiments. Standard deviations are indicated.


A
Ethanol
Ethanol
Adh
Acetaldehyde
Ald
Acetate
Acetate
Acs
Acetyl CoA
Glyoxylate cycle
TCA cycle
B
wt
sir2Δ
(○,●) Cell number (cell/ml)
(□,■) Glucose, (△,▲) Ethanol (g/l)
Time (h)
C
wt
sir2Δ
Ethanol (g/l)
Time (h)

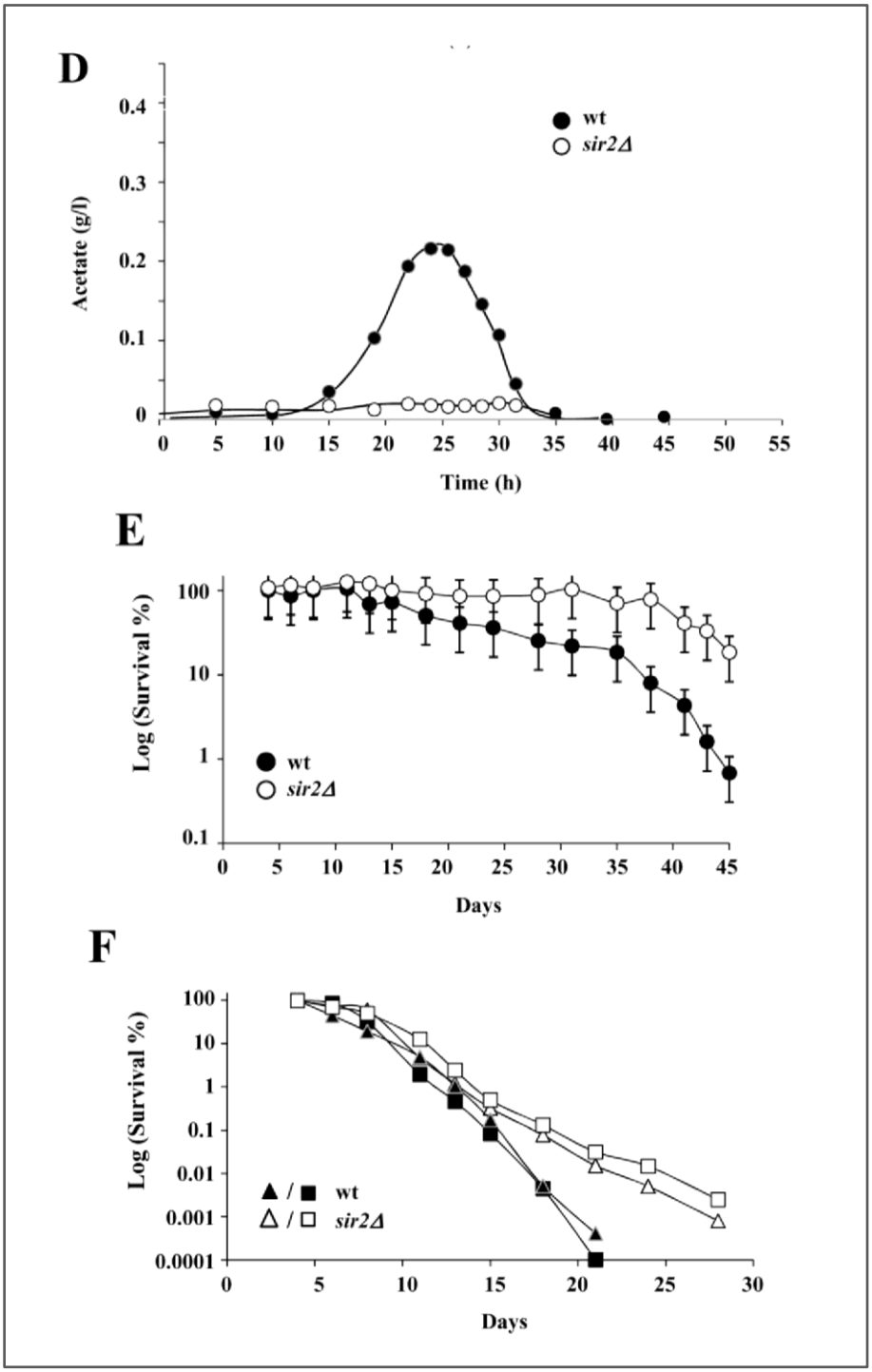

D
Acetate (g/l)
0.4
0.3
0.2
0.1
0
wt
sir2Δ
0 5 10 15 20 25 30 35 40 45 50 55
Time (h)
E
Log (Survival %)
100
10
1
0.1
wt
sir2Δ
0 5 10 15 20 25 30 35 40 45
Days
F
Log (Survival %)
100
10
1
0.1
0.01
0.001
0.0001
▲ / ■ wt
△ / □ sir2Δ
0 5 10 15 20 25 30
Days

Fig. 2.1. *SIR2* inactivation affects the extracellular abundance of ethanol and acetate produced by glucose fermentation. (A) Scheme of the metabolic steps allowing ethanol utilization. Adh: alcohol dehydrogenase; Ald: aldehyde dehydrogenase; Acs: acetyl-CoA synthase. (B) Wild type (wt) and *sir2*Δ cells were grown in YEP/2% glucose (YEPD) and cell growth was monitored by counting cell number over time. Extracellular concentrations of ethanol and glucose (B) and acetate (D) were also measured in medium samples collected at different time-points. One representative experiment is shown. (C) Bar charts of ethanol concentrations measured in the media of cultures described in (B). Data were obtained from mean values determined in three independent experiments. Standard deviations are indicated. (E) CLS of wt and *sir2*Δ mutant cells grown on YEPD. 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 Materials and methods). Error bars are the standard deviation of three replicates. (F) CLS of wt and *sir2*Δ mutant cells grown on minimal medium/2% glucose (with a four-fold excess of supplements). At every time-point, viability was measured as in (E). Two representative experiments are shown.

In parallel, since ethanol and acetate can be utilized by *S.cerevisiae* as sole carbon and energy source under aerobic conditions, we also examined the growth behavior of the *sir2*Δ mutant during exponential growth on these nonfermentable substrates. A marked decrease in the Td of the *sir2*Δ mutant was detected for cells growing on YEP/ethanol compared with the wt, while for cells grown on glucose no significant differences were observed (Table 2.1). Similar results were obtained for YNB-based medium (data not shown). Given that ethanol enters the cells by passive diffusion, a faster growth can be indicative of a faster ethanol catabolism in agreement with the faster depletion of ethanol in the *sir2*Δ culture reported in Fig. 2.1, Fig. S2.1 and by [12]. When cells were grown on acetate, pH 5.8, the Td of the wt and of the *sir2*Δ strain was similar. Nevertheless, lowering the pH to 4 favored the mutant strain growth with respect to the wt that displayed a severe growth defect (Fig. S2.2A). Uptake of acetate is linked to a proton symport mechanism accompanied by passive diffusion of the undissociated acid [44, 45]. The acetic/acetate couple forms a buffer system: at pH 4, acetic acid (pKa 4.75) is substantially undissociated while at pH 5.8 the amount of the charged acetate anion considerably increases. Consequently, at pH 4 the uptake of acetate is facilitated because passive diffusion is elevated. Once

inside the cell (pH close to neutral), acetic acid dissociates causing intracellular acidification that, in turn, is thought to have negative effects on yeast metabolic activity explaining why acetate is generally toxic at pH 4 [37, 45]. The fact that the *sir2*Δ strain grows on acetate at pH 4 also suggests an increased resistance of these cells to acetic acid. Consistently, *sir2*Δ cells showed less sensitivity to acetic acid stress at low pH (Fig. S2.2B).

2.4.2. Lack of Sir2 correlates with a more efficient acetate utilization

In order to better define the metabolic consequences of the *SIR2* inactivation and to obtain reliable quantitative measurements of metabolite productions, we carried out experiments in chemostat. A prototrophic *sir2*Δ strain was generated in a GRF18 background (Table S1) since auxotrophic strains are less suited for quantitative physiological analyses [46]. In this genetic background, the effects of *SIR2* inactivation were similar of those reported in Fig.2.1 and Table 2.1 (data not shown). Aerobic glucose-limited chemostat cultures at a low dilution rate (0.15 h^{-1}) were set up for prototrophic *sir2*Δ and wt strains. In both cultures the high biomass yield, the absence of fermentation products and CO_2 production (biomass and CO_2 were the only products), all indicated that the metabolism was fully respiratory (data not shown) [30]. To this starting condition, where the catabolite repression is alleviated and the enzymes for catabolism of C2 compounds are partially derepressed [47], an ethanol pulse was applied with the simultaneous switch-off of the glucose influx/efflux. In such a way, cells grow using ethanol as the sole carbon source. As shown in Fig.2.2A, Table 2.2, the ethanol specific consumption rate (qEtOH, mmol g^{-1} DW h^{-1}) for the *sir2*Δ strain was higher than the wt one indicating that *SIR2* inactivation accelerates ethanol catabolism. Moreover, following the ethanol pulse, an immediate secretion of acetate was observed in both cultures (Fig. 2.2B) implying an increased metabolic flux through acetaldehyde dehydrogenase towards acetate (Fig. 2.1A) in line with other ethanol pulse experiments [48, 49]. Acetate accumulation in the medium is due to an imbalance between its production rate from acetaldehyde and its conversion rate into acetyl-CoA (Fig. 2.1A). Interestingly, the *sir2*Δ strain not only secreted less acetate than the wt, but

after a transient accumulation, the concentration of acetate started to decrease quickly despite high levels of ethanol in the medium (Fig. 2.2A and B). After 2 h from the pulse in the mutant, ethanol and acetate were consumed in parallel (ethanol-acetate cometabolism) and in the medium the latter was exhausted first (Fig. 2.2A and B). This indicates that in the *sir2*Δ strain, acetate is metabolized faster than it is produced implying an increased metabolic flux downstream from the acetate. Indeed, when an acetate pulse was applied to the aerobic glucose-limited chemostat cultures set up for the *sir2*Δ and wt strains, the acetate specific consumption rate (qAc, mmol g^{-1} DW h^{-1}) of the *sir2*Δ strain was higher than the wt one (Table 2.2) supporting the assumption of a more efficient acetate metabolism in the mutant. Acetate metabolism requires acetate activation by acetyl-CoA synthetase isoenzymes Acs1 and Acs2, to acetyl-CoA which can be used to fuel the glyoxylate and TCA cycles.

Table 2.2: *SIR2* inactivation increases ethanol and acetate specific consumption rates.

	Strain	
	wt	*sir2*Δ
qEtOH (mmol g^{-1} DW h^{-1})	2.56 ± 0.08	4.24 ± 0.05
qAc (mmol g^{-1} DW h^{-1})	0.65 ± 0.04	0.79 ± 0.03

Ethanol (qEtOH) and acetate (qAc) specific consumption rates were determined in ethanol and acetate pulse experiments, respectively. Data represent the average ± standard deviation of three independent replicates.

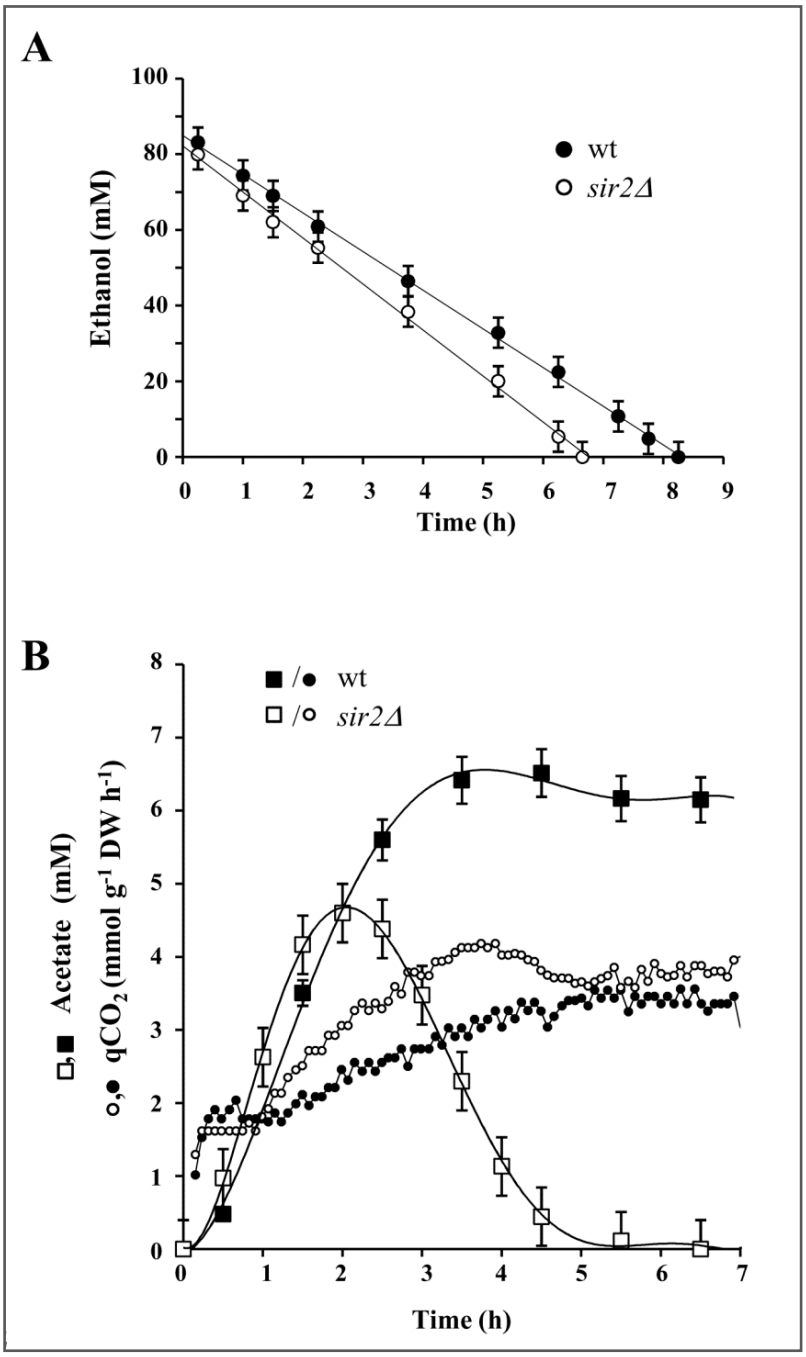


Fig. 2.2. *SIR2* inactivation affects acetate metabolism. Wild type (wt) and *sir2*Δ cells were grown in glucose-limited chemostat cultures (0.15 h^{-1} dilution rate) and at

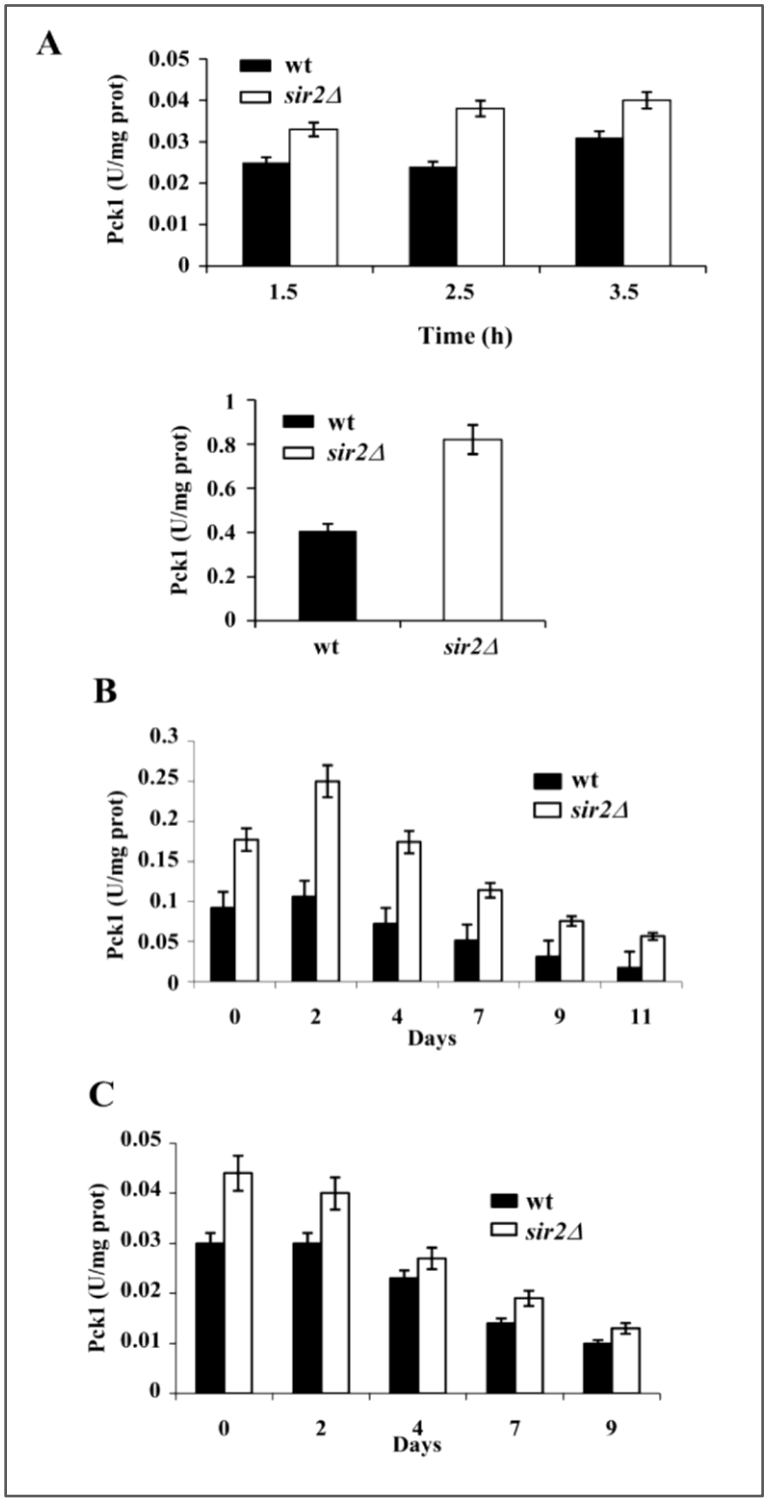
the steady-state (time 0) an ethanol pulse was applied (about 90 mM final concentration). Extracellular ethanol (A) and acetate (B) were measured in the medium at different time-points after ethanol addition. Standard deviations are indicated. In parallel, the concentration of carbon dioxide in the off-gas was monitored. qCO_2: carbon dioxide specific production rate. Data are reported as a function of the time after ethanol addition.

In addition, acetyl-CoA is used for synthesis of macromolecules which requires active gluconeogenesis [50]. It has been reported that increase in Acs activity did not result in enhanced acetate utilization [51, 52] indicating that reactions downstream from Acs mainly control the *in vivo* rate of acetate activation. In this context, off-gas analyses during the ethanol pulse gave some information about the reactions downstream from acetyl-CoA that were influenced by *SIR2* inactivation. No significant difference in the oxygen specific consumption rate (qO_2, mmol g^{-1} DW h^{-1}) between the wt and the *sir2*Δ strains was found (Fig. S2.3). This is indicative of a similar mitochondrial respiratory activity. On the contrary, a higher CO_2 production rate was measured in the mutant during acetate accumulation and utilization (Fig. 2.2B) suggesting that decarboxylation/s along the pathway of the acetate metabolism is/are increased.

2.4.3. Lack of Sir2 correlates with enhanced gluconeogenesis

Phosphoenolpyruvate carboxykinase (Pck1) catalyzes the rate-limiting step in gluconeogenesis by converting a C4 compound such as oxaloacetate (OAA) to a C3, phosphoenolpyruvate. Pck1 is subjected to different layers of regulation including posttranslational acetylation at K514 by Esa1 which is crucial for its enzymatic activity, for the ability of yeast cells to grow on nonfermentable carbon sources and for CLS extension under a severe form of CR such as water starvation. Interestingly, Sir2 is the enzyme responsible for Pck1 deacetylation [53]. In the *sir2*Δ strain, during the ethanol pulse and during the growth in batch on ethanol, Pck1 enzymatic activity was higher than that of the wt (Fig. 2.3A and B). In addition, chronological aging *sir2*Δ cells had higher levels of Pck1 enzymatic activity compared with the

wt (Fig. 2.3C and D). Since *SIR2* loss of function increases acetylated Pck1 [53], these results prompted us to investigate whether the increase of Pck1 enzymatic activity detected in the *sir2*Δ cells was associated with an increase of the acetylated form of the enzyme. To this end, wt and *sir2*Δ strains expressing a 3HA-tagged version of the endogenous Pck1 were generated. As shown in Fig. 2.3D and E and S2.4, *SIR2* deletion did not affect Pck1-3HA levels, while the amount of acetylated Pck1-3HA increased in the *sir2*Δ strain compared with the wt. This suggests that *SIR2* deletion correlates with an *in vivo* increase of the acetylated enzymatic active Pck1 which, in turn, may favor the gluconeogenic flux. During growth on C2 compounds, the glyoxylate cycle is generally assumed to be the exclusive source of OAA (derived from two molecules of acetyl-CoA) which enters the gluconeogenic pathway [50]. Measurements of the enzymatic activity of isocitrate lyase (Icl1), which is one of the unique enzymes of the glyoxylate cycle, showed higher levels in the *sir2*Δ mutant compared with the wt during both the ethanol pulse and batch growth (Fig.2.4A). This further supports the notion of an increased assimilation of C2 units by the way of the glyoxylate/gluconeogenic pathway linked to *SIR2* inactivation and also gives some experimental evidence of the model proposed by [53] concerning the role played by the Pck1 acetylation state in the control of the gluconeogenic flux. Gluconeogenesis switches the direction of metabolite flow towards the essential biosynthetic precursor, glucose-6-phosphate, which is also needed for glycogen and trehalose stores. In particular, trehalose has been proposed to be a key determinant of the quiescent metabolic state as a fuel reserve that enables yeast to survive starvation and then rapidly proliferate upon return to favorable growth conditions [54]. During stationary phase, in both the wt and *sir2*Δ strains the trehalose content increased but in the mutant this disaccharide is more abundant (Fig. 2.4B), in line with transcriptional data showing down-regulation of genes involved in utilization/breakdown of trehalose [12]. Enhanced intracellular trehalose levels might contribute to the reported stress-resistant phenotype of the mutant [12] and to improve its survivability and CLS.


A
Pck1 (U/mg prot)
0.05
0.04
0.03
0.02
0.01
0
wt
sir2Δ
1.5
2.5
3.5
Time (h)
1
0.8
0.6
0.4
0.2
0
wt
sir2Δ
B
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
7
9
11
Days
C
0.05
0.04
0.03
0.02
0.01
0
0
2
4
7
9
Days

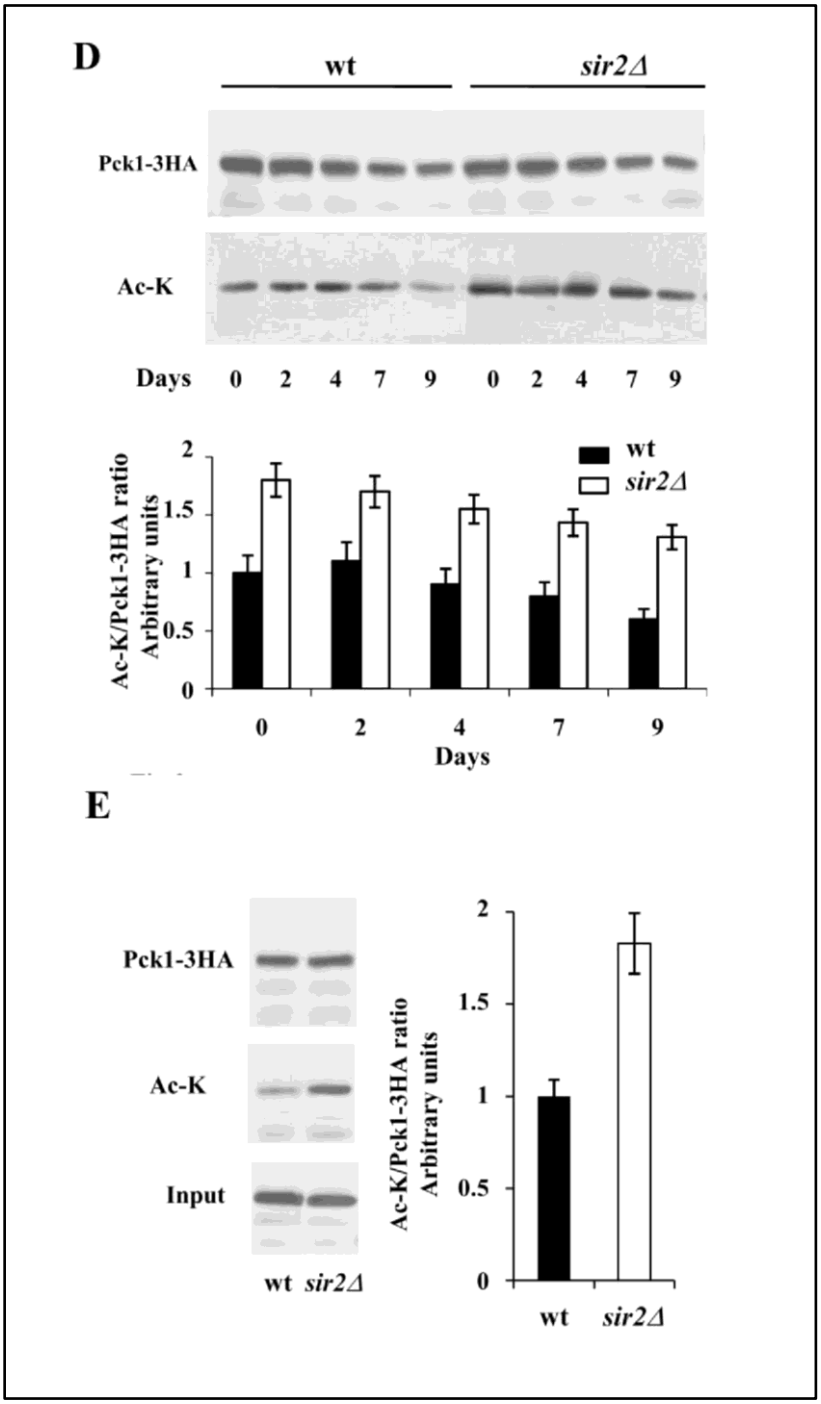
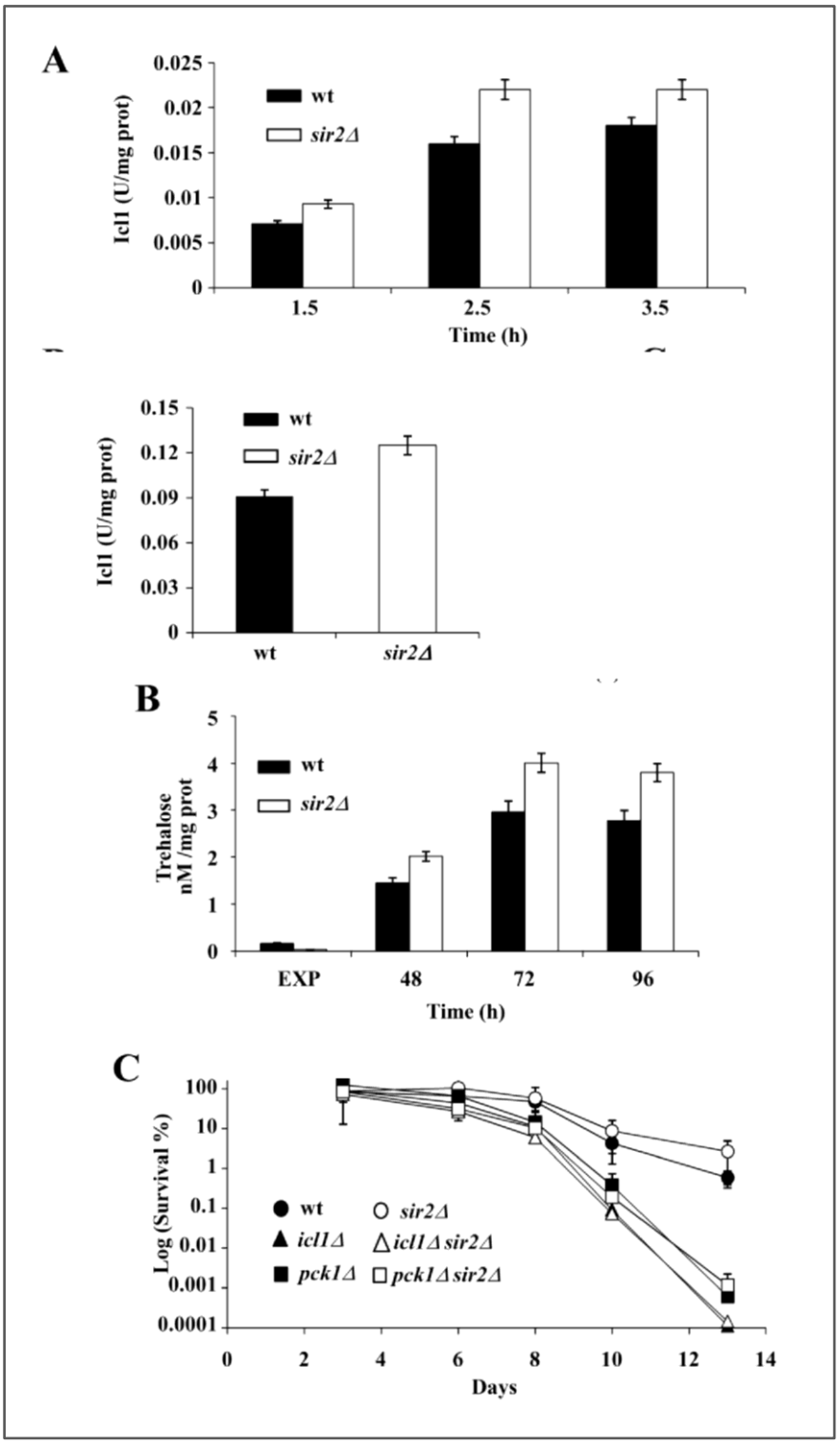

D
wt
sir2Δ
Pck1-3HA
Ac-K
Days 0 2 4 7 9 0 2 4 7 9
Ac-K/Pck1-3HA ratio
Arbitrary units
2
1.5
1
0.5
0
wt
sir2Δ
0 2 4 7 9
Days
E
Pck1-3HA
Ac-K
Input
wt sir2Δ
Ac-K/Pck1-3HA ratio
Arbitrary units
2
1.5
1
0.5
0
wt
sir2Δ

Fig. 2.3. *SIR2* inactivation increases acetylated Pck1 in concert with increased enzymatic activity. (A) Pck1 enzymatic activity was measured at the indicated time-points after the ethanol addition to glucose-limited chemostat cultures of Fig. 2.2 (left panel) and in wt and *sir2*Δ cells exponentially growing on ethanol in batch (right panel). Pck1 enzymatic activity was also measured at the indicated time-points during chronological aging in YEPD (B) and minimal medium/2% glucose (C). Day 0, diauxic shift. Error bars are the standard deviation of three replicates. (D) Wt and *sir2*Δ cells expressing 3HA-tagged Pck1 were grown in minimal medium. During chronological aging at the indicated time-points total extracts (shown in Fig. S2.4) were collected, immunoprecipitated with anti-HA antibody and subjected to Western analysis. Immunodecoration was performed with anti-HA and anti-Ac-K antibodies. Representative blots are shown. For each lane, band intensities were quantified by densitometry and the ratio of Ac-K to Pck1-HA was plotted (lower panel). Standard deviations are indicated ($P \leq 0.01$). Day 0, diauxic shift. (E) Total extracts collected from wt and *sir2*Δ cells expressing 3HA-tagged Pck1, exponentially growing on ethanol were immunoprecipitated with anti-HA antibody and subjected to Western analysis. Immunodecoration was performed with anti-HA and anti Ac-K antibodies. Input, total protein extracts probed with anti-HA antibody. Band intensities were quantified as in (D) and the ratio Ac-K/Pck1-HA plotted (left panel). Standard deviations are indicated ($P \leq 0.01$).

Starting from the aforementioned results, we analyzed the effects on CLS and on extracellular ethanol and acetate levels following *PCK1* or *ICL1* deletions in the *sir2*Δ background. As depicted in Fig. 2.4C, loss of *PCK1* significantly reduced CLS in agreement with [53] but interestingly this was also the case for *ICL1* deletion, further underlying a connection between gluconeogenesis and chronological longevity. Moreover, *SIR2* deletion did not influence the CLS of *pck1*Δ mutants and did not influence the CLS of *icl1*Δ mutants either (Fig. 2.4C). Concerning extracellular ethanol and acetate, the effect produced by both the single *PCK1* and *ICL1* deletions was opposite of that elicited by *SIR2* deletion. In fact, in both single *pck1*Δ and *icl1*Δ cultures, ethanol was exhausted later than in the wt one (Fig. 2.4D) and acetate was detected at higher levels (Fig. 2.4E). In addition, *pck1*Δ and *icl1*Δ cells showed more sensitivity to acetic acid stress at low pH (Fig. S2.5). Similar to the effect on CLS, *PCK1* and *ICL1* were epistatic to *SIR2* in

the pathway involved in the metabolism of ethanol and acetate, since *SIR2* deletion did not affect either ethanol consumption (Fig. 2.4D), acetate levels (Fig. 2.4E) or acetic acid sensitivity (Fig. S2.5) that were detected in the *pck1*Δ and *icl1*Δ mutants. We next performed a media-swap experiment among wt, *icl1*Δ and *sir2*Δ cultures. The three strains were grown on minimal medium and, at Day 1 after the diauxic shift, cultures were centrifuged and media exchanged. The media exchange did not influence the chronological survival of the *sir2*Δ strain (Fig. 2.4F and G) whose CLS was the same despite more ethanol and acetate present in the *icl1*Δ pre-conditioned medium, (Fig. 2.4D and E). On the contrary, the *sir2*Δ pre-conditioned medium had a beneficial effect on the CLS of both wt and *icl1*Δ strains (Fig. 2.4F and G). Interestingly, resuspension of *icl1*Δ cells in the *sir2*Δ pre-conditioned medium increased their CLS to the same extent as that of the wt maintained in its original medium (Fig. 2.4F and G). These results further show that the *SIR2* deletion not only decreases the level of extracellular factors which contribute negatively to CLS, but also enables the cells to better cope with them. With regard to acetic acid, its role during CLS, it is a matter of debate and a clear consensus has not been reached yet [55]. Although preventing acidification of the culture media which is mainly due to acetic acid is sufficient to extend CLS [11, 42], it is unlikely that the physiological extracellular accumulation of acetic acid during CLS standard experiments may be the toxic determinant in itself.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Icl1 (U/mg prot)
0.025
0.02
0.015
0.01
0.005
0
wt
sir2Δ
1.5
2.5
3.5
Time (h)
Icl1 (U/mg prot)
0.15
0.12
0.09
0.06
0.03
0
wt
sir2Δ
wt
sir2Δ
B
Trehalose nM /mg prot
5
4
3
2
1
0
wt
sir2Δ
EXP
48
72
96
Time (h)
C
Log (Survival %)
100
10
1
0.1
0.01
0.001
0.0001
wt
sir2Δ
icl1Δ
icl1Δ sir2Δ
pck1Δ
pck1Δ sir2Δ
0
2
4
6
8
10
12
14
Days

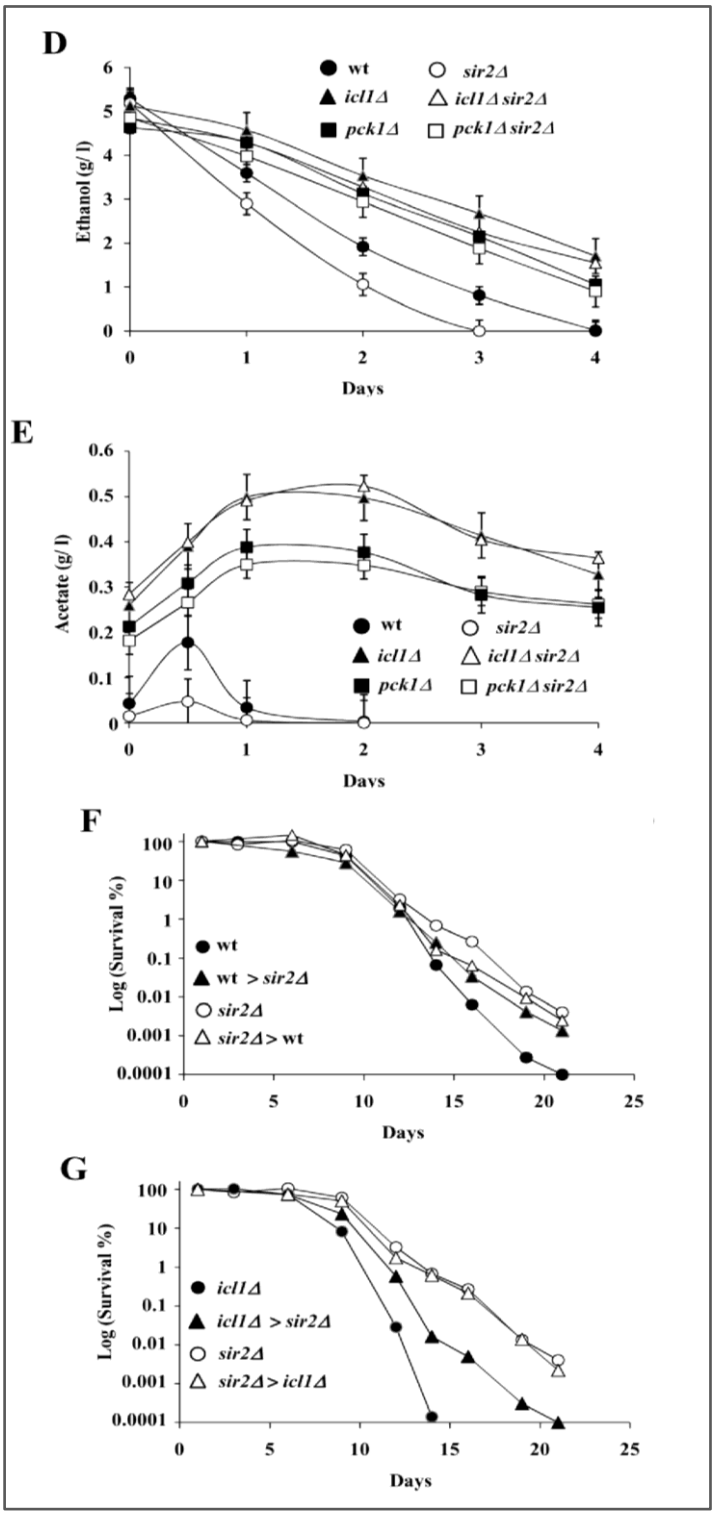
D
wt
sir2Δ
icl1Δ
icl1Δsir2Δ
pck1Δ
pck1Δsir2Δ
Ethanol (g/ l)
Days
E
Acetate (g/ l)
Days
F
wt
wt > sir2Δ
sir2Δ
sir2Δ> wt
Log (Survival %)
Days
G
icl1Δ
icl1Δ > sir2Δ
sir2Δ
sir2Δ> icl1Δ
Log (Survival %)
Days

Fig. 2.4. *SIR2* inactivation increases acetyl-CoA flux towards glyoxylate-requiring gluconeogenesis. (A) Icl1 enzymatic activity was measured at the indicated time-points after the ethanol addition to glucose-limited chemostat cultures of Fig. 2.2 (left panel) and in wt and *sir2*Δ cells exponentially growing on ethanol in batch (right panel). (B) Intracellular trehalose content of the indicated strains grown as described in Fig. 2.1B. Measurements were performed during the exponential phase (EXP) up to the stationary one. Histograms were obtained from mean values determined in three independent experiments. Standard deviations are indicated. (C) CLS of the indicated strains grown on minimal medium. One representative experiment is shown. In parallel, extracellular concentrations of ethanol (D) and acetate (E) were measured in medium samples collected at different time-points. Day 0, diauxic shift. Data were obtained from mean values determined in three independent experiments. Standard deviations are indicated. (F and G) CLS of wt, *icl1Δ* and *sir2Δ* mutant cells grown as in (C) which have been harvested and resuspended in their cell free original media or subjected to cell free media-swap at Day 1 after the diauxic shift. One representative experiment is shown.

To sum up, collectively these data indicate that a lack of Sir2 deacetylase activity positively affects acetate metabolism by enhancing the glyoxylate-requiring gluconeogenesis. This also has a stimulatory effect on ethanol catabolism in order to support the more rapid metabolic flux downstream from the acetate activation into acetyl-CoA. It follows that i) *sir2*Δ cells grow better than the wt on ethanol and acetate and ii) in the post-diauxic phase, following glucose exhaustion, these cells avoid acetate accumulation in the medium and deplete extracellular ethanol faster. This C2 compound indirectly has CLS-shortening effects by being metabolized to acetate. In fact, it has been previously reported that the addition of ethanol to water can prevent CLS extension of chronologically aging cells associated with their transfer to water [12]. This is a severe form of CR which dramatically extends CLS and such a salutary effect is more pronounced in cells lacking Sir2 [12 and Fig. 2.5]. In line with this, ethanol add-back wt and *sir2*Δ cultures had a reduced CLS compared with cells incubated in water alone (Fig. 2.5**).** Moreover, inhibition of ethanol oxidation to acetate by pyrazole, an inhibitor of alcohol dehydrogenase 2 [39], abrogated the pro-aging

signaling seen with ethanol producing a CLS extension similar to that observed in water (Fig. 2.5).

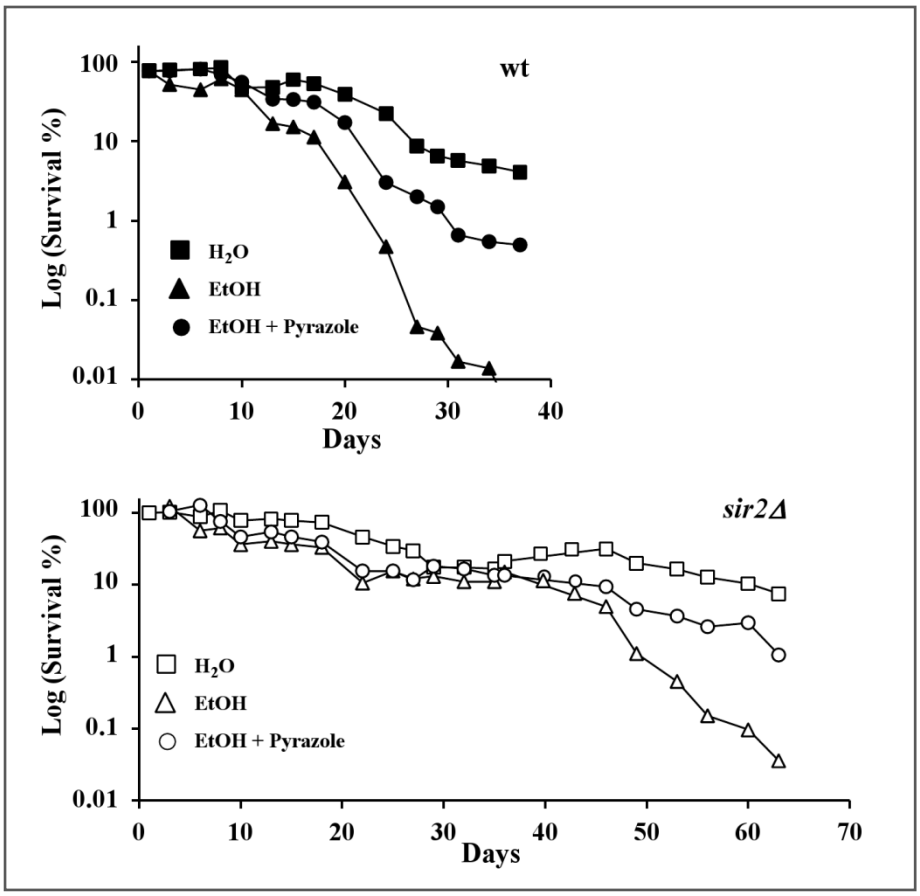

Fig. 2.5. Pyrazole suppresses the CLS-shortening effect of ethanol. Wt and *sir2Δ* cells grown in minimal medium were switched to water, water/ethanol (6 g/l) or water/ethanol/pyrazole (50 mM) at Day 1. Every 48 h, cultures were resuspended in fresh water and each time, ethanol and pyrazole were added when indicated. At every time-point, viability was measured by counting CFUs on YEPD plates. Survival of cells in their exhausted medium was also monitored as control (reported in Fig. 2.1F). One representative experiment is shown.

Enhancement in the intracellular flux of central metabolic pathways such as glycolysis/gluconeogenesis, can be rarely ascribed to changes in a single enzyme since flux control is shared by multiple steps and not usually localized in only one step. Nevertheless, a particular enzymatic reaction can exert a high degree of control over the flux along a pathway and, hence, strongly influence the rate of the flux itself. Thus, except for enzymes subjected to allosteric regulation or feedback inhibition, it is reasonable to speculate that if the rate of this crucial step could be increased, the overall pathway flux might also increase. In this context, probably the major signal which triggered the metabolic changes leading to an improved assimilation of C2 units by the glyoxylate-requiring gluconeogenesis in the *sir2*Δ mutant is the increase in the main flux-controlling step of the gluconeogenesis, namely the Pck1 enzymatic activity. This increase can be brought about by the increase in the acetylated active form of Pck1 due to the lack of the Sir2-targeted deacetylation. Furthermore, the enhancement in the direction of metabolite flow towards glucose-6-phosphate in the *sir2*Δ may not only reduce pro-aging extracellular signaling from ethanol/acetic acid but also increase the accumulation of trehalose creating a beneficial environment that is important for the long-term survival of non-dividing cells.

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2.6 Supplementary materials

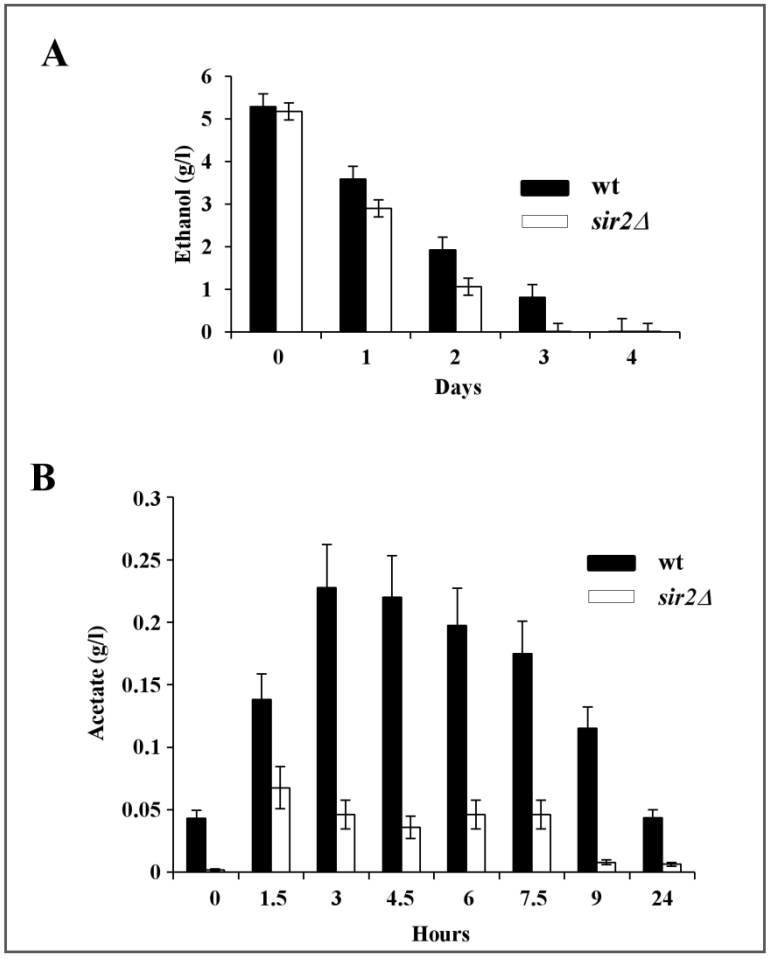

Fig. S2.1 Effects of *SIR2* inactivation on acetate concentration in the CLS-conditioned minimal medium. Wt and *sir2*Δ cells were grown in minimal medium/ 2% glucose and followed up to stationary phase. Bar charts of ethanol (A) and acetate (B) concentrations measured at the indicated time-points in the media of both cultures. Day 0, diauxic shift. Error bars are the standard deviation of three replicates.

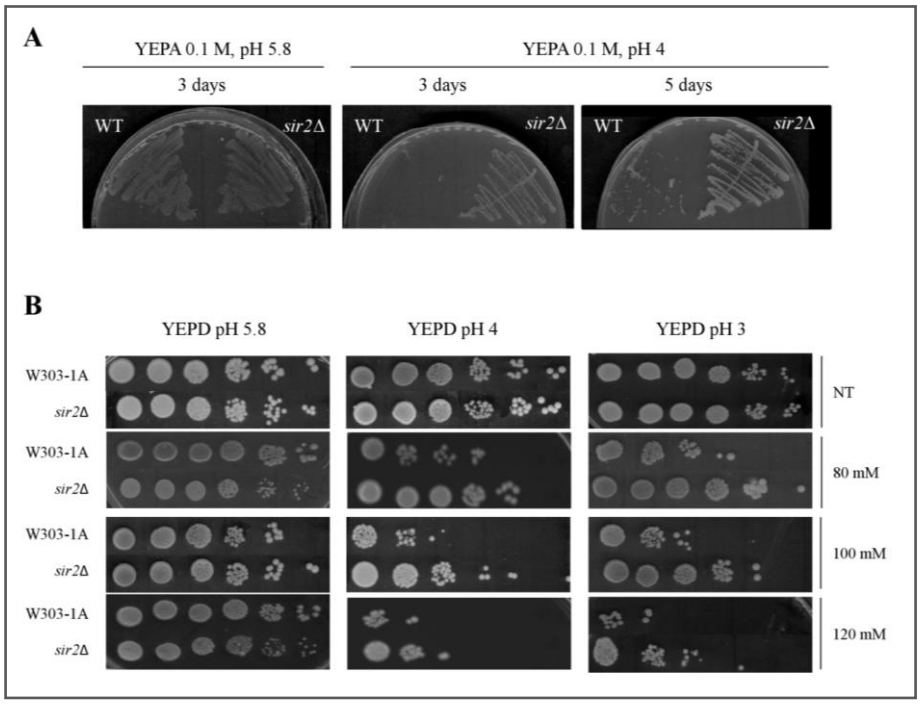

Fig. S2.2 Growth of *sir2*Δ cells is favored in conditions where acetic acid is present. (A) Wild-type and *sir2*Δ cells were streaked onto YEP/0.1 M acetate (YEPA) plates where pH was set to pH 5.8 or 4. Plates were incubated at 30°C for the indicated days. (B) Exponentially growing cells were harvested, resuspended in YEPD at pH 5.8, pH 4 and pH 3 with or without (NT) the indicated concentrations of acetic acid. After 200 minutes at 30°C with shaking, cells (5ml from a concentrated suspension and from 10-fold dilutions) were spotted onto YEPD plates and incubated at 30°C for 3 days. One significant experiment out of 3 is shown.

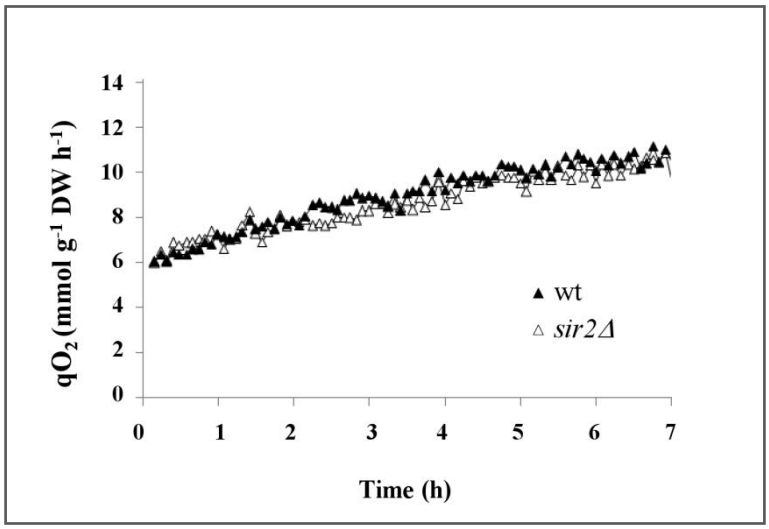

Fig. S2.3 Time profile of the oxygen comsumption rate as a response to an ethanol pulse. Specific oxygen consumption rate of glucose-limited chemostat cultures described in Fig. 2.2 after ethanol addition. The concentration of oxygen was monitored in the off-gas over time.

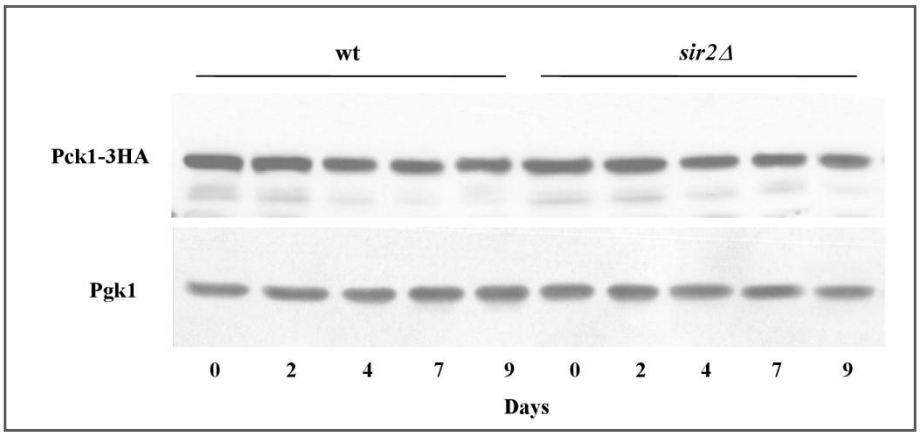

Fig. S2.4 *SIR2* inactivation does not affect Pck1 level during chronological aging. Wt and *sir2*Δ cells expressing 3HA-tagged Pck1 were grown in minimal medium. During chronological aging at the indicated time-points total extracts were prepared and subjected to Western analysis. Immunodecoration was performed with anti-HA antibody. The same filter was also probed with anti-Pgk1 antibody (loading control). T_0, diauxic shift. Representative blots are shown.

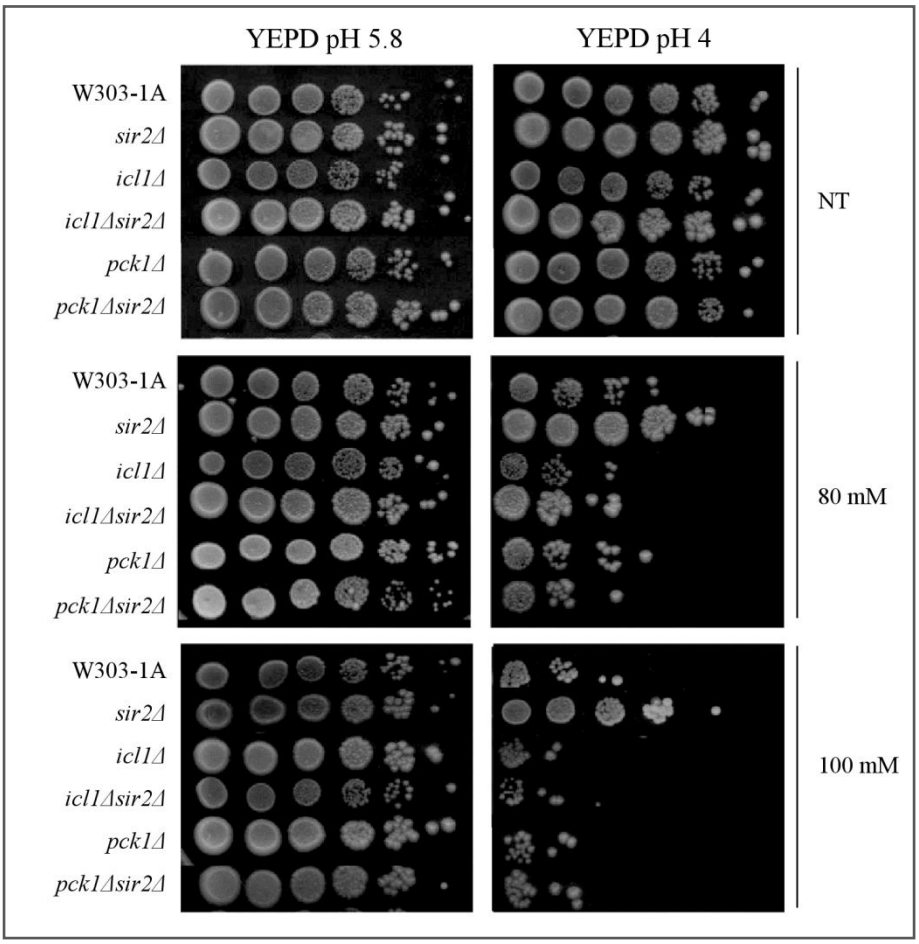

Fig. S2.5 *PCK1* and *ICL1* are epistatic to *SIR2* for acetic acid sensitivity. The indicated strains exponentially growing were harvested, resuspended in YEPD at pH 5.8 and pH 4 with or without (NT) the indicated concentrations of acetic acid. After 200 minutes at 30°C with shaking, cells were spotted as in Fig. S2.2B onto YEPD plates and incubated at 30°C. One representative experiment is shown.

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3

Lack of Ach1 CoA-transferase triggers apoptosis and decreases chronological lifespan in yeast

3.1 Abstract

ACH1 encodes a mitochondrial enzyme of *Saccharomyces cerevisiae* endowed with CoA-transferase activity. It catalyzes the CoASH transfer from succinyl-CoA to acetate generating acetyl-CoA. It is known that *ACH1* inactivation results in growth defects on media containing acetate as a sole carbon and energy source which are particularly severe at low pH. Here, we show that chronological aging *ach1*Δ cells which accumulate a high amount of extracellular acetic acid display a reduced chronological lifespan. The faster drop of cell survival is completely abrogated by alleviating the acid stress either by a calorie restricted regimen that prevents acetic acid production or by transferring chronologically aging mutant cells to water. Moreover, the short-lived phenotype of *ach1*Δ cells is accompanied by reactive oxygen species accumulation, severe mitochondrial damage, and an early insurgence of apoptosis. A similar pattern of endogenous severe oxidative stress is observed when *ach1*Δ cells are cultured using acetic acid as a carbon source under acidic conditions. On the whole, our data provide further evidence of the role of acetic acid as cell-extrinsic mediator of cell death during chronological aging and highlight a primary role of Ach1 enzymatic activity in acetic acid detoxification which is important for mitochondrial functionality.

3.2 Introduction

In the single-celled yeast *Saccharomyces cerevisiae*, the replicative and chronological aging paradigms have been described. In the latter, chronological lifespan (CLS) is the mean and maximum survival period of a population of non-dividing cells in postmitotic stationary phase. Viability over time is defined as the ability to resume mitotic growth upon return to rich fresh medium [1]. This growth arrest simulates the postmitotic quiescent state of multicellular organisms.

Yeast cells respond to nutrient scarcity by inducing a series of metabolic, physiological and morphological changes which mainly increase stress resistance in order to survive starvation [2]. Moreover, in this context, unfit cells can undergo apoptosis for the benefit of the whole population [3,4]. Apoptosis is a highly regulated cellular "suicide" program whose activation can rely on different exogenous or endogenous stimuli [5]. Chronological aging is an example of an endogenous, physiological trigger [6], while treatment of yeast cells with a harsh environmental stress, such as acetic acid, is an example of an exogenous one [7]. Acetic acid which is also a by-product of the yeast metabolism and in some settings has been reported to restrict CLS [8,9]. In both chronological aging and acetic acid-induced apoptosis, mitochondria play an active and fundamental role [10–13]. In addition, among the different mitochondrial proteins involved in the execution of the acetic acid- induced apoptotic program, Dnm1 has been shown to be also implicated in chronological aging. This protein is required for mitochondrial fission and its lack of function impairs not only mitochondrial apoptotic fragmentation but also increases CLS [14], underlying a connection among mitochondrial dynamics, apoptosis and aging.

Mitochondria are also important organelles for yeast carbon metabolism and become essential for growth on non-fermentable substrates such as acetate. Acetate metabolism requires acetate activation to acetyl-CoA by acetyl-CoA synthetase isoenzymes, the mitochondrial Acs1 and cytosolic Acs2 which are known as the gluconeogenic and glycolytic isoforms, respectively [15,16]. Once generated, acetyl-CoA can be used to fuel the glyoxylate and TCA cycles and also for the synthesis of macromolecules which requires

active gluconeogenesis [17]. The concentration of cellular acetyl-CoA is primarily controlled by the balance between its synthesis and utilization in the different metabolic pathways.

The mitochondrial enzyme Ach1 was initially assumed to act as acetyl-CoA hydrolase, probably involved in reducing mitochondrial accumulation of acetyl-CoA during growth on acetate to avoid toxic effects [18,19]. However, hydrolysis of a high energy thioester bond has no apparent metabolic advantage since would result in losing two ATP molecules which have been consumed for acetate activation in the ester. Similarly, the ortholog Acu-8 from *Neurospora crassa* had been classified as an acetyl-CoA hydrolase [20], giving no further possible explanation for the physiological role of this "energy wasting" process. Thus, the existence of such an enzyme has been denoted as a biochemical conundrum [18]. Successively, the mitochondrial enzyme CoaT from *Aspergillus nidulans*, which is involved in propionyl-CoA detoxification in the presence of acetate, was characterized [21]. This protein shows a high aminoacidic identity to Ach1 and Acu-8, but displays a CoA-transferase activity being able to transfer the CoASH moiety from propionyl-CoA to acetate [21]. A re-characterization of Ach1 has been performed indicating that this enzyme acts as a CoA-transferase by catalyzing the transfer of the CoASH moiety from succinyl-CoA to acetate. Thus, it could detoxify mitochondria from acetate by an enzymatic reaction which would save one ATP [21].

In this work we provide evidence that *ACH1* inactivation severely impairs mitochondrial functions. This influences the acetate metabolism, the CLS which is restricted, and the occurrence of apoptosis.

3.3 Materials and methods

3.3.1. Yeast strains and growth conditions

All haploid 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*): *ach1*Δ (*ach1*Δ::*KlLEU2*), *yca1*Δ (*yca1*Δ::*URA3*) [22] and *ach1*Δ *yca1*Δ (*ach1*Δ::*KlLEU2 yca1*Δ::*URA3*). The accuracy of gene replacement was verified by PCR with flanking and internal primers. Standard methods were used for DNA manipulation and yeast transformation. 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 or 0.05% w/v (Calorie Restriction, CR) glucose. Auxotrophies were compensated for with a four-fold excess of supplements [23]. For shift experiments in acetate-containing medium, cells were grown in minimal medium/2% glucose up to exponential phase (10^7 cells/ml), centrifuged and resuspended in fresh acetate medium. For acetate medium, precalculated amounts of 0.2 M acetic acid and 0.2 M sodium acetate solutions were mixed and added to minimal medium to obtain the required pH and molarity. All strains were inoculated at the same cellular density (culture volume no more than 20% of the flask volume) and growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described [24]. Duplication times (Td) were obtained by linear regression of the cell number increase over time on a semi-logarithmic plot.

For growth assays on agar plates, exponentially growing cells were dropped (5 μl from a concentrated solution of 10^8 cells/ml and from serial 10-fold dilutions) onto rich medium (YEP, 1% w/v yeast extract, 2% w/v bacto peptone) agar plates supplemented with acetic acid (YEPA) at the indicated pH and molarity. Plates were incubated at 30°C for 3-5 days.

3.3.2. Metabolite measurements

At designated time-points, aliquots of the yeast cultures were centrifuged 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). Values represent the average of three independent experiments.

3.3.3. CLS determination

Survival experiments in expired medium were performed on cells grown in minimal medium (with a four-fold excess of supplements) containing 2% glucose as described by [1,23]. 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, when cells stopped dividing and cell density reached a plateau value. Subsequent age-points were taken every 2-3 days. Cells were plated onto rich medium/2% glucose (YEPD) plates and viability was scored by counting colony-forming units (CFUs). The number of CFUs at Day 3 was considered the initial survival (100%). For survival experiments in water, post-diauxic cells (at Day 1) 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 [1]. Survival experiments in water containing acetic acid were performed essentially as described by [23] for survival in water/ethanol. In our setting, acetic acid (10 mM) substituted for ethanol and the pH of the water was adjusted to 2.8 for both the control and acetic acid add-back cultures [8]. Viability was measured as described above. Survival experiments in expired medium were also performed on cells grown in minimal medium (with a four-fold excess of supplements) containing 0.05% glucose. Index of respiratory competence (IRC) was also measured according to [25] by plating identical samples on YEPD plates and on rich

medium/3% glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.

3.3.4. Tests for oxidative stress and cell death markers

Reactive oxygen species (ROS) were detected with dihydrorhodamine 123 (DHR123, Sigma) and dihydroethidium (DHE, Sigma) [26]. TdT-mediated dUTP nick end labeling (TUNEL, Roche) and Annexin V (ApoAlert Annexin V Apoptosis Kit, Clontech) assay for apoptotic markers as well as propidium iodide (PI, Sigma) staining for necrotic ones were performed as described [27]. Caspase activity was measured with 50 μM FITC-VAD-fmk (CaspACE, Promega) as described [22]. The mitochondrial membrane potential was assessed by staining with rhodamine 123 (RH123) and 3,3”-dihexyloxacarbocyanine iodide ($DiOC_6$) (both from Molecular Probes, Invitrogen), according to [28,29]. A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350F ccd camera was used. Digital images were acquired using FW4000 software (Leica).

3.3.5. Statistical analysis

All values are presented as the mean ± standard error of the means (SEM). Differences in measurements were assessed by Student’s *t*-test. The level of statistical significance was set at a P value of ≤ 0.001.

3.4 Results and discussion

3.4.1. Loss of Ach1 restricts CLS and increases apoptosis

Since acetic acid is a factor whose presence in the growth medium promotes chronological aging and Ach1 is a mitochondrial CoA-transferase which has been proposed to be involved in acetic acid detoxification [21], we decided to analyze the effects of *ACH1* inactivation on CLS.

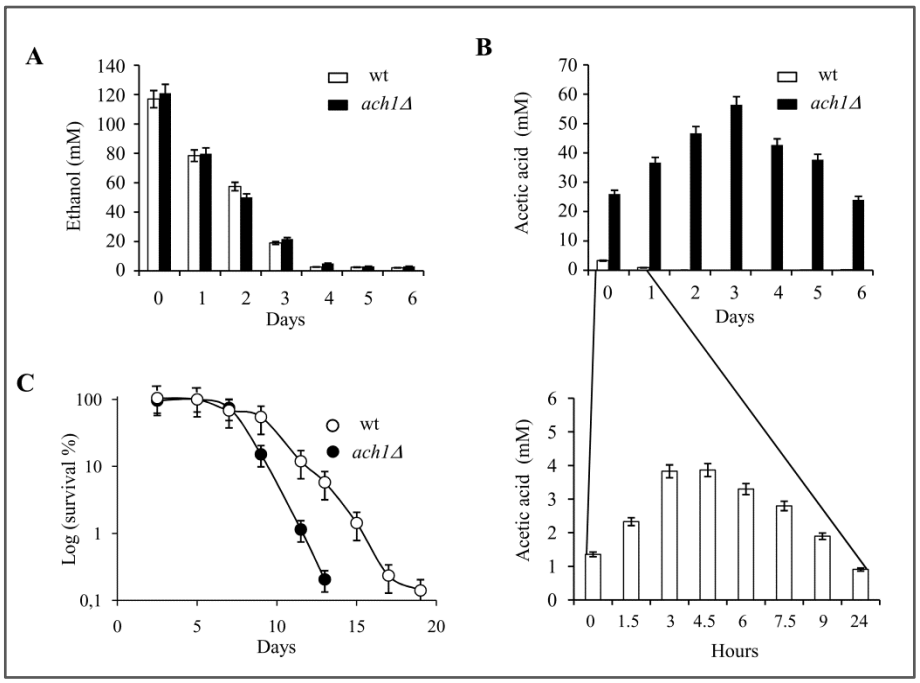


Fig. 3.1. *ACH1* inactivation shortens CLS in concert with increased extracellular acetic acid. Wild type (wt) and *ach1*Δ mutant cells were grown in minimal medium (with a four-fold excess of supplements) containing 2% glucose and followed up to stationary phase. Histograms of ethanol (A) and acetic acid (B) concentrations measured in the medium of both cultures at the indicated time-points. Day 0, diauxic shift. Inset: time-scale blow-up. Error bars are the standard deviation of three replicates. (C) CLS of wt and *ach1*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 (see Materials and Methods). Error bars are the standard deviation of three replicates.

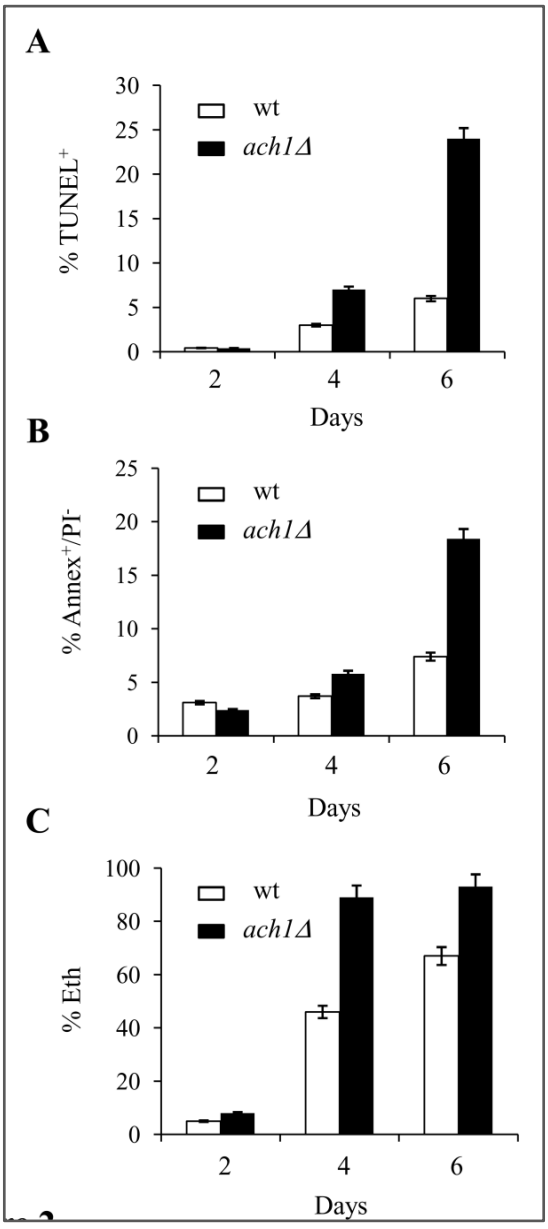


Fig. 3.2. Chronological aging *ach1Δ* cells display an early insurgence of apoptosis. Chronological aging cultures of Figure 1 at the indicated time-points were assessed for DNA fragmentation by TUNEL assay (A), phosphatidylserine externalization and membrane integrity by Annexin V/ propidium iodide (PI) co-staining (Annex V^+/PI^-, B) and intracellular superoxide by dihydroethidium conversion into fluorescent ethidium (Eth, C). Summary graphs of the percentage of positive cells for each test are indicated. Evaluation of about 1000 cells for each sample in three independent experiments was performed. Standard deviations are indicated.

In the context of a standard CLS experiment [1], after the diauxic shift, when cells switched from fermentation- to a respiration-based metabolism, we also measured extracellular ethanol and acetic acid concentrations (see par.3.3 Material and Methods). As a by-product of glucose fermentation, the wild type (wt) strain and *ach1*Δ mutant produced a similar maximal amount of ethanol which, during the post-diauxic phase, decreased at a similar rate in

both culture media (Fig. 3.1A). Acetic acid concentration, in the wt culture, initially increased and then rapidly decreased because it is utilized by the cells for the respiratory metabolism during the post-diauxic phase (Fig. 3.1B).

On the contrary, *ach1*Δ mutant showed a prolonged accumulation of this C2 compound. In fact, high levels of acetic acid were still present 6 days following the entry in post-diauxic phase (Fig. 3.1B) indicating a severe impairment in its utilization in line with a previous study [21]. Moreover, *ACH1* inactivation significantly reduced CLS (Fig. 3.1C).

Yeast cells undergo apoptosis during chronological aging as well as upon exposure to acetic acid [3,6,7,30]. Thus, we assessed different apoptotic features in the short-lived *ach1*Δ cells. DNA fragmentation was detected by TUNEL assay, exposure of phosphatidylserine at the outer leaflet of the plasma membrane and membrane integrity were evaluated by combined Annexin V/PI staining which allows for the identification of early apoptotic events (Annexin V^+) and necrotic cell death (PI^+) [31]. At Day 6, when survival of *ach1*Δ cells began to decrease (Fig. 3.1C), in these cells DNA strand breakage occurred and the percentage of $TUNEL^+$ was four-fold higher (24 ± 1%) as compared to wt ones (6 ± 1%) (Fig. 3.2A). At the same time-point, Annexin V staining, under conditions where plasma membrane was not compromised, as indicated by exclusion of PI co-staining (data not shown), detected about 18.4 ± 3.1% of Annexin V^+ *ach1*Δ cells in comparison to 7.3 ± 1.9% in the wt (Fig. 3.2B). All these data indicate that the *ach1*Δ mutant is subject to a much faster chronological aging process accompanied by an early insurgence of apoptosis.

3.4.2. In the *ach1*Δ mutant mitochondrial functionality is impaired

ROS accumulation is an important endogenous trigger which has been associated with apoptosis during chronological aging and acetic acid treatment (see [5] for a review). ROS content, measured as the superoxide-driven conversion of non-fluorescent DHE into fluorescent ethidium (Eth),

was already significantly enhanced in the *ach1*Δ mutant (89 ± 3%) as compared to the wt strain (46 ± 4%) at Day 4 (Fig. 3.2C) revealing an endogenous situation of higher oxidative stress in the mutant.

Mitochondria are key organelles in superoxide generation. This radical can directly induce oxidative damage or can be converted to other ROS which, in turn, induce aging-associated damage [13]. In addition, a direct correlation between reduced CLS and dysfunctional mitochondria has been reported [11,32]. Consequently, we decided to analyze whether *ACH1* inactivation could affect mitochondrial functionality. *S. cerevisiae* can grow by either fermentation on glucose as carbon source or by respiration by using different non-fermentable substrates such as glycerol. The growth on the latter can take place only when mitochondria are functional. This feature is exploited to evaluate whether mitochondria are extensively damaged at a point when the cell is still viable. The percentage of viable cells which are competent to respire defines the index of respiratory competence (IRC) [25]. At Day 1, both the wt and *ach1*Δ chronologically aging cells had an IRC of about 100% (Fig. 3.3A) indicating that all the cells are respiration-competent. During the following days, this value never dropped below about 80% for the wt, while it decreased quickly for the *ach1*Δ mutant reaching about 15-20% by Day 6 which is indicative of a time-dependent loss of mitochondrial functionality.

In parallel, mitochondrial morphology was examined by using $DiOC_6$ dye. In fact, at low concentrations (20-100 ng/ml), this dye accumulates specifically at mitochondrial membranes and can be observed by fluorescence microscopy. However, cells with low mitochondrial membrane potential will fail to accumulate $DiOC_6$ [29]. Balanced fusion and fission of mitochondria results in tubular mitochondrial morphology, as was the case for wt cells, whereas for *ach1*Δ cells punctiform formations were observed at Day 2 (Fig. 3.3B). The conversion of mitochondrial morphology from tubular structures to punctuate ones is also referred to as mitochondrial fragmentation. It is presumed to occur by excessive mitochondrial fission [3] and has been observed in yeast apoptosis induced by different stimuli including acetic acid treatment [3]. Moreover, at Day 4, $DiOC_6$ staining was greatly reduced in *ach1*Δ cells (Fig. 3.3B) revealing a reduction in mitochondrial membrane potential. Taken together these data indicate that

the early insurgence of apoptosis in chronological aging *ach1*Δ cells is preceded by a severe damage of mitochondria.

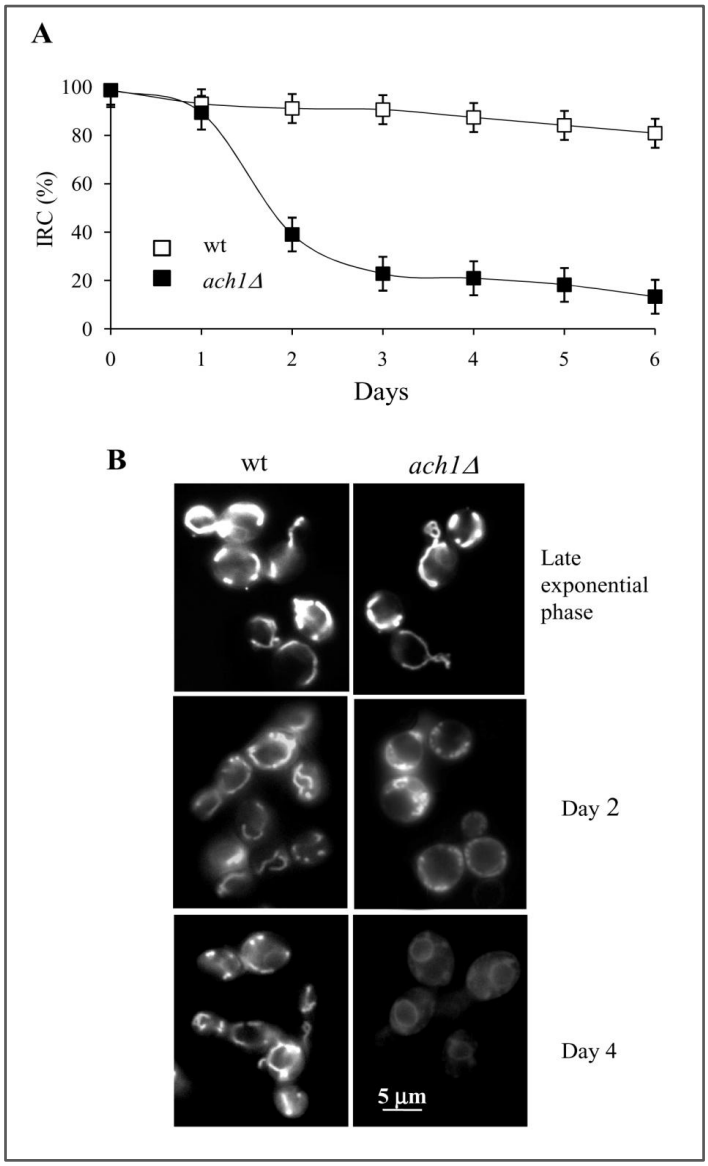


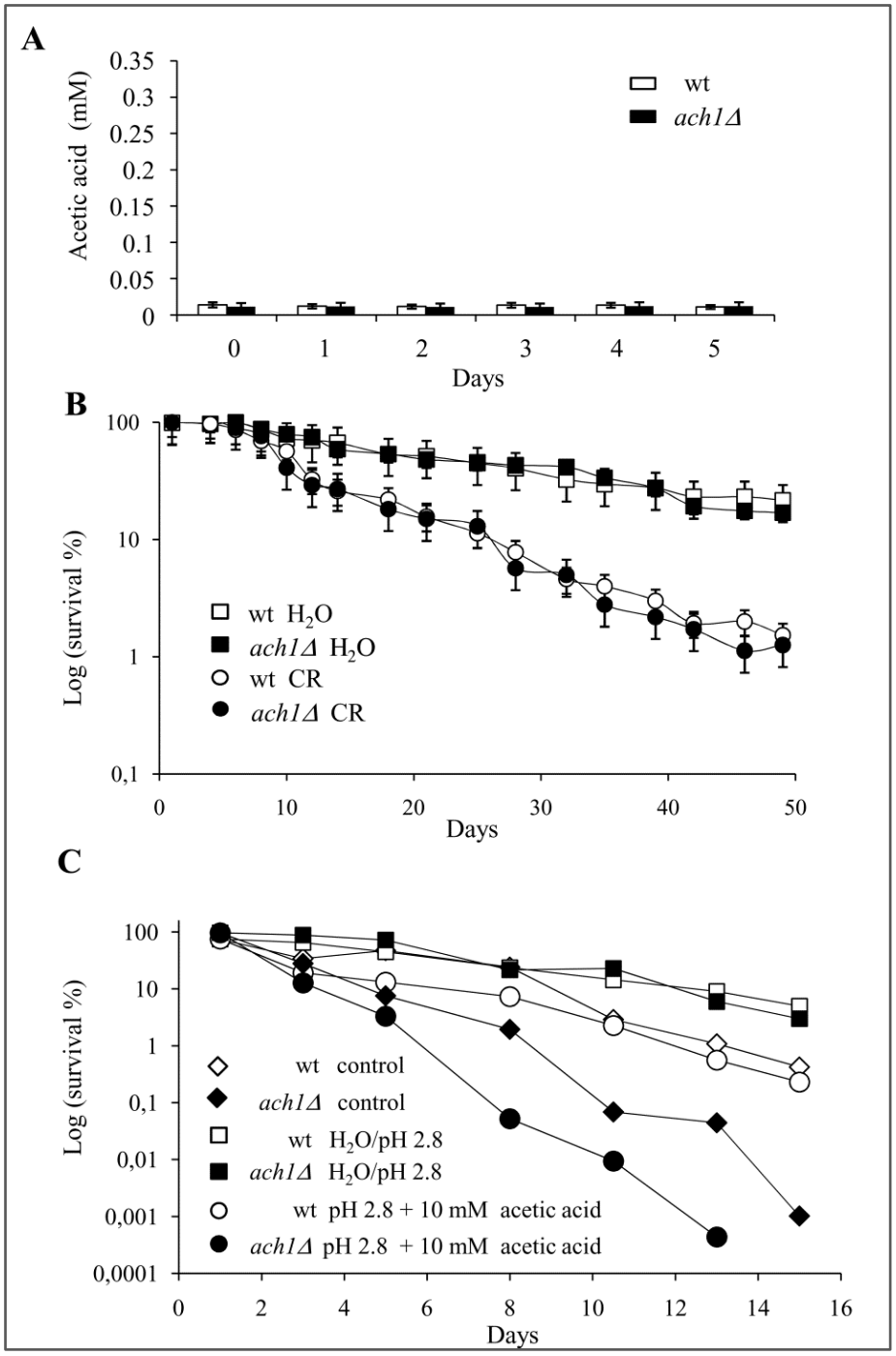
Fig. 3.3. Chronological aging *ach1Δ* cells display compromised mitochondrial activity. Chronological aging cultures of Figure 3.1 at the indicated time-points were serially diluted, plated onto YEPD and YEPG plates and the index of respiratory competence (IRC) was determined (A). Standard deviations of three independent experiments are indicated. (B) Same cells stained with $DiOC_6$ to visualize

mitochondrial membranes. Morphologies of the mitochondria of cells in late exponential phase are also reported. Representative images are shown. Bar: 5μm.

3.4.3. Acetic acid is responsible for the reduced CLS of *ach1*Δ mutant

In order to determine whether the reduced CLS of *ach1*Δ mutant was linked to the excess of acetic acid in the extracellular environment, we first examined the effects of lowering glucose concentration from 2 to 0.05% in the initial culture medium. This is a growth condition of CR which reduces acetic acid production in the chronological aging culture and extends CLS [8]. As expected, almost undetectable extracellular acetic acid was present in wt cultures grown in CR (Fig. 3.4A) which displayed an enhanced survival relative to wt cultures grown on 2% glucose (Fig. 3.4B and Fig. 3.1C). Growth in a CR regimen for the *ach1*Δ mutant produced undetectable extracellular acetic acid as well (Fig. 3.4A). Additionally, it was also sufficient to avoid completely the deleterious effect on cell viability associated with growth on 2% glucose. In fact, CR *ach1*Δ cells had a CLS comparable to CR wt cells (Fig. 3.4B and Fig. 3.1C).

Fig. 3.4. CR suppresses the CLS-shortening effect of *ACH1* inactivation. Wild type and *ach1*Δ cells were grown on 0.05% glucose (CR) and extracellular acetic acid concentration was measured for both cultures at the indicated time-points. Day 0, diauxic shift (A). In parallel, cell survival in the exhausted medium was determined by counting CFUs on YEPD plates (B). Wild type and *ach1*Δ chronological aging cells grown on 2% glucose were switched to water (extreme CR) at Day 1. Every 48 h, cultures were resuspended in fresh water and at every time-point, viability was measured by counting CFUs on YEPD plates (B). Survival of cells in their exhausted medium was also monitored as control (reported in Fig. 3.1C and 3.4C). Error bars are the standard deviation of three replicates. (C) Wild type and *ach1Δ* chronological aging cells grown on 2% glucose were switched to water adjusted to pH 2.8 and water/pH 2.8 containing 10 mM acetic acid at Day 1. Every 48 h, cultures were resuspended in fresh water/pH 2.8 and each time 10 mM acetic acid was added. At every time-point, viability was measured as in (B). Survival of cells in their exhausted medium was also monitored (control). One representative experiment is shown.


A
Acetic acid (mM)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
wt
ach1Δ
0
1
2
3
4
5
Days
B
Log (survival %)
100
10
1
0,1
wt H2O
ach1Δ H2O
wt CR
ach1Δ CR
0
10
20
30
40
50
Days
C
Log (survival %)
100
10
1
0,1
0,01
0,001
0,0001
wt control
ach1Δ control
wt H2O/pH 2.8
ach1Δ H2O/pH 2.8
wt pH 2.8 + 10 mM acetic acid
ach1Δ pH 2.8 + 10 mM acetic acid
0
2
4
6
8
10
12
14
16
Days

We next monitored survival of cells switched from the expired medium to water. Incubation in water is an extreme form of CR which is known to dramatically extend CLS [23]. Wt and *ach1*Δ mutant cells were grown on 2% glucose and after the diauxic shift switched to water (see par.3.3 Materials and Methods). As shown in Fig. 3.4B, both wt and *ach1*Δ cells, incubated in water, increased CLS to the same extent. Thus, the short-lived phenotype of the *ach1*Δ mutant seems to be mainly due to the toxicity of acetic acid which is accumulated in the environment of chronologically aging *ach1*Δ cells.

Starting from the aforementioned results, we wondered whether the addition of acetic acid could influence the chronological survival of *ach1*Δ cells. It has been previously reported that the addition of acetic acid (10 mM) to low pH (2.8) water can prevent CLS extension of chronologically aging cells associated with their transfer to water [8]. Wt and *ach1*Δ cultures grown in 2% glucose were transferred to low pH water supplemented with acetic acid after the diauxic shift and cell viability monitored. In line with acetic acid pro-aging role, acetic acid add-back cultures had a reduced CLS compared to cells incubated in water alone (Fig. 3.4C). Notably, acetic acid affected chronological survival of *ach1*Δ cells to a higher extent relative to wt cells. In fact, acetic acid add-back wt cultures displayed a CLS similar to that of wt cells aged in their exhausted medium in agreement with [8], while for the acetic acid add-back mutant cultures the CLS was much more reduced (Fig. 3.4C). Together all these data are fully consistent with the notion that the extracellular acetic acid is responsible for the reduced CLS of the *ach1*Δ mutant and also suggest that the lack of Ach1 makes cells more sensitive to acetic acid.

3.4.4 The *ach1*Δ mutant displays an apoptotic caspase-dependent phenotype

Since *ACH1* deletion results in growth defects on media containing acetic acid as a sole carbon and energy source which are particularly severe under acidic conditions [21], to further refine our investigation, we examined whether such a growth impairment could be ascribed to mitochondrial damages. For this purpose, wt and *ach1*Δ cells exponentially growing on 2%

glucose were harvested and transferred to 50 mM acetic acid medium, pH 4.5.

In this way, cells were released from glucose repression and were able to express all the genes involved in acetate catabolism and assimilation [33], including *ACH1* [19]. Moreover, the combination 50 mM acetic acid/pH 4.5 is still a permissive growth condition for wt cells but not for the mutant [21]. Analyses were performed 4 h and 16 h after the metabolic shift corresponding to time-windows during which gene expression changes required for acetate metabolism [33] and a significant CoA-transferase activity [21] were respectively detected. At these time-points, wt and *ach1*Δ cells were incubated with RH123 in order to visualize active mitochondria, and with the ROS-sensing dye, DHR123. After 4 h, in both strains similar mitochondrial patterns were observed with RH123 associated with DHR123-negative staining indicating that mitochondria retain their membrane potential and no ROS accumulation took place (Fig. 3.5A). This was still the case for wt cells 12 h later, while in the mutant a severe reduction in mitochondrial membrane potential and increase in ROS-accumulating cells (about 80%) were observed (Fig. 3.5A-B). These features were also accompanied by the appearance of Annexin V^+ (about 29%) and $TUNEL^+$ (about 37%) cells (Fig. 3.5B) suggesting the onset of apoptosis.

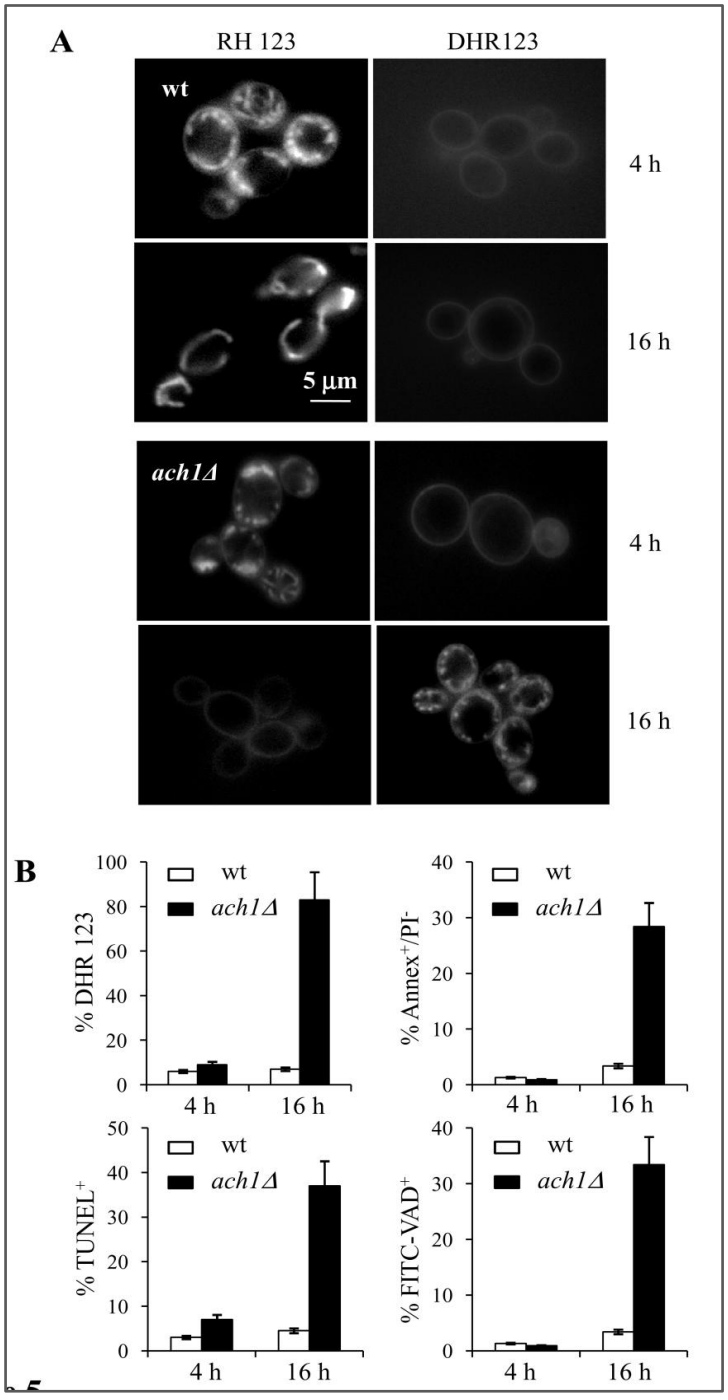
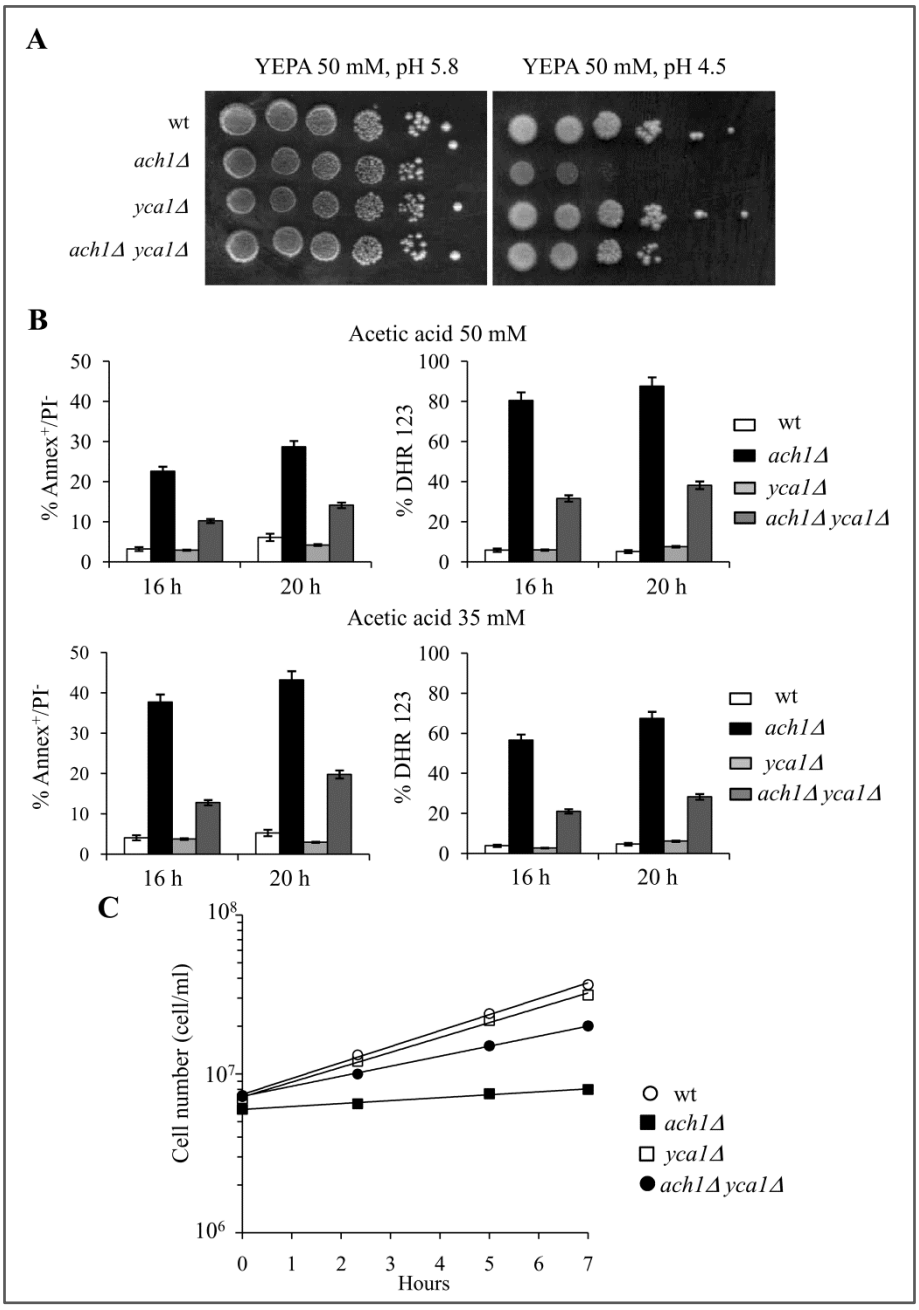

A
RH 123
DHR123
wt
4 h
16 h
5 µm
ach1Δ
4 h
16 h
B
wt
ach1Δ
% DHR 123
% Annex+/PI-
% TUNEL+
% FITC-VAD+
4 h
16 h

Fig. 3.5. The presence of acetic acid induces apoptosis in *ach1*Δ cells. Wild type and *ach1*Δ cells exponentially growing on 2% glucose were shifted to acetate (50 mM) medium, pH 4.5 as sole carbon source. At the indicated time-points after the shift, cells were stained with Rhodamine 123 (RH123) to visualize functional mitochondria and with DHR123 for the presence of intracellular ROS (A). Representative images are shown. Bar: 5μm. The same cultures were analyzed for apoptotic markers by Annexin V/PI co-staining and TUNEL assay, and for caspase activity by FITC-VAD-fmk. Summary graphs of the percentage of ROS-accumulating cells, of Annexin V^+/PI^-, of $TUNEL^+$ and $FITC\text{-}VAD^+$ are reported (B). Evaluation of about 500 cells for each sample in three independent experiments was performed. Standard deviations are indicated.

Both caspase-dependent and caspase-independent cell death pathways have been described in yeast [26]. The Yca1 metacaspase is a yeast functional orthologue of mammalian caspases which mediates the apoptotic process triggered by several intrinsic and extrinsic inducers including acetic acid [34,35]. To evaluate whether *ach1*Δ cells shifted to the acetate medium showed an endogenous metacaspase activity, the FITC-labeled caspase inhibitor VAD-fmk, which binds to activated caspase, was used. 16 h after the shift, about 32 ± 2.3% of *ach1*Δ cells were FITC-positive (Fig. 3.5B) suggesting that apoptosis in *ach1*Δ cells occurs through a caspase-dependent cascade. Consequently, we analyzed the effects of *YCA1* inactivation in the *ach1*Δ background. Initially, we assessed cellular growth on 50 mM acetic acid media whose pH had been adjusted to 5.8 or 4.5: a permissive and a restrictive growth condition for the *ach1*Δ mutant, respectively [21]. As depicted in Fig. 3.6A, both wt and *yca1*Δ cells grew on all the acetic acid-containing media while acidification of the medium affected severely the *ach1*Δ mutant viability. Notably, this effect was almost completely prevented by deleting *YCA1* (Fig. 3.6A). Changes in the pH of glucose-containing media did not influence the growth of all strains (data not shown). Moreover, on 50 mM acetic acid medium, pH 4.5, the measurement of apoptotic markers (Annexin V^+/PI^-) and ROS showed that *YCA1* inactivation partly rescued the *ach1*Δ mutant from apoptosis, as well as from ROS accumulation (Fig. 3.6B). Decreases in Annexin V^+/PI^- cells and ROS were

also observed in the *ach1*Δ*yca1*Δ mutant compared to the *ach1*Δ mutant in 35 mM acetic acid medium, pH 4.5 (Fig. 3.6B). This was accompanied by an improvement of cellular growth (Fig. 3.6C) confirming the results obtained for 50 mM acetic acid medium. Together all these data point to an involvement of Yca1 in the caspase-dependent apoptosis of the *ach1*Δ mutant promoted by acetic acid.

Fig. 3.6. Effects of *YCA1* inactivation on *ach1*Δ cells. (A) The indicated strains exponentially growing on 2% glucose were spotted (10-fold serial dilutions) onto plates containing 50 mM acetic acid (YEPA) as a carbon source at pH 5.8 and pH 4.5. Plates were incubated at 30°C for 4 days. One significant experiment out of 3 is shown. (B) The indicated strains exponentially growing on 2% glucose were shifted to 35 mM and 50 mM acetic acid-containing media, pH 4.5. At the indicated time-points after the shift, cells were analyzed for apoptotic markers by Annexin V/PI co-staining and stained with DHR123 for the presence of intracellular ROS. Summary graphs of the percentage of Annexin V^+/PI^- and of ROS-accumulating cells are reported. Evaluation of about 500 cells for each sample in three independent experiments was performed. Standard deviations are indicated. (C) Growth curves of the indicated strains grown in 35 mM acetic acid-containing medium, pH 4.5. Growth was measured as increase in cell number over time. One representative experiment is shown.


A
YEPA 50 mM, pH 5.8
YEPA 50 mM, pH 4.5
wt
ach1Δ
yca1Δ
ach1Δ yca1Δ
B
Acetic acid 50 mM
% Annex+/PI-
% DHR 123
16 h
20 h
wt
ach1Δ
yca1Δ
ach1Δ yca1Δ
Acetic acid 35 mM
C
Cell number (cell/ml)
Hours

3.5 Discussion

Acetic acid, a well known by-product of yeast glucose fermentation, has been identified as a cell-extrinsic mediator of cell death during chronological aging [8]. Data reported in this paper provide more experimental evidence of a role for acetic acid toxicity as a determinant of CLS and of the positive feed-forward connection between mitochondrial damage and apoptosis. In fact, we show that inactivation of *ACH1* encoding a mitochondrial CoASH transferase which catalyses the transfer of the CoASH moiety from succinyl-CoA to acetate [21], reduces CLS. The short-lived phenotype relies on an excess of extracellular acetic acid which is accumulated in the medium of chronologically aging *ach1*Δ cells. In fact, the faster chronological aging process can be completely abrogated either by transferring chronologically aging *ach1*Δ cells from the expired medium to water, thus alleviating the acid stress experienced by the cells, or by growing them under a CR regimen (0.05% glucose) and consequently avoiding acetic acid production ([8]; this work). Since 0.05% is a glucose concentration which is well below what is normally needed to relieve glucose repression [36], yeast metabolism switches from fermentation to respiration. Acetyl-CoA is synthesized directly from pyruvate by oxidative decarboxylation, catalyzed by the mitochondrial pyruvate dehydrogenase complex and in such a way glycolysis is coupled to the TCA cycle [37].

In parallel with the faster drop of cell survival, chronologically aging *ach1*Δ cells undergo an early insurgence of apoptosis. In addition, *ach1*Δ cells also undergo apoptosis when they are inoculated in 35 mM or 50 mM acetate medium at pH 4.5; a growth condition which has a detrimental effect on the viability of this mutant. It is well known that acetic acid represents a compound commonly used to induce yeast apoptosis when applied in the presence of glucose at pH 3 [7,12,38]. Here, we show that acetic acid alone triggers apoptosis in glucose-derepressed *ach1*Δ cells. Uptake of acetate is linked to a proton symport mechanism (subjected to glucose-repression) accompanied by passive/facilitated diffusion of the uncharged/undissociated acid through the Fps1 aquaglyceroporin channel (see [39] for a review). Acetic acid displays toxicity at low extracellular pH primarily due to the

undissociated acid diffusion. In fact, the acetic/acetate couple forms a buffer system; at pH values below the pKa of the acid (4.75) the undissociated form prevails and diffuses through the plasma membrane. Once inside the cell (pH close to neutral), the acid dissociates causing anion accumulation and intracellular acidification that, in turn, is thought to have negative effects on yeast metabolic activity. Additionally, free radical production is also affected leading to severe oxidative stress [40]. In line with this, *ach1*Δ cells on acetate display ROS accumulation and a strong reduction in mitochondrial membrane potential similar to that elicited by acetic acid in glucose-repressed wild type cells [12] supporting the notion of a role for Ach1 as a mitochondrial detoxifying enzyme [21]. Moreover, as in the case of the acetic acid-induced apoptosis of glucose-repressed cells, occurrence of apoptotic markers in the *ach1*Δ mutant is accompanied by caspase activation. So far, four proteases have been described in the yeast apoptotic scenarios: the separase Esp1 [41], the nuclear HtrA-like protein Nma111 [42,43], Kex1 [44] and the metacaspase Yca1 [45]. Here, evidence is provided that Yca1 is involved in the caspase-dependent apoptosis of the *ach1*Δ mutant promoted by acetic acid since *YCA1* deletion decreases apoptotic markers, as well as ROS accumulation in the *ach1*Δ mutant and, conversely, improves its cellular growth on acetic acid containing media at low pH. Such an involvement is fully consistent with the requirement of this metacaspase for the ROS-dependent acetic acid-induced apoptosis [46].

A pattern of endogenous severe oxidative stress is also observed in chronologically aging *ach1*Δ cells in concert with elevated levels of extracellular acetic acid. Here, a precocious increase of mitochondrial superoxide which is well known to target primarily several mitochondrial enzymes [47,48] and to play a major role in chronological aging [1,13,49], is associated with loss of respiratory competence which precedes apoptotic death suggesting that Ach1 is required to protect mitochondrial function during chronological aging. Fleck and Brock [21] proposed that under extracellular acidic conditions, the diffusional entry of the undissociated acid through the plasma membrane into the cell might also lead to an influx of acetic acid over the mitochondrial membrane. This would result in acetate accumulation and mitochondrial acidification affecting negatively mitochondrial functionality. Ach1 enzymatic activity would prevent this acetate accumulation by a CoASH transfer from succinyl-CoA (produced by

the TCA cycle) to acetate generating acetyl-CoA. Ach1 has a low Km for both succinyl-CoA and acetate [21]. In addition, the CoA-transferase reaction saving ATP, compared with the acetate activation to acetyl-CoA by acetyl-CoA synthetase, would be favoured in a condition where in order to counteract intracellular acidification induced by acetic acid, cells are already consuming energy [40]. Taken together our data are fully consistent with this hypothesis since they indicate that mitochondria are the main target of acetic acid toxic effects in *ach1*Δ cells in extracellular acidic conditions such as acetate medium at pH 4.5 and during chronological aging (pH about 3) [8]. This implies that Ach1 can function as a mitochondrial detoxifying enzyme. Moreover, the mitochondrial damage resulting from Ach1 loss of function can account for the growth impairment on acetate and the CLS decrease.

3.6 References

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4

The influence of ethanol and acetic acid on CLS

4.1 Introduction

Studies on the manipulation of intrinsic nutrient-signaling pathways in yeast have uncovered the strong impact of growth conditions on the chronological aging process. Apart from the influence of CR, a condition which is obtained by lowering glucose concentration in the medium [1–4], other extrinsic components of the culture media, have been implicated in the regulation of CLS (as described in Chapter 1) such as the by-products of the fermentation process, ethanol and acetic acid [5–8], even though the mechanisms by which they reduce CLS are yet unclear.

In yeast, ethanol is the main product resulting from alcoholic fermentation during aerobic growth on glucose media and it is in turn used as a carbon source during the post-diauxic phase [9]. Nevertheless, many toxic effects have been observed following ethanol presence. High ethanol concentrations (5% v/v) are even known to negatively affect the metabolic activity of yeast cells especially by inhibiting cell growth and fermentation [10,11]. Ethanol is also known to cause damage of cell membranes by increasing membrane fluidity and by inhibiting the transport systems across the plasma membrane [11–13]. Anyway, the severity of these effects is dependent on the alcohol concentration, that can raise up to result in cell death [14]. Recently, it has also been suggested a correlation between extracellular ethanol accumulation and loss of peroxisomal function since ethanol was seen to suppress the synthesis of certain proteins localized at peroxisomes [15]. Few years ago, physiological ethanol accumulation after fermentation has been proposed as an extrinsic factor that promotes aging, significantly decreasing CLS of strains in severe CR condition (where lifespan is known to be widely extended) [5,8]. Moreover, removal of ethanol from the exhausted medium in a standard CLS experiment causes a significant increase in lifespan [8]. In a recent study on mutants lacking active gluconeogenesis, it has also been proposed that the extracellular ethanol concentration accumulated following glucose fermentation was inversely proportional to CLS [16]. In addition, as reported in Chapter 2, by inhibiting alcohol dehydrogenase activity during the post diauxic phase, we have also stated that these effects are caused by the catabolism of ethanol more than by its simple presence [5].

Acetic acid is another by-product of the yeast fermentative metabolism and is a well known inducer of apoptotic cell death leading to ROS production [17]. When available as the sole carbon and energy source, acetate is activated to acetyl-CoA by the ACS proteins and then oxidized in the TCA cycle for energy production. In parallel, to replenish the cell with biosynthetic precursors, it is also used to produce succinate by entering the glyoxylate cycle, which involves the key enzyme isocitrate lyase Icl1 [18]. In addition, active gluconeogenesis is required for the synthesis of macromolecules [19]. In a recent study, Burtner and colleagues stated that, more than ethanol, it is acetic acid to be the most important extracellular factor affecting CLS [7,20] but a clear consensus on this has not been reached yet [21]. In line with Burtner and collegues, long-lived mutants (*ade4*Δ cells) do not accumulate acetic acid in the culture medium during growth on glucose, while short-lived *atg16*Δ cells (Atg16 is involved in the autophagic process) accumulate acetic acid at higher level than wild type cells, inversely correlating also in this case the amount of acetic acid released by cells with CLS extension [22]. Moreover, we found in our mutant strains (see Chapter 2 and 3) that the long-lived *sir2*Δ cells did not accumulate extracellular acetate, while the short-lived *ach1*Δ cells did it at a higher extent than wild type cells [5,6]. We have also observed that *PCK1* or *ICL1* inactivation leads to an increase in the levels of both ethanol and acetic acid and to a parallel CLS reduction [5]. These two genes code for key enzymes of gluconeogenesis and glyoxylate cycle respectively, pathways that are necessary for the catabolism of ethanol and acetate. This further underlines the tight interconnection between the cell metabolism and lifespan extension. Anyway, this correlation between CLS and the metabolite levels is not always true: in fact, it was reported that exchanging media between some short- and long-lived strains (containing higher and lower ethanol and acetic acid concentrations respectively) was not sufficient to influence CLS extension [23,24], thus underlining that the effects on CLS are the result of a complex and not yet clear interplay of extrinsic as well as intrinsic factors. Moreover, it was also recently demonstrated that buffering aging cultures to pH 6 is sufficient to increase CLS, since this intervention neutralizes the toxic effect of acetic acid. In fact, when dissociated, the acetate anion cannot be easily taken up from the environment, because it enters the cell only with the intervention of specific transporters, while the

undissociated protonated acetic acid is able to freely cross the plasma membrane [25,26].

As a consequence, an essential aspect to be considered when dealing with acetic acid (and weak organic acid in general) is pH. Actually, every weak organic acids in aqueous solutions exist in a pH-dependent equilibrium between undissociated and dissociated states depending on the pKa of the particular acid (pKa of acetic acid is 4,75). At low extracellular pH weak acids are mainly in the undissociated form: the uncharged species have high membrane permeability and are therefore able to (re)enter the cell via passive diffusion. The high pH of the yeast cytosol (pH 6-7) causes their dissociation, releasing protons and anions. This leads to intracellular acidification, anion accumulation and inhibition of some cellular metabolic activities. Interestingly, it has been observed that at least during the wine fermentation process, enolase is the glycolitic enzyme most affected by acetic acid, thus resulting in an alteration of glycolysis and consequently in the fermentation yield [27,28]. Since charged acid anions and protons do not readily diffuse across the membranes, a vast array of export mechanisms exists to maintain pH and ion homeostasis, as described in Chapter 1, whose function depends on the pH and on the concentration of different acid species across the membranes [25]. Recently, for the first time, Leontieva and Blagosklonny described a "yeast-like" chronological senescence model in mammalian cells [29]. They showed that human tumor cells maintained in stationary culture lose their viability by a process accelerated by medium acidification caused in part by lactate accumulation: this mirrors the acidification and the accumulation of ethanol and acetic acid in the medium of *S. cerevisiae* aging cells. Moreover, they demonstrated that the "yeast-like" chronological senescence in mammalian cells is delayed and attenuated by the inhibition of the conserved mTOR signaling pathway, strengthening the parallel with yeast phenotype: rapamycin, the well-known TOR inhibitor, prevents chronological senescence essentially by decreasing lactate production. On the whole, this paper gives light to the place of yeast studies in the aging research and its relevance via common signaling pathways to cancer and organismal aging [30,31].

In this Chapter, we focused on the physiological role played by ethanol and acetic acid in the CLS and on the possible mechanisms by which both these

compounds influence longevity in yeast. In particular, we pointed out that it is the metabolism, and not the simple presence, of these two compounds to cause the known detrimental effects on CLS.

4.2 Materials and methods

4.2.1. Yeast strains and growth conditions

All yeast strains used in this part of my work are listed in Table 4.2. All deletion strains were generated by PCR-based methods [32]. The accuracy of all gene replacements and correct deletions/integrations was verified by PCR with flanking and internal primers. Standard methods were used for DNA manipulation and yeast transformation.

Table 4.2. Yeast strains used in this study.

Strain	Relevant genotype
W303-1A	*MAT*a *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*
*sir2*Δ	W303-1A *sir2*Δ*::URA3*
*icl1*Δ	W303-1A *icl1Δ::KlLEU2*
*ach1*Δ	W303-1A *ach1*Δ*::KlLEU2*
*ach1*Δ *sir2*Δ	W303-1A *ach1*Δ*::KlLEU2 sir2*Δ*::URA3*

Yeast cells were grown in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/l) with 2% glucose and auxotrophies were compensated for with a four-fold excess of supplements [8]. 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 [33]. Doubling time (Td) was obtained by linear regression of the cell number increase over time on a semilogarithmic plot.

4.2.2. CLS determination

Survival experiments in expired medium were performed on cells grown in minimal medium (with a four-fold excess of supplements) with 2% glucose as described by [34]. 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 24 h (Day 1, first age-point) after the diauxic shift (Day 0). CLS was measured according to [8] by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 1 was considered the initial survival (100%).

Survival experiments in water were performed on cells grown in minimal medium with 2% glucose (with a four-fold excess of supplements) as described by [8]. Briefly, at Day 1, cultures were transferred into fresh sterile water. Where indicated, every 48 h, ethanol or acetic acid were added to the culture at the indicated concentrations after washing. Viability was then measured as described above. Where indicated, pyrazole (Sigma-Aldrich) was added at the final concentration of 50 mM [5].

For CLS determination in media-swap experiments, cells were grown in minimal medium/glucose (with a four-fold 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 [5].

4.3 Results and discussion

In order to unravel the physiological role played by ethanol and acetic acid and by their metabolism on longevity, initially in a standard CLS experiment in conditions of extreme CR we treated wild type cells with the physiological amount of acetic acid which is released in the extracellular medium following yeast fermentation, as reported in Fig. 2.1D [5]. So wild type cells were grown in minimal medium containing 2% glucose and at Day 1 after diauxic shift cells were switched to low pH water in the presence or absence of 5 mM acetic acid and their viability was analyzed over time. The pH of the water was set at pH 2.8, a value which mimics that measured in the medium during chronological aging. At this pH, far below the pKa of acetic acid (4.75), almost all the acid is present in the undissociated form and can thus enter the cell by passive diffusion. As a control, cells were treated also with 10 mM acetic acid, a concentration which has been already reported to reduce CLS, as reported in Fig. 3.4C [6] and [7]. As shown in Fig. 4.1, incubation in water dramatically extended CLS of wild type cells as also reported in Fig. 3.4C [6], in Fig. 2.5 [5] and in [8], while the addition of 5 mM acetic acid had a negative effect on cell survival. This suggests that the concentration of extracellular acetic acid accumulated during chronological aging, even if very low, can have a pro-aging role. Addition of 6 g/L ethanol (130 mM), which is the concentration detected in the exhausted medium (see Fig. 2.1C [5]) to low pH water prevented the CLS extension of chronological aging cells associated with their transfer to water (Fig. 4.1), in line with Fig. 2.5 [5]. Interestingly, by comparing the CLS curves of acetic acid add-back cultures and the ethanol add-back ones with the CLS curve of the cells in their exhausted medium, it can be observed that, although their clear detrimental effect on cell survival, no one of them is able to reproduce on calorie restricted cells the CLS limitation driven by the exhausted medium, suggesting that it is a combined effect of both metabolites to influence CLS.

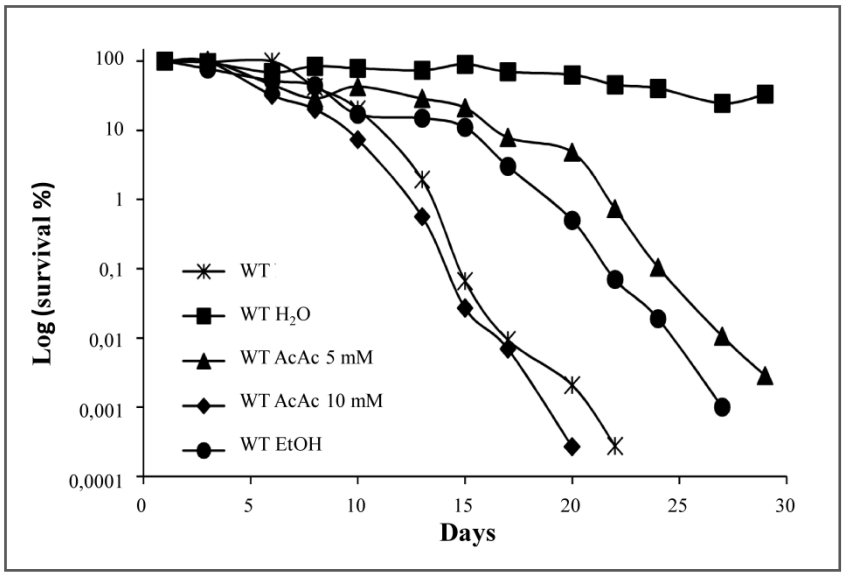

Fig. 4.1. Effect of ethanol and acetic acid on calorie-restricted cells. Wild type chronological aging cells grown on 2% glucose were switched to low pH water and water containing the indicated concentrations of acetic acid or ethanol (6 g/L) at Day 1. Every 48 h, cultures were resuspended in fresh low pH water and each time acetic acid or ethanol was added again. At every time-point, viability was measured by counting CFUs on YEPD plates. Survival of cells in their exhausted medium was also monitored as a control (WT). One representative experiment is shown. Day 0 = diauxic shift.

In Fig. 4.1, it is shown that cells shifted in water supplemented with 10 mM acetic acid displayed a similar, or even a bit reduced, CLS if compared with cells in their expired medium and a significantly shorter CLS than cells treated with 5 mM of acetic acid. Moreover, we have previously observed that *ach1*Δ mutant cells accumulated up to 60 mM in the post diauxic phase in their expired medium, as reported in Fig. 3.1B [6], due to the severe impairment in acetic acid utilization and to their dysfunctions in mitochondrial activity. In that context, we also hypothesized that the short lived phenotype of *ach1*Δ cells was due to the detrimental effects of such a high acetic acid concentration.

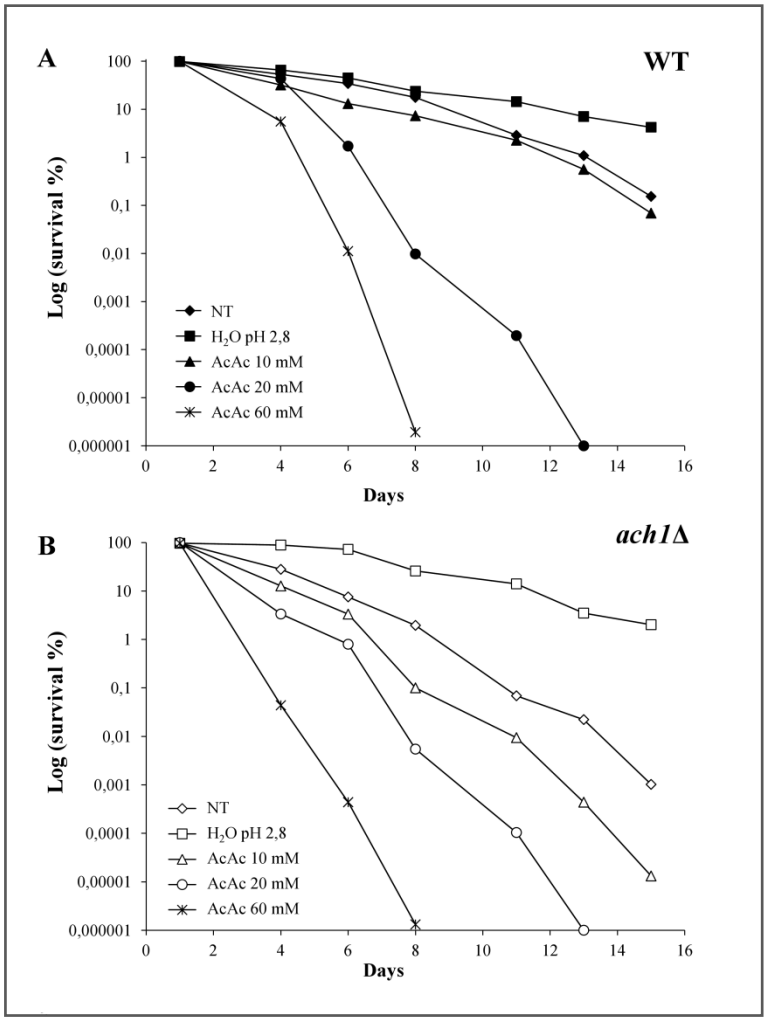

Fig. 4.2. Acetic acid toxicity on CLS extension. Wild type (A) and *ach1*Δ (B) chronological aging cells grown on 2% glucose were switched to water adjusted to pH 2.8 and water (pH 2.8) containing the indicated concentrations of acetic acid at Day 1. Every 48 h, cultures were resuspended in fresh water (pH 2.8) and each time acetic acid was added again. At every time-point, viability was measured by counting CFUs on YEPD plates. Survival of cells in their exhausted medium was also monitored as a control. Representative experiments are shown. Day 0 = diauxic shift.

As a consequence, we repeated the experiment on wild type and *ach1*Δ cells in conditions of extreme CR by adding 10, 20 and 60 mM acetic acid. We then followed cell viability over time. In Fig. 4.2A, it can easily be observed that acetic acid induced a dose-dependent negative effect in shortening the lifespan of these calorie-restricted cells. By comparing wild type and *ach1*Δ curves in Fig. 4.2A and Fig. 4.2B, it is also evident how in both strains, independently from mitochondrial functionality, CLS was dramatically decreased to a similar extent with the highest concentrations of acetic acid, condition in which cell death was induced in few days. This indicates that at these concentrations this metabolite becomes toxic for the cells probably because it interferes with the normal cellular activities, for example by inducing excessive intracellular acidification [35]. Of relevance, with regard to the 10 mM concentration, a difference in the effect of acetic acid on the CLS arose, as reported in Fig. 3.4C [6]: CLS of mutant cells was significantly shorter with respect of wild type cells treated in the same conditions, suggesting that at this concentration the cell ability to cope with acetic acid presence plays a key role.

To test if the metabolism of acetic acid in the cell could influence the effect of this metabolite on CLS, we took into consideration *sir2*Δ cells which, differently from *ach1*Δ cells, display a more efficient acetate metabolism (Chapter 2 and [5]). In fact, actually during CLS standard conditions, *sir2*Δ cells did not even accumulate extracellular acetate, as reported in Fig. 2.1D [5]. Thus, wild type and *sir2*Δ cells were grown in minimal medium containing 2% glucose and, at Day 1 after the diauxic shift, shifted to water (pH 2.8) supplemented with the physiological concentration of 5 mM acetic acid.

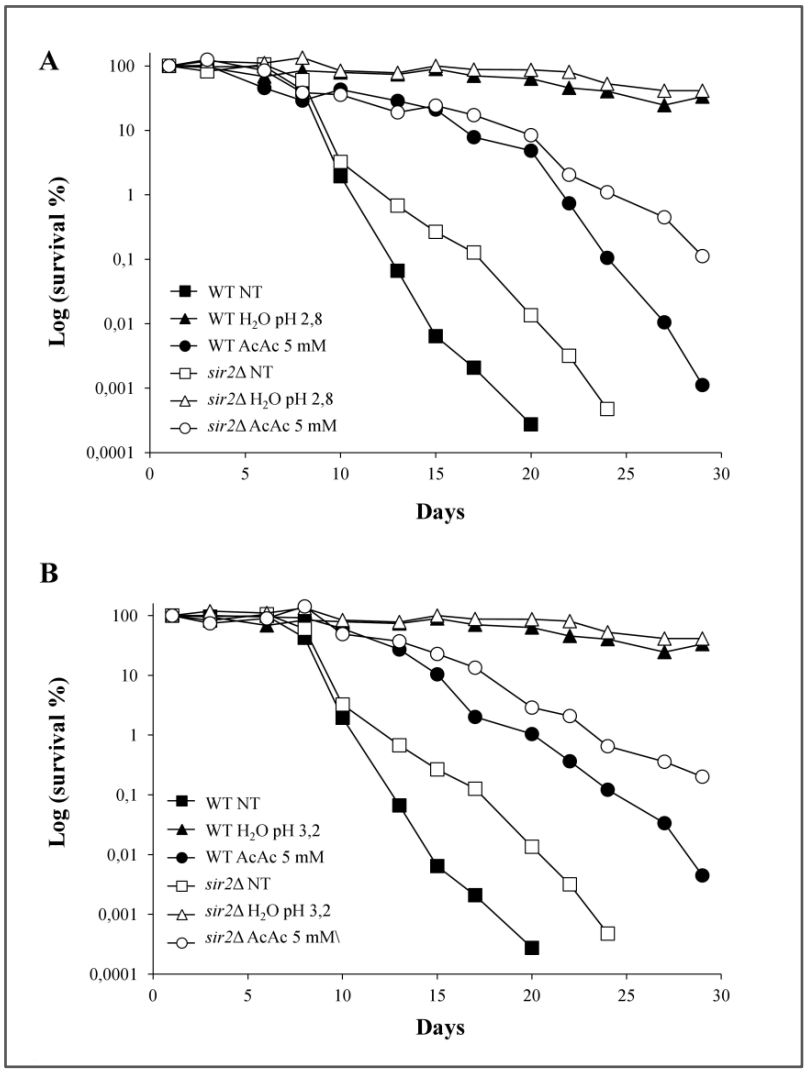

Fig. 4.3. Physiological concentration of acetic acid shortens CLS of calorie restricted *sir2*Δ cells. (A) Wild type and *sir2*Δ chronological aging cells grown on 2% glucose were switched to water adjusted to pH 2.8 and water (pH 2.8) containing 5 mM acetic acid at Day 1. Every 48 h, cultures were resuspended in fresh water (pH 2.8) and each time acetic acid was added again. At every time-point, viability was measured by counting CFUs on YEPD plates. Survival of cells in their exhausted medium was also monitored as a control. (B) The same experiment as in (A) was repeated by adjusting the pH of the water to 3.2. Representative experiments are shown. Day 0 = diauxic shift.

The pH of the water was also set to 3.2, the pH that we found in the culture medium of our strains at Day 1 after the diauxic shift, which is the moment in which physiological accumulation of acetic acid can be observed for wild type cells. This pH value remains much higher than the pKa of acetic acid; so the amount of the undissociated acetic acid able to permeate the membranes is almost similar to that at pH 2.8. As reported in Fig. 4.3A, the CLS shortening effect due to acetic acid was lower for *sir2*Δ cells than for wild type cells. This is consistent with an increased capability of mutant cells to metabolize acetate, which would thus be less able to prevent the CLS extension of calorie restricted cells.

On the basis of these results, we also analyzed the effects of combining *SIR2* and *ACH1* inactivation. Thus, we deleted the *SIR2* gene in *ach1*Δ cells. Double mutant cells do not behave differently than wild type cells when grown in minimal medium (with a four-fold excess of amino acids) with 2% glucose, with no changes in their doubling time nor in the cell density reached upon glucose exhaustion (data not shown). We then measured cell survival in a context of a standard CLS experiment. Surprisingly, double mutant *ach1*Δ*sir2*Δ cells displayed an even shorter CLS than *ach1*Δ single mutant cells, as reported in Fig. 4.4. As already stated, *SIR2* inactivation leads to an increased acetate utilization which in turn has a stimulatory effect on ethanol catabolism. This correlates with an enhanced glyoxylate/ gluconeogenetic flux [5]. The lack of Ach1 severely compromises mitochondrial functionality [6]. We hypothesized that the shorter CLS of the double mutant strain can be due to the fact that when cells are in the post diauxic phase, lack of Sir2 pushes the equilibrium of ethanol conversion towards acetaldehyde first and then to acetate and acetyl-CoA, as reported in Fig. 1.14. In particular, acetaldehyde diffuses freely across mitochondrial membranes entering inside mitochondria, where it is then converted to acetate. Acetate which is made inside mitochondria also from ethanol. This would result in an acetate accumulation. In a context of cells lacking Ach1, whose enzymatic activity is required to prevent acetate accumulation by a CoASH transfer from succinyl-CoA (provided by the TCA cycle) to acetate generating acetyl-CoA, this would worsen the already compromised mitochondrial functionality, leading to precocious cell death.

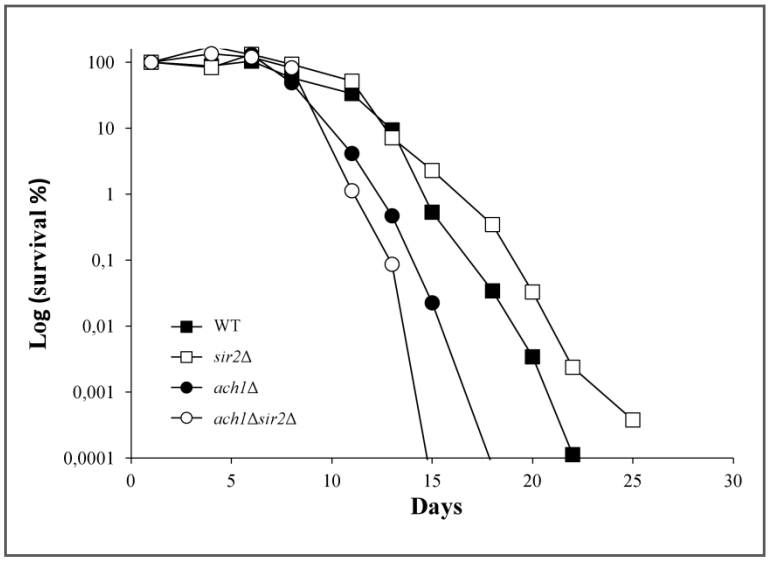

Fig. 4.4. *ach1*Δ*sir2*Δ mutant cells are short-lived mutants. CLS of wt, *sir2*Δ, *ach1*Δ and *ach1*Δ*sir2*Δ mutant cells grown on minimal medium/2% glucose (with a four-fold excess of supplements). At every time-point, viability was determined by counting CFUs on YEPD plates. One representative experiment is shown. Day 0 = diauxic shift.

Thus, not only ethanol or acetic acid, but also the intermediate of their reaction, acetaldehyde, might be involved in CLS, as it has also been suggested recently through some experiments on wine yeast aging cells [36,37]. Consequently, we took into consideration pyrazole, a selective irreversible inhibitor of the cytosolic alcohol dehydrogenase Adh2 [5,38]: this compound impairs the catabolism of ethanol by blocking its conversion to acetaldehyde. In a context of a CLS experiment in extreme CR conditions (water), we have previously demonstrated that pyrazole is able to inhibit the shortening effect of ethanol on CLS, thus indicating that it is its metabolism to be detrimental for lifespan extension [5]. In order to confirm such data in a non physiological condition, we added pyrazole directly to the exhausted medium of the wild type culture. As shown in Fig. 4.5A, also in this case pyrazole treatment significantly increased CLS of wild type cells. As a further refinement of our analysis, we added pyrazole in the exhausted medium of *icl1*Δ mutant cells. These mutants, having the glyoxylate cycle

activity impaired [5], accumulate higher amounts of ethanol and acetic acid following the diauxic shift (as reported in Fig. 2.4E [5]); in addition, they display a reduced CLS (as reported in Fig. 2.4C [5]). When we added pyrazole to their exhausted medium, a significant lifespan extension was observed, further underlining that it is not ethanol presence but some aspects of its metabolism to limit the survival of chronological aging yeast cells.

Finally, we performed some media-swap experiments between wild type and *icl1*Δ cultures. To this end, both strains were grown in minimal medium and at different moments after the diauxic shift, cultures were centrifuged and cells resuspended in exhausted media obtained at different times after the diauxic shift (so containing different amounts of ethanol and acetic acid). In particular, as reported in Fig. 4.5B, we switched wild type cells into the pre-conditioned medium of *icl1*Δ cells collected after 12h and 24h from the diauxic shift: in both conditions, higher amounts of both metabolites were present, as reported in Fig. 2.4D and 2.4E [5]. After the swap, we found that the CLS of wild type cells resulted shorter compared with that measured in their original exhausted medium. This detrimental effect on viability of wild type cells swapped to *icl1*Δ pre-conditioned medium (at Day 1 after diauxic shift) was abolished by adding pyrazole and CLS was increased to the same extent of that of the wild type cells maintained in their original medium in the presence of pyrazole, as reported in Fig. 4.5A.

Conversely, when *icl1*Δ mutant cells were shifted into wild type pre-conditioned media taken at 12h and at Day 1 after the diauxic shift, a significant CLS extension was observed according to the different concentrations of extracellular ethanol and acetate, as reported in Fig. 2.4D and 2.4E [5]. On the whole, our data point to a dose-dependent influence of acetic acid and ethanol on CLS by inversely correlating their amount in the medium with CLS extension and to the fact that this effect is mainly due to their metabolism more than to their simple presence.

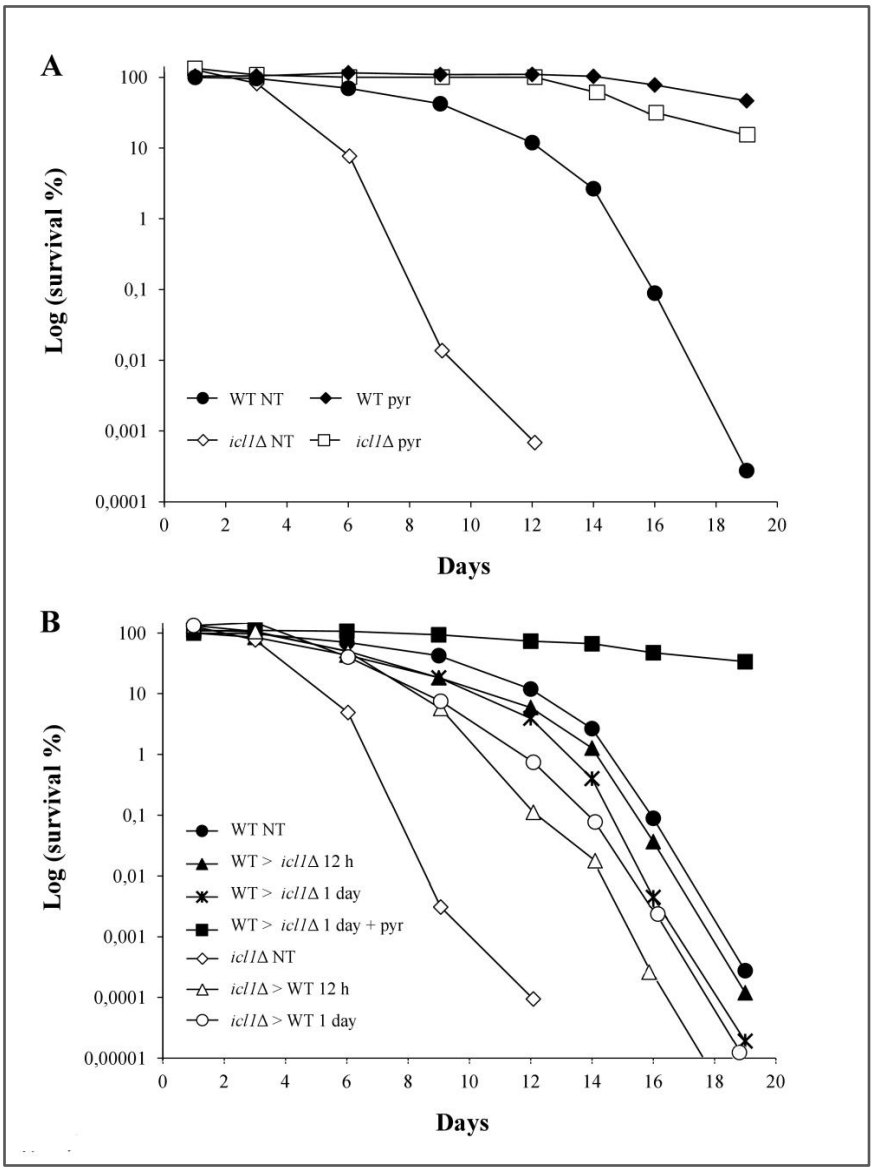

Fig. 4.5. The amount of ethanol and acetic acid in the medium modulates CLS. (A) Wt and *icl1*Δ cells were grown in minimal medium. At Day 1 after the diauxic shift, pyrazole was added every 48 h at the final concentration of 50 mM. At every time-point, viability was measured by counting CFUs on YEPD plates. Survival of cells in their exhausted medium was also monitored as control. One representative experiment is shown. (B) Wt and *icl1*Δ mutant cells grown as in (A) have been harvested and resuspended at Day 1 in their cell free original media or subjected to

cell free media-swap with exhausted media collected 12 h and at Day 1 after the diauxic shift. Resuspension in media collected at Day 1 was performed also in the presence of pyrazole. Every 48 h viability was measured as in (A). One representative experiment is shown. Day 0 = diauxic shift.

4.4 References

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Riassunto

Con il passare del tempo, tutti gli organismi viventi vanno incontro a un progressivo declino fisiologico, che correla con un aumento del rischio di sviluppare diverse malattie. Finora, molti fattori sono stati coinvolti nel processo di invecchiamento, quali lo stress ossidativo, l'accorciamento delle estremità telomeriche, le disfunzioni mitocondriali, l'instabilità genomica o cambiamenti epigenetici; nonostante tutti gli sforzi fatti, però, nessuno di tali fattori è stato ancora riconosciuto come causa primaria di questo complesso fenomeno. È stato ampiamente dimostrato che il lievito gemmante *Saccharomyces cerevisiae* è un utile modello sperimentale il processo di invecchiamento per identificare quei *pathway* la cui controparte può essere ritrovata negli eucarioti superiori e in particolare nell'uomo. In lievito, sono stati descritti due paradigmi di invecchiamento: replicativo e cronologico. Quest'ultimo si riferisce all'invecchiamento di cellule in uno stato di quiescenza, in cui la *chronological lifespan,* CLS, è definita come il periodo di tempo in cui cellule di lievito non proliferanti sono in grado di mantenere il loro potenziale replicativo. La CLS può venir modulata da diversi fattori, sia intrinseci che estrinseci. Il primo gruppo comprende le vie di trasduzione del segnale coinvolte nella risposta a stress e ai nutrienti, come le vie di TOR/Sch9 e Ras/PKA. Nel secondo gruppo, invece, sembrano giocare un ruolo essenziale due prodotti del metabolismo cellulare: l'acetato e l'etanolo. In particolare, la loro presenza nel terreno di crescita risulta avere un effetto pro-*aging*, anche se l'esatto ruolo che essi svolgono è ancor oggi un tema dibattuto.

Inoltre, un ruolo chiave nella regolazione del processo di invecchiamento è generalmente riconosciuta a Sir2, il capostipite delle Sirtuine, una famiglia di deacetilasi istoniche NAD-dipendenti, altamente conservate. Però, diversamente dalle altre famiglie di deacetilasi, le Sirtuine accoppiano la deacetilazione delle proteine con la scissione di una molecola di NAD^+, una caratteristica che le rende elementi chiave nel legame tra il controllo dell'omeostasi cellulare e l'invecchiamento. Inoltre, le Sirtuine possono influenzare l'attività di diversi enzimi metabolici nell'uomo modulando il loro stato di acetilazione, rafforzando ulteriormente la stretta relazione tra lo stato metabolico della cellula e la funzione delle Sirtuine.

All'interno di questo contesto, questo lavoro di tesi si propone di approfondire il ruolo di alcuni regolatori sia intrinseci che estrinseci dell'invecchiamento cronologico. Tra i fattori intrinseci, ci siamo focalizzati su Sir2, che gioca un ruolo pro-*aging* nella CLS. Abbiamo osservato che la mancanza di tale deacetilasi influenza alcuni aspetti del metabolismo cellulare, in particolar modo per quanto riguarda etanolo e acetato. Tra i fattori estrinseci, e in particolare focalizzandosi sull'acetato, ci siamo concentrati su Ach1, un enzima mitocondriale la cui funzione non è ancora stata particolarmente caratterizzata, ma che può giocare un ruolo chiave nel metabolismo dell'acido acetico a livello mitocondriale.

Per analizzare l'interconnessione tra l'attività di Sir2, l'invecchiamento e il metabolismo cellulare, abbiamo effettuato esperimenti in *batch* con fonti di carbonio fermentabili e non-fermentabili durante l'invecchiamento cronologico e in chemostato (culture limitate da glucosio sottoposte a un *pulse* di etanolo e acetato), condizioni in cui sono stati analizzati i parametri di crescita così come il contenuto di metaboliti. Durante l'invecchiamento cronologico, abbiamo anche analizzato la CLS. Dato che in cellule prive di Sir2 abbiamo osservato un catabolismo aumentato di acetato e etanolo, ci siamo focalizzati sulla gluconeogenesi (in particolare su Pck1, l'enzima che catalizza la reazione limitante) e sul ciclo del gliossilato (in particolare su uno dei due enzimi unici di tale ciclo, Icl1). Infatti, entrambi i processi sono fondamentali quando le cellule crescono su acetato e etanolo. Misurando l'attività di Pck1 e Icl1, abbiamo osservato che in cellule *sir2*Δ entrambe queste attività enzimatiche risultano aumentate. In particolare, la maggior attività di Pck1 correla con un maggior livello di acetilazione di tale proteina, fornendo anche evidenza sperimentale a un modello in cui era stato proposto che Pck1, acetilata da Esa1, potesse essere oggetto di eventi di deacetilazione mediati da Sir2. Inoltre, per verificare se l'attività di questi enzimi fosse collegata al fenotipo di una maggiore sopravvivenza cronologica di cellule *sir2*Δ, sono stati anche inattivati *ICL1* e *PCK1* in cellule *sir2*Δ per analizzare la longevità dei ceppi portanti la doppia inattivazione e i livelli di metaboliti durante l'invecchiamento cronologico: sia cellule *icl1*Δ che *pck1*Δ accumulano alti livelli di acetato e di etanolo extracellulare e sono risultati avere una ridotta CLS. Inoltre, coerentemente con un aumento del flusso gluconeogenetico, i livelli di trealosio in cellule *sir2*Δ erano più alti rispetto a cellule *wild type*. Nel complesso, abbiamo

dimostrato che l'inattivazione di *SIR2* influisce positivamente sul metabolismo dell'acetato, aumentando la gluconeogenesi e il ciclo del gliossilato, da cui la prima via dipende. Nel contesto dell'invecchiamento, questo implica livelli inferiori di fattori extracellulari che hanno un'influenza negativa sulla CLS e un accumulo maggiore di trealosio, che ha invece una funzione protettiva, creando così un ambiente favorevole alla sopravvivenza a lungo termine di cellule non proliferanti.

Successivamente, come detto in precedenza, a proposito dell'analisi dei fattori intrinseci, ci siamo focalizzati sull'acetato e in particolare su Ach1, un enzima mitocondriale la cui esatta funzione non è ancora stata caratterizzata. Negli anni '90, Ach1 fu identificato come idrolasi mitocondriale, nonostante non fosse chiara la ragione fisiologica dell'idrolisi di acetil-CoA. Recentemente, è stato identificato in *Aspergillus nidulans* un enzima con alta identità di sequenza con Ach1, coinvolto nel processo di detossificazione delle cellule in presenza di propinato. A partire da questa scoperta, è nata l'ipotesi che anche in *S. cerevisiae* questo enzima potesse catalizzare una reazione trasferasica attivando, più che idrolizzando, l'acetil-CoA a partire dall'acetato. In questo lavoro di tesi, abbiamo caratterizzato il fenotipo di cellule *ach1*Δ, con l'intento di comprendere meglio il ruolo di tale proteina nel metabolismo dell'acetato, così come le possibili implicazioni sulla funzionalità mitocondriale e sulla CLS. Abbiamo osservato che durante l'invecchiamento cronologico, cellule *ach1*Δ accumulano livelli maggiori di acetato extracellulare che correla con una CLS più corta. Questo fenotipo è strettamente dipendente dai livelli di acetato extracellulare, in quanto abbiamo potuto ristabilire la vitalità cellulare nel momento in cui lo stress dovuto alla presenza di acido è stato abolito o tramite un regime di restrizione calorica (dove non vi è produzione di acido acetico) o tramite il trasferimento di cellule *ach1*Δ in acqua durante l'invecchiamento cronologico. Inoltre il fenotipo di ridotta vitalità del cellule *ach1*Δ è accompagnato da un accumulo di ROS, da un'attività mitocondriale compromessa e da una precoce attivazione del *pathway* apoptotico dipendente dalla caspasi Yca1. In accordo con questa compromessa attività mitocondriale, abbiamo anche osservato che cellule *ach1*Δ mostrano severi problemi di crescita in terreni contenenti acetato come unica fonte di carbonio, sottolineando il fatto che l'attività enzimatica di Ach1 svolge un ruolo primario nella detossificazione da acido acetico, che risulta importante

per la funzionalità mitocondriale. Funzionalità mitocondriale che a sua volta gioca un ruolo essenziale nella sopravvivenza cellulare durante l'invecchiamento cronologico.

Dal momento che abbiamo osservato una correlazione inversa tra i livelli di etanolo e acetato extracellulare e la CLS, ulteriori esperimenti sono stati effettuati per chiarire il ruolo di questi due fattori pro-*aging*. I dati ottenuti supportano l'ipotesi che a livello fisiologico non è la loro mera presenza a influenzare la CLS ma che sia piuttosto il loro metabolismo. Così, entrambi questi composti C2 agiscono come fonti di carbonio che prevengono l'entrata delle cellule in uno stato analogo a quello derivato da condizioni di restrizione calorica, unica in cui le cellule sono in grado di mantenere una sopravvivenza a lungo termine.

Appendix I:
List of abbreviations

Ac-K	acetylated lysine
Acetyl-CoA	acetyl coenzyme A
AceCS	acetyl coenzyme A synthase (nomenclature for human proteins)
ACS	acetyl coenzyme A synthase
ACH	acetyl coenzyme A hydrolase
Acyl-CoA	acyl coenzyme A
ADH	alcohol dehydrogenase
ADP	adenosine di-phosphate
AMP	adenosine mono-phosphate
ATP	adenosine tri-phosphate
CFU	colony-forming unit
CLS	chronological lifespan
CO_2	carbon dioxide
CoASH	coenzyme A (reduced form)
CR	calorie restriction
DHE	dihydroethidium
DHR123	dihydrorhodamine 123
$DiOC_6$	3,3”-dihexyloxacarbocyanine iodide
DR	dietary restriction
ERC	extrachromosomal rDNA circles
Eth	ethidium
G_0	phase of quiescence of the cell cycle
GRAS	Generally regarged as safe
HAT	histone acetyl transferase
HDAC	histone deacetylase
ICL	isocitrate liase
IRC	index of respiratory competence
K_m	Michaelis constant
mAb	monoclonal antibody
MnSOD	manganese dependent superoxide dismutase
NAD^+/NADH	nicotinamide adenine dinucleotide (oxidized and reduced form)
$NADP^+$/NADPH	nicotinamide adenine dinucleotide phosphate (oxidized and reduced form)

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OAADPr	O-acetyl ADP-ribose
OAA	oxaloacetate
PDS	post diauxic shift
PEPCK (Pck)	phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
pH_c	cytoplasmic pH
PI	propidium iodide
PKA	protein kinase A
pKa	acid dissociation constant
PCR	polymerase chain reaction
qAc	acetate specific consumption rate
qCO_2	carbon dioxide specific production rate
qEtOH	ethanol specific consumption rate
rDNA	ribosomal DNA
RH123	rhodamine 123
RLS	replicative lifespan
ROS	reactive oxygen species
RTG	retrograde response
S6K	ribosomal protein S6 kinase
SIR	silent information regulator
SOD	superoxide dismutase
STRE	stress responsive element
TCA cycle	tricarboxylic acid cycle
Td	duplication time
TUNEL	TdT-mediated dUTP nick end labeling
YEP(D)	yeast extract – peptone (destrose)
YNB	yeast nitrogen base
TOR	target of rapamycin

Appendix II:
Publications and congresses

Publications

Casatta N, Porro A, Orlandi I, Brambilla L and Vai M.
'Lack of Sir2 increases acetate consumption and decreases extracellular pro-aging factors'.
Biochimica et Biophysica Acta - Molecular Cell Research, 1833 (2013) 593-601.

Orlandi I, Casatta N and Vai M.
'Lack of Ach1 CoA-transferase triggers apoptosis and decreases chronological lifespan in yeast'
Frontiers in Oncology 2 (2012) 67; doi: 10.3389/fonc.2012.00067.

International Congresses

Casatta N, Orlandi I, Porro A, Brambilla L and Vai M.
'Role of the NAD-dependent histone deacetylase Sir2 in *Saccharomyces cerevisiae* metabolism and ageing'.
8th IMYA Meeting. University of Kent, Canterbury, UK, 05/2011.

Casatta N, Orlandi I and Vai M.
'Lack of Ach1 CoA-transferase triggers apoptosis and decreases chronological lifespan in yeast'.
9th IMYA Meeting. Università La Sapienza, Roma 09/2012.