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Acetyl-CoA Metabolism and Aging: Risks and Resources

Cognome / Surname: Pellegrino Coppola
Nome / Name: Damiano
Matricola / Registration number: 701542

Tutore / Tutor: Dr. Ivan Orlandi
Supervisor: Prof. Marina Vai
Coordinatore / Coordinator: Prof. Marco Vanoni

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Contents

Abstract

Abstract in Italian

I composti intermedi generati dal metabolismo del carbonio sono precursori fondamentali per la biosintesi di lipidi e carboidrati, la cui importanza nella regolazione della longevità è sempre più rilevante. Per comprendere il ruolo di tali composti nel contesto dell'invecchiamento diventa necessario indagare le relazioni tra i pathway che generano i precursori metabolici e come i flussi dei singoli metaboliti impattano sulla sopravvivenza cellulare. Per affrontare questa tematica, il mio progetto di dottorato sfrutta il lievito gemmante *Saccharomyces cerevisiae*, organismo modello ampiamente affermato per lo studio di meccanismi molecolari evolutivamente conservati. In particolare, si è studiato l'invecchiamento cronologico, ossia quello a cui vanno incontro cellule di lievito quiescenti in fase stazionaria e che rappresenta un efficace modello per lo studio dell'invecchiamento di cellule di mammifero post-mitotiche quali neuroni e miociti. In questo contesto, un metabolita di fondamentale interesse è l'acetil-CoA, ossia la forma attivata dell'acetato tramite un legame tioestere con il coenzima A. L'acetil-CoA rappresenta un importante snodo per il metabolismo, anche in relazione alla sua localizzazione. Ad esempio, l'acetil-CoA mitocondriale, generato per decarbossilazione ossidativa del piruvato nel mitocondrio, entra nel TCA per foraggiare il metabolismo biosintetico e quello energetico. L'altro grande pool di acetil-CoA è quello nucleocitosolico, rilevante nel metabolismo lipidico: l'acetil-CoA può essere infatti utilizzato per convogliare unità di carbonio alla biosintesi di macromolecole lipidiche, inclusi acidi grassi i quali in seguito, tramite β-ossidazione nei perossisomi, possono fornire nuovamente acetil-CoA. Inoltre, lo stesso pool nucleocitosolico è anche usato come donatore di gruppi acetili, per regolare tramite l'acetilazione attività enzimatiche e stato della cromatina, quindi lo stato funzionale della cellula. Procedendo, tre punti diventano rilevanti: 1) la regolazione del flusso dei precursori dell'acetil-CoA tra compartimenti cellulari; 2) il controllo del metabolismo attraverso il meccanismo di (de)acetilazione; 3) il ruolo degli acidi grassi, intimamente connessi con l'acetil-CoA. Questi punti sono stati indagati generando mutanti *ad hoc* tramite la delezione di geni, esaminando diversi aspetti sia metabolici che funzionali e adoperando approcci nutrizionali mirati. Complessivamente, i risultati hanno permesso chiarire come il

ruolo dell'acetil-CoA non sia rilegato ad una specifica fase di crescita o ad un particolare metabolismo ma abbia un valore più ampio. La sua corretta produzione e distribuzione tra i compartimenti cellulari, favorita anche dalla somministrazione di molecole di interesse nutrizionale, garantisce una modulazione efficace del metabolismo anche attraverso modificazioni post-traduzionali, incrementando la longevità cellulare. Infine, i mitocondri sono emersi come organelli estremamente sensibili alla gestione di acetil-CoA, quindi come sensori per monitorare lo stato cellulare nel tempo. In conclusione, i dati ottenuti sottolineano la complessità del metabolismo dell'acetil-CoA durante l'invecchiamento, e come la gestione di questo metabolita chiave sia strettamente legata alla sopravvivenza cellulare.

Abstract in English

Carbon metabolism intermediates are known to be fundamental precursors in the biosynthesis of carbohydrates and lipids, whose metabolism is being increasingly acknowledged as a relevant lifespan regulator. Therefore, to understand the importance of these compounds in the context of aging, it is necessary to deepen the relations among the pathways involved in the production of metabolic precursors and how fluxes of single key metabolites impact on cellular survival. In order to reach this goal, we employ the budding yeast *Saccharomyces cerevisiae*, a model organism successfully used to study evolutionarily conserved molecular mechanisms. In particular, the interest is in chronological aging, defined as the aging process of quiescent yeast cells during the stationary phase, that is, after all carbon sources are consumed. Currently, this model is extensively used as a paradigm for the aging process of mammalian post-mitotic cells such as neurons and myocytes. In this context, a particularly interesting metabolite is acetyl-CoA, the activated form of acetate due to a thioester bond with coenzyme A. Acetyl-CoA is indeed important for many cellular events, depending on its cellular localization. For instance, mitochondrial acetyl-CoA, generated from the uptake of pyruvate in the mitochondria, enters the TCA cycle and fosters both biosynthesis and energy metabolism. The other great distinct pool, represented by the nucleocytoplasmic acetyl-CoA, provides instead the fundamental bricks for the biosynthesis of lipid macromolecules, including fatty acids, which in turn, if processed by β-oxidation, can provide again acetyl-CoA. In addition, the available nucleocytosolic pool is also used as a source of acetyl groups to modulate, through post-translational modifications, enzyme activities and chromatin state, therefore the functional state of a cell. Proceeding through steps, three key points become relevant in the context of acetyl-CoA and aging: 1) the regulation of the flux of acetyl-CoA precursors among compartments; 2) the control of metabolism through (de)acetylation; 3) the role of fatty acid metabolism, intimately linked to acetyl-CoA. These key points were investigated generating *ad hoc* mutants through gene deletion, analysing different metabolic and functional aspects and using peculiar nutritional approaches. Overall, results gave insights on the role of acetyl-CoA, highlighting that the importance of this

metabolite is not restricted to a particular growth phase or metabolism but has a broader value. A correct production and distribution of acetyl-CoA among compartments, supported by the administration of nutritionally relevant molecules, guarantees an efficient modulation of metabolism with the contribute of post-translational modifications, increasing longevity. Also, mitochondria emerged as extremely sensitive on how acetyl-CoA is managed during aging, therefore as sensors to monitor the cellular state through time. In conclusion, the obtained data underline the complexity of acetyl-CoA metabolism during aging, and how the management of this key metabolite is strictly related to cellular survival.

1 Introduction

1.1 Preamble

Aging, commonly known as the process of "becoming older", can be described as a time-dependent functional decline in living organisms. Aging is characterized by a progressive accumulation of genetical and biochemical alterations that lead to variations in the physiology of an organism and ultimately a loss of fitness. Over the past years, aging has been increasingly linked to specific damages, with every single damage defined as a *hallmark of aging* according to the following pattern: (a) it should manifest spontaneously during normal aging; (b) its experimental aggravation should accelerate aging; (c) its experimental amelioration should retard the normal aging process (López-Otín *et al.*, 2013). Different hallmarks have been found, and each one can be brought back to triggering factors on a subcellular scale. Indeed, it is true that typical aging alterations establish a fertile field for the onset of morbidity and therefore that aging and diseases are correlated and may have common molecular origins. Nevertheless, a difference is underlined in the measures currently used in relation to aging, *lifespan* and *healthspan*. While lifespan refers to the whole life length, the healthspan refers exclusively to the healthy life length. In this sense, efforts are being made to both enhance lifespan and improve the quality of life through physical, nutritional and pharmacological approaches in order to "compress morbidity" and promote healthy aging (Fries, 1980; Fries *et al.*, 2011; de Cabo *et al.*, 2014). Due to experimental, economical and ethical

limits, comparative studies among different organisms have always been a necessity in the research field of aging. This possibility is promoted by the important discovery that resembling aging-related biological events or factors and aging hallmarks can be observed in a plethora of organisms ranging from the unicellular eukaryote yeast to multicellular eukaryotes such as humans, underlyning the presence of general aging mechanisms that are evolutionarily conserved. From this principle, a brief description of the main non-human aging model organisms and their relation to the known general aging mechanisms will be presented, with a particular emphasis towards the unicellular budding yeast *Saccharomyces cerevisiae*, which contributed more than 60 years ago to one of the first rigorous, scientific definitions of aging (Mortimer & Johnston, 1959).

1.2 Model organisms

In the aging-research field, a comparative aging biology study has been developed, based on experimental approaches that are carried out using evolutionarily divergent organisms, including non-mammalian models ranging from insects to unicellular organisms such as yeasts. Currently, the four main model organisms are the mouse *Mus musculus*, the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and the budding yeast *S. cerevisiae* (Bitto *et al.*, 2015), whose contribution for the comprehension of aging has been fundamental. In addition, recent studies of aging in non-human primates also had a remarkable impact, as well as newly introduced model organisms that present peculiar longevity features (Austad, 2009; Petralia *et al.*, 2014; Bitto *et al.*, 2015).

1.2.1 Laboratory mouse

Mice from the species *M. musculus* are the main non-primate mammalian model organism, and today many strains are well-characterized, as well as different genetic engineering techniques, such as the homologous recombination system or the CRISP/Cas9 system, to produce transgenic mice. The main strain used in many aging studies has been the C57BL/6, which can live approximately 2 years (Lees, H. *et al.* 2016). Anyhow, the use of a

single strain has oriented numerous results only in relation to a specific genetic background. Known this limitation, the good practice which is increasingly spreading is the use of genetically heterogeneous mice such as the UM-HET3 (Bitto *et al.*, 2015), in order to include in the studies the variability value of genetic diversity.

1.2.2 Laboratory fruit fly

D. melanogaster is a non-mammalian, invertebrate eukaryote with lifespan studies dating back to 1916 (Loeb & Northrop, 1916). The fly life cycle is composed by distinguishable stages, namely embryo, larva, and pupae, followed by the reproductive adulthood. The wild type strain has a lifespan of ~2-3 month. The particularity of this fly is that it shows many homologous structures that resemble mammalian ones, including a nervous system which is intesively used as a model to study cognitive functions and neurodegenerative diseases (Fontana *et al.*, 2010; Bitto *et al.*, 2015).

1.2.3 Laboratory worm

C. elegans was first described as a model for studying ageing nearly 40 years ago (Klass, 1977). It is a facultative hermaphrodite with a life cycle composed by four larval stages that precede the reproductive adult form, characterized by the fixed number of 959 cells. Nearly all laboratories use the wild type strain N2, with a lifespan of ~20 days. In addition, this model also offers the opportunity to study healthspan by investigating both functionality and degeneration of tissues. Importantly, it was with this model organism that the influence of single genes on lifespan was demonstrated, and it extensively contributed to the discovery that the insulin/IGF-1-like signalling pathway is evolutionarily conserved as a major aging regulator (Fontana *et al.* 2010; Kenyon, 2011), as illustrated in Figure 1.1.

1.2.4 Laboratory yeast and yeast aging paradigms

Among the innumerable yeast species, the first choice for investigating cellular and molecular aging factors in a rather quick, simple and econom-

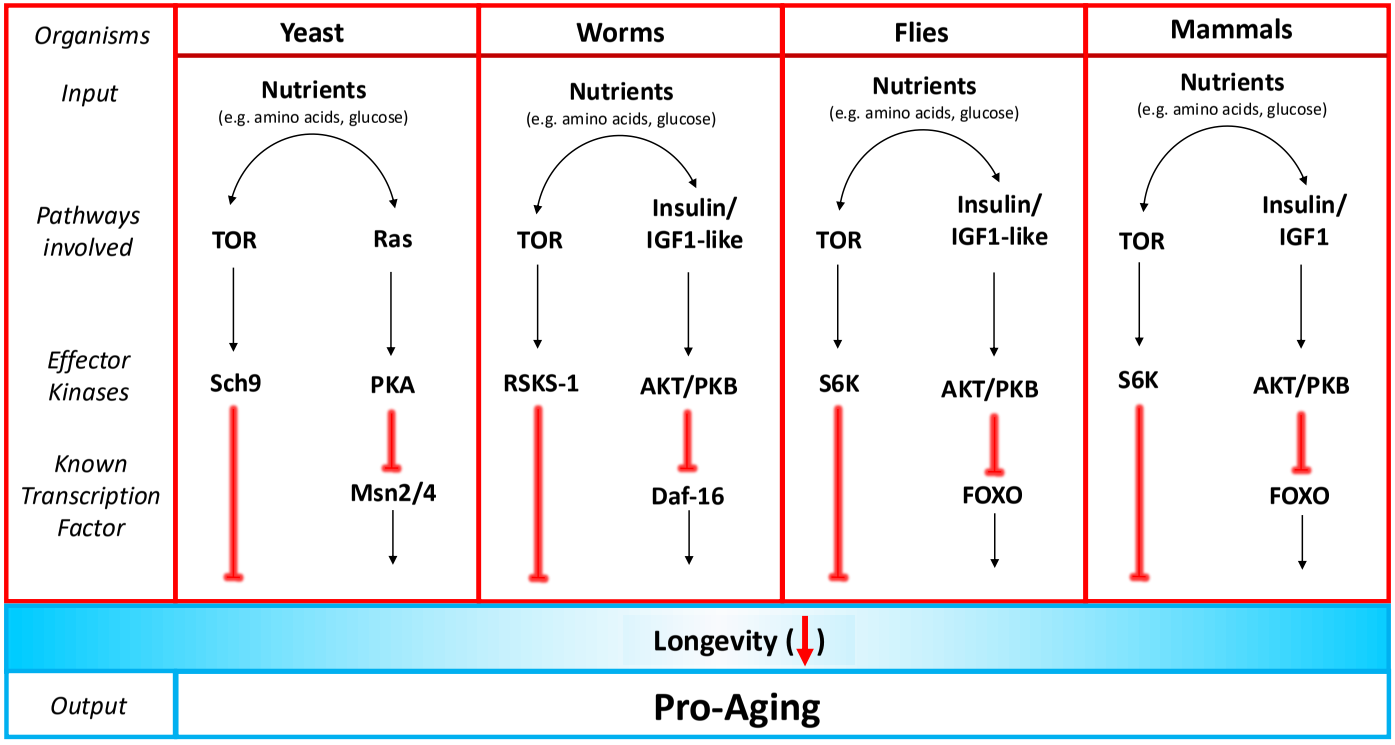

Figure 1.1 – Response to nutrients is evolutionarily conserved among phyla and is regarded as a common aging regulator. As shown, input, pathways involved, relative kinases and responses are organized in a resembling fashion (adapted from Longo *et al.*, 2005). The Target of Rapamycin (TOR) and the Insulin/IGF-1-like signalling (IIS) or Ras/cAMP/PKA in yeast represent nutrient-sensing pathways which are involved, for instance, in amino acids or glucose metabolism, respectively. These pathway are related to cell growth and proliferation, and their activation has been linked to organismal aging.

ical, however extremely trustworthy way is to employ the budding yeast *S. cerevisiae*. Even if 850 milion years separate humans from the unicellular *S. cerevisiae* (Table 1.1, adapted from Lees H. *et al.*, 2016), it strikingly bears many human homologue genes and structurally conserved pathway. In the last years, *S. cerevisiae* has been successfully used as a model for aging of both proliferating and non-proliferating cells, due to the conception of the replicative and chronological aging paradigms, respectively (Fabrizio & Longo, 2003; Steinkraus *et al.*, 2008). In this sense, the obstacle represented by the problem that aging in humans involves both mitotically active and post-mitotic cells was avoided by dissecting this feature in two radical, singular aging aspects. Replicative aging is related to the replicative potential of proliferating cells, and the yeast replicative lifespan (RLS) is defined as the number of daughter cells generated by an asymmetrically dividing mother in the presence of nutrients (Steinkraus *et al.* 2008). Chronological aging is defined as the aging process of quiescent yeast cells during stationary phase, that is, after all carbon sources are consumed. Chronological lifespan (CLS), i.e. viability over time, is defined as the ability of yeast aging cells to resume mitotic growth in presence of nutrients (Fabrizio & Longo, 2003). Currently, this model of aging is extensively used as a paradigm for the aging process of mammalian post-mitotic cells such as neurons and myocytes (Longo, 1999; MacLean *et al.* 2001). Both model are depicted in Figure 1.2.

Based on the lessons from comparative biology, a certain number of evolutionarily conserved longevity pathways has been identified, with the exception of some aging genes that can still not be ascribed in any known evolutionarily shared mechanism. Among the conserved longevity pathways, of particular relevance are the Target of Rapamycin (TOR) pathway, the Insulin/IGF-1-like signalling (IIS) pathway and the stress resistance pathways, as well as the Sirtuins regulation mechanism (Sutphin & Kaeberlein, 2011). As a matter of fact, all the cited pathways can be linked to a multifactorial nature of aging and a common series of downstream targets that are involved in the regulation of senescence such as:

- mitochondrial functionality and reactive oxygen species (ROS) production (Chen *et al.* 2005, Szklarczyk *et al.* 2014; Pan, 2011);

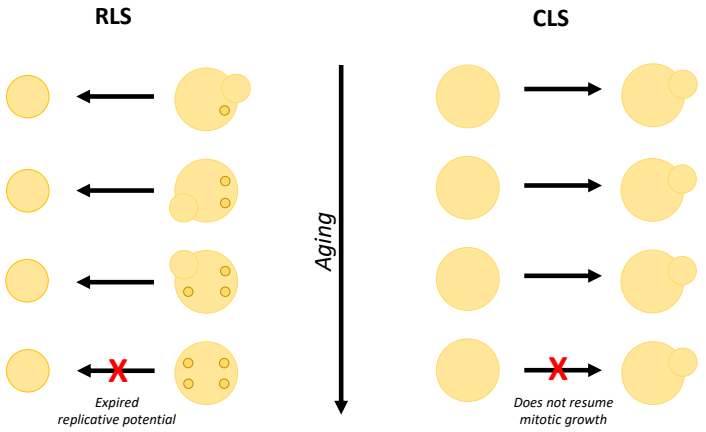

Figure 1.2 – RLS and CLS models. During aging, replicative potential expires and mother cells cannot generate daughters anymore, even if nutrients are present. On the other hand, chronological aging affects the capacity to resume mitotic growth in presence of nutrients.

Organism	Evolutionary conservation with human genome (%)	Time since divergence (million years)	Number of cells in the organism	Generation time
Yeast	46	850	1	2 h
Worms	43	600	959	3 days
Flies	61	600	$>10^6$	10 days
Mice	95	75	$>10^9$	12 weeks
Dogs	84	90-100	$>10^{11}$	~3 years
Rhesus monkeys	97.5	6	$>10^{11}$	~12 years
Humans	99.5	na	$>10^{12}$	~25 years

Table 1.1. Various model organisms compared to humans are presented, including details on multicellularity, generation time and evolutionary divergence (adapted from Lees *et al.*, 2016).

- autophagy (Rubinsztein *et al.* 2011; He *et al.* 2013; Choi *et al.* 2013);
- genome stability and epigenetic regulation (Vijg & Suh, 2013; Moskalev *et al.*, 2013; Booth & Brunet, 2016);
- metabolism regulation and management of storage nutrients (Longo *et al.*, 2005; Fontana *et al.*, 2010; Solon-Biet *et al.* 2015).

In addition, high-throughput techniques and wide-screenings are now bringing to light other interesting elements that may potentially participate in the regulation of aging (Fabrizio *et al.*, 2010). Anyway, the downstream targets reported above are those found impaired in the aging process of different organisms. For this reason, mitochondrial dysfunctions, altered autophagy, epigenetic alterations and genome instability, as well as deregulated management of nutrient metabolism are commonly regarded as *hallmarks of aging*.

1.3 Hallmarks of Aging

1.3.1 Mitochondrial dysfunctions

Mitochondria are essential double-membrane organelles found in all eukaryote organisms that very probably arose from an endosymbiont bacterium living inside the ancestor of eukaryote cells. They are involved in a plethora of cellular events such as energy production, cells signaling, biosynthesis of precursors and programmed cell-death. Inside mitochondria, the respiratory chain can be found in the inner membrane: here is where ATP is produced according to the functional needs, and where ROS are produced by electron leakage (Figure 1.3). As cells or organisms age, electron leakage increases at the expense of the generation of ATP (Green *et al.*, 2011), producing ROS that further promote mitochondrial dysfunctionality, as pictured according to the mitochondrial free radical theory of aging (Harman, 1972). ROS production can increase greatly in neurodegenerative conditions (Parkinson's disease, Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis; Adam-Vizi *et al.*, 2006), and in general oxidized cell constituents, such as DNA, proteins and lipids

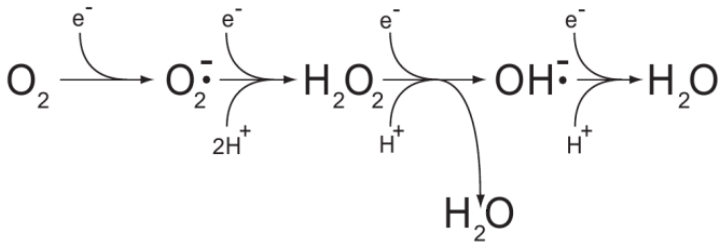

Figure 1.3 – Chain model for ROS production. The ROS group includes many oxygen-containing molecules, such as the radical superoxide (O_2^-), the non-radical hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), which are represented in this redox step-based generation model. Both electron leakage or enzymatic reactions can participate in the production of ROS, with the production steps varying in relation to the reactive molecule that is considered.

accumulate in aged cells and in age-related disorders (Scherz-Shouval & Elazar, 2007). Also mitochondrial DNA (mtDNA) accumulates mutations during aging, and mtDNA instability was linked to degenerative processes in different model organisms, including mammalians (Szklarczyk *et al.*, 2014). Further, mtDNA mutations induce deficiencies in mitochondrial complexes causing a shortage of ATP supply and an impairment in the NAD^+/NADH balance, resulting in the induction of problems in anabolic and catabolic biochemical pathways (Hyun *et al.*, 2006; Imai & Guarente, 2014). Moreover, mitochondrial dysfunctions can promote aging independently by ROS, as shown in mice deficient for polymerase γ (Edgar *et al.*, 2009; Hiona *et al.*, 2010). In general, this can occur through alterations in mitochondria signaling, biogenesis or clearance. Different pharmacological approaches brought to light compunds such as resveratrol or the sirtuin activator SRT1720 which improve mitochondrial respiration in a PGC-1α-dependent manner (Baur & Sinclair, 2006; Lagouge *et al.*, 2006; Minor *et al.*, 2011). Despite being a unicellular organism, *S. cerevisiae* has provided valuable insights on how mitochondria can influence longevity. For istance, rho^0 cells, characterized by the lack of mtDNA, lack critical respiratory chain catalytic subunits that are encoded in the mitochondrial genome and have and extremely reduced CLS (Ocampo *et al.*, 2012). On the contrary, enhancing respiratory activity has been shown to increase

RLS and CLS (Barros *et al.*, 2010). Mitochondrial effects on both replicative and chronological aging are reported in Table 1.2. The sole presence of functional respiration has a positive effect on lifespan. Also, the capacity to limit the production of ROS to a benefical level supports mitohormesis, a theory for which a low level of toxicity brought by ROS triggers an improvement in cellular fitness. In agreement, elevated mitochondrial ROS during growth are needed for full extension of CLS (Pan, 2011). Overall, mitochondrial dysfunctions can accelerate aging through an increase in ROS and a decrease in NAD^+, or by alterations in energy production. A strong influence on mitochondrial efficiency and biogenesis is given by nutrients, as reported in Table 1.2. This holds true for different model organisms (reviewed in Ruetenik & Barrientos, 2015). Again, nutrients can also modulate the damage to mtDNA, as recently shown by the U.S. CALERIE research project, based on the application of calorie restriction (CR), defined for mammals as a decrease of the 20-40% in the calorie intake. In the trial studies, a decrease of the 25% in the calorie intake was applied, and CR patients were shown to have less mtDNA damage, more mtDNA content, and an increased expression of antioxidant enzymes. This system also seems to involve a reorganization in the acetylation of mitochondrial proteins, as a consequence of the increase in oxidative metabolism relaying on the supply of mitochondrial acetyl-CoA (Pougovkina *et al.*, 2014; Kauppila *et al.*, 2016). Therefore, nutritional approaches able to manage acetyl-CoA dynamics and fluxes among compartments potentially represent a factor able to relocate the scale needle in favour of healthy aging. One solid example regards L-carnitine and the related acyl group shuttle, whose effect will be presented in the study described in *Chapter 2*.

Physiological conditions that affect yeast ageing	Mitochondrial effect	Lifespan effect	References
Defective mother/-doughter segregation	Organelle misdistribution and accumulation of senescence factors	Decreased RLS	Lai *et al.*, 2002; Piper *et al.*, 2002
Activation of retrograde signaling	Higher α-ketoglutarate and amino acid production, glutamate accumulation	Increased RLS	Kirchman *et al.*, 1999; Borghouts *et al.*, 2004
Absence of mtDNA	Cannot support normal oxidative phosphorylation and must use ATP derived solely from glycolysis	Decreased CLS	Ocampo *et al.*, 2012
Inhibition of autophagy	Accumulation of protein aggregates	Decreased CLS	Alvers *et al.*, 2009
Histone deacetylase activation	NAD^+ regeneration in mitochondria	Increased RLS, but not CLS Some results are strain-specific	Lin *et al.*, 2002; Jiang *et al.*, 2002
NAD^+ levels	Normal respiratory activity regenerates NAD^+	Increased RLS and CLS	Lin *et al.*, 2002; Anderson *et al.*, 2003a; Anderson *et al.*, 2003b; Tahara *et al.*, 2007

Table 1.2. *(Continues next page.)*

Physiological conditions that affect yeast ageing	Mitochondrial effect	Lifespan effect	References
Respiratory deficiency, petite strains	Lower NAD^+ levels and enhanced ROS production	Reduced CLS Strain-specific effects on RLS	Kirchman *et al.*, 1999; Powell *et al.*, 2000; Lin *et al.*, 2002
Increased respiration	Higher NAD^+, decreased ROS production and oxidized products No overt changes in antioxidant levels	Increased RLS and CLS	Jiang *et al.*, 2000; Lin *et al.*, 2000; Barros *et al.*, 2004; Lorin *et al.*, (2006); Tahara *et al.*, 2007
Glucose restriction	Dependent on respiratory enhancement and changes in NAD redox state	Increased RLS and CLS	Jazwinski, 2000; Jiang *et al.*, 2000; Lin *et al.*, 2000; Barros *et al.*, 2004; Tahara *et al.*, 2007
Amino acid restriction	Activates retrograde signaling	Increased RLS	Jiang *et al.*, 2000
Copper supplementation	May favor cytochrome c oxidase assembly	Increased RLS in respiratory, but not fermentative, media	Osiewacz, 2002; Kirchman & Botta, 2007

Table 1.2. Mitochondrial metabolism, signaling and distribution have been acknowledged as important factors in the regulation of lifespan, either from a replicative or chronological paradigm perspective (adapted from Barros *et al.*, 2010).

1.3.2 Altered Autophagy

Autophagy is an evolutionarily conserved cellular mechanism through which cells recycle material and eliminate dysfunctional/damaged cellular components (from molecules to aggregates to organelles), and has been strongly linked to aging: in particular, inhibiting autophagy has degenerative and pro-aging effects, while enhancing autophagy has protective and anti-aging effects (Rubinsztein *et al.*, 2013; López-Otín *et al.* 2013, Knuppertz & Osiewacz, 2016). Further, autophagy is one of the main protein quality control systems and together with the proteosomal-mediated degradation and the chaperone-mediated folding guarantees protein homeostasis, which is altered during aging (Koga, 2011). Three different types of autophagy can be found within a cell: (i) macroautophagy, where portions of cytoplasm are sequestered into double-membrane vesicles and targeted for degradation in the lysosome; (ii) microautophagy, where invaginations at the lysosomal membrane participate in the direct sequestration of proteins and organelles; (iii) chaperone-mediated autophagy (CMA), where single individual soluble proteins are intercepted by a cytosolic chaperone complex and targeted one by one to the lysosome lumen for degradation (Figure 1.4). The collected information will be centred on macroautophagy, the main investigated form of autophagy, and will from herein referred solely as "autophagy". Multiple studies reported that autophagy proteins (ATG) have a reduced expression in aged tissues and that autophagy decreases in an age-dependent manner (Rubinsztein *et al.*, 2013). This decrease was found not just in normal conditions, such as in the normal human brain aging (Lipinski *et al.*, 2010), but also in age-related pathological diseases, such as the neurodegenerative Alzheimer's disease (Decuypere *et al.*, 2011). In agreement, the knockout of essential *ATG* genes in mice is lethal (Levine & Kroemer, 2008), while tissue-specific knockout generates age-associated marks (Rubinsztein *et al.*, 2013). For instance, the central nervous system-specific knockout of *ATG5* or *ATG7* causes progressive accumulation of ubiquitinylated proteins as inclusion bodies in neurons, which enhances neurodegeneration (Hara *et al.*, 2006; Komatsu *et al.*, 2006); the liver- and spleen-specific knockout of *ATG7* causes accumulation of peroxisomes, deformed mitochondria and ubiquitin-positive inclusions in hepatocytes and hepatocytes death, bring-

Figure 1.4 – The main forms of autophagy. With the exception of chaperone-mediated autophagy (CMA), the other forms are conserved also in yeast. Further, additional autophagy mechanisms are now being investigated (Orenstein & Cuervo, 2010).

ing hepatopathy (Komatsu *et al.*, 2010); the islet cell-specific knockout of *ATG7* causes accumulation of ubiquitinated protein aggregates colocalized with p62, mitochondrial swelling, endoplasmic reticulum distension and vacuolar changes which support the degeneration of the islets and impaired glucose tolerance with reduced insulin secretion, overall resulting in insulin-dependent diabetes (Jung *et al.*, 2008; Ebato *et al.*, 2008; Wu *et al.*, 2009). In agreement, loss-of-function of *atg1*, *atg7*, *atg18*, and *bec-1* (orthologue of the mammalian Beclin-1) also decreases the lifespan of *C. elegans* (Tóth *et al.*, 2008), while deficient expression in *Atg1*, *Atg8a*, and *dSesn* (orthologue of the mammalian Sestrins) reduces the lifespan of *D. melanogaster* (Simonsen *et al.*, 2008; Lee *et al.*, 2010). Moreover, an unbiased screen searching for chronological aging factors in *S. cerevisiae* led to the identification of 10 *ATG* mutants with autophagy defects and a decreased CLS (Matecic *et al.*, 2010). On the other hand, enhancing autophagy through genetic or pharmaceutical approaches was found to increase lifespan in different organisms. The *daf-2* (Insulin/IGF-1 receptor) loss-of-function mutant in *C. elegans* had enhanced autophagy and lifespan (Meléndez *et al.*, 2003); transgenic expression of *Atg8a* in the brain of *D. melanogaster* counteracted the age-associated loss of *Atg8a* expression, increasing autophagy in neurons and lifespan (Simonsen *et al.*, 2008); the administration of the well-known TOR inhibitor rapamycin to mice extended lifespan by restoration of chaperone-mediated autophagy and macroautophagy in livers of aged animals (Harrison *et al.*, 2009). In *S. cerevisiae*, autophagy was found to be inversely correlated to histone acetylation: in particular, an increased level of nucleocytoplasmic acetyl-CoA (given by the sum of nucleic and cytosolic acetyl-CoA) leads to hyperacetylation of histones, reduced expression of *ATG* genes, autophagy inhibition, and ultimately a shortened lifespan (Eisenberg *et al.*, 2014). In agreement, spermidine, which acts as a histone acetylase inhibitor, reduces histone acetylation while it upregulates the expression of *ATG* genes, induces autophagy and extends longevity (Eisenberg *et al.*, 2009). Also, TORC1 is activated by amino acids and represses autophagy: in addition to rapamycin treatments, also nutrient deprivation such as nitrogen starvation is able to induce autophagy through TORC1 inactivity and extend lifespan in yeast (Powers *et al.* 2006), *C. elegans* (Kenyon, 2010), *D. melanogaster* (Bjedov *et al.*, 2010), and mice (Harrison *et al.*, 2009). Further, selective

forms of autophagy have been identified to degrade specific targets based on a receptor-recognition process, and among these forms mitophagy is dedicated to mitochondria (Figure 1.4). In *S. cerevisiae*, Atg32 is an essential receptor protein for mitophagy, with orthologues in other organisms, such as BCL2-L-13 in mammals (Murakawa *et al.*, 2015). In both yeast and mammalian cells, the removal of depolarized mitochondria, as the ones with defective oxidative phosphorylation, is an example for the role of selective autophagy as part of the quality control network (Priault *et al.*, 2005; Nowikovsky *et al.*, 2007; Narendra *et al.*, 2008; Twig *et al.*, 2008; Graef & Nunnari, 2011), defining mitophagy as a longevity-promoting process (Richard *et al.*, 2013). All the reported findings suggest a key role for diet and nutrition in the modulation of autophagy. This vision is further supported by dietary restriction (DR), which has been defined as a reduction in food consumption in the absence of malnutrition (Masoro, 2005; Kennedy *et al.*, 2007). DR was found to increase lifespan in many different species, including yeast, worms, flies, and rodents, as well as in a non-human primate, the rhesus macaque, underlyning an evolutionarily conserved longevity effect (Colman *et al.*, 2009; Rubinsztein *et al.*, 2013). Several nutritional and genetic approaches have been used to investigate the factors involved in DR, but a specific, singular mechanism has still to be identified, as it may be more likely that DR can act through different pathways, involving nutrient-sensing mechanisms (amino acids and TOR; glucose and IIS or Ras/cAMP/PKA in yeast) as well as energy availability-sensing mechanisms ruled by AMPK and the Sirtuins (Bitto, *et al.*, 2015). This concept is well depicted in Figure 1.5.

1.3.3 Genome instability and epigenetic alterations

Genome instability

Accumulation of DNA damage is an inevitable consequence of normal aging and a common trait in progeria syndromes (Burtner & Kennedy, 2010; Moskalev *et al.*, 2012). Genome stability is continuously challenged by both extrinsic factors (either physical, chemical, or biological) and intrinsic damages (mutations, chromosomal events, telomere shortening). However, the cell possess a network of specific repair mechanisms and

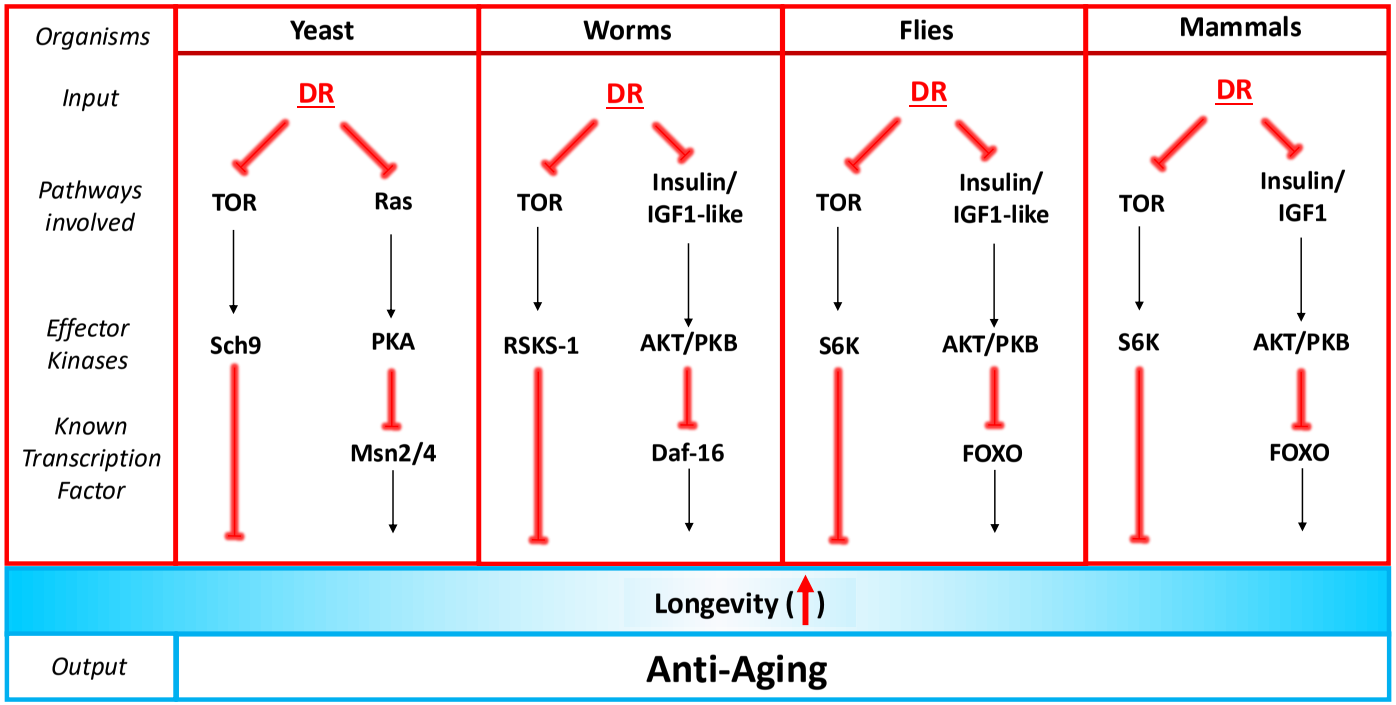

Figure 1.5 – Effects of DR on longevity. DR can act through different pathways, involving nutrient sensing-mechanisms as well as energy-sensing mechanisms (adapted from Longo *et al.* 2005). In particular, the controlled down-regulation of TOR and IIS or Ras/cAMP/PKA in yeast through DR was found to extend longevity, as opposed to the enhancing of anabolic pathways which accelerates aging.

stability systems to ensure the integrity of both nuclear and mitochondrial DNA. All forms of DNA alterations always risk to affect essential genes and/or transcriptional pathways and the resulting dysfunctional cells, if not eliminated by apoptosis, impair cellular and organismal homeostasis. Further, in the case of multicellular organisms with stem cells, this could compromise their ability to regenerate tissues (Jones & Rando, 2011). There is evidence that reduction of DNA damage/mutations increases lifespan and that species differing in DNA damage/mutation load also differ in longevity (Moskalev *et al.*, 2013). Moreover, compromising the DNA repair genes in different model organisms, including *M. musculus*, *C. elegans*, *D. melanogaster* and *S. cerevisiae* has detrimental effects on lifespan (Moskalev *et al.*, 2013). On the other side, efforts have been made in trying to enhance the DNA protecting systems through genetic manipulations, but the data obtained are controversial, and also gave differences that are gender-specific. In a *Drosophila* model, the constitutive overexpression of PARP-1 in the nervous system reduced lifespan in males but enhanced lifespan in females (Shaposhnikov *et al.*, 2011). A reduction of both RLS or CLS in yeast was obtained by mutating *RAD* genes, as well as other DNA repair-related genes (reviewed in Moskalev *et al.*, 2013). Further, pharmacological approaches have been tried, with positive effects on the lifespan of model organisms. For example, the administration of aspirin or rapamycin in mice or dimethyl sulfoxide in *C. elegans* all had positive effects on lifespan (Hsu & Li, 2002; Strong *et al.*, 2008; Harrison *et al.*, 2009; Jia *et al.*, 2010; Wang *et al.*, 2010; Limson & Sweder, 2010; Chen *et al.*, 2011). Efforts were also made in *S. cerevisiae*, where the amidic form of nicotinic acid (NA), nicotinamide (NAM), known to stimulate the repair of DNA, increased both RLS and CLS in yeast (Tsuchiya *et al.*, 2006; *Chapter 3*). Summing up, four criteria can be proposed to evaluate the relationship between DNA damage and aging, as depicted in Figure 1.6. Further research is still necessary to determine to what extent accumulation of DNA damages contributes to the overall aging process and the consequent anti-aging strategy.

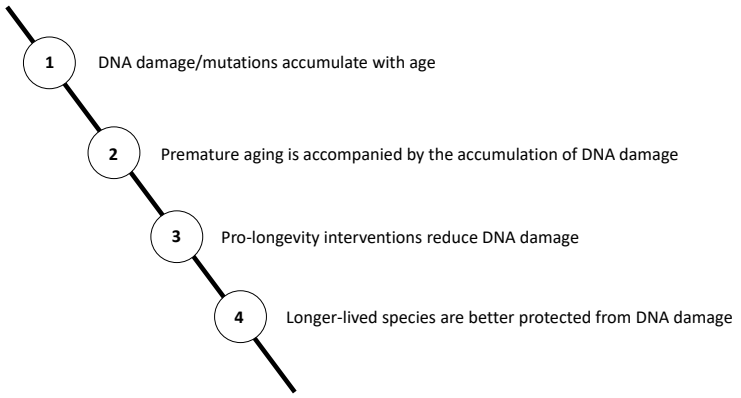

Figure 1.6 – The relation between DNA damage and aging. Four criteria are presented as key points that link DNA damage and DNA repair to aging (adapted from Moskalev *et al.*, 2013).

Epigenetic alterations

Epigenetic changes affect gene expression without mutating the DNA sequence and involve alterations such as reversible DNA methylation and post-translational modification of histones. Epigenetic alterations affect all cells throughout life (Talens *et al.*, 2012) and many age-associated epigenetic marks have been identified. Regarding DNA, methylation of adenine or cysteine can occur, depending on the organism. In mammals, methylation of CpG dinucleotides occurs, producing 5-methylcytosine, a heritable and conserved regulatory mark found in the 5' regulatory regions of many genes and that is generally associated with transcriptional repression. Further, another form of DNA methylation yelding 6-methyladenosine was discovered in *C. elegans*, *D. melanogaster* and mammals (Ratel *et al.*, 2006; Greer *et al.*, 2015; Zhang *et al.*, 2015; Luo *et al.*, 2015; Wu *et al.*, 2016), while recent gas chromatography/mass spectrometry experiments identified cysteine methylation also in different yeast strains, with a genome-wide DNA methylation that is evaluated to be 1 to 2 orders of magnitude lower than that in mammalian cells (Tang *et al.*, 2012). In mammals, the pattern of DNA methylation changes in the genome over time, with a strong proba-

bility that it could have a major role in the progression of aging (Benayoun *et al.* 2015; Zampieri *et al.*, 2015). Age-related DNA hypomethylation has been observed in a variety of species, including mammals, but recent studies have shown that many loci are subject to age-related hypermethylation (Maegawa *et al.*, 2010), and patient with progeroid syndromes recapitulate these findings (Shumaker *et al.*, 2012). Indeed, efforts have been made to accurately predict age on the base of DNA methylation (Horvath *et al.*, 2015). However, no direct experimental demonstration that altering methylation can modulate lifespan has been obtained so far (López-Otín *et al.*, 2013).
Regarding histone post-translational mdifications, age-associated marks such as increase in H4K16 acetylation, decrease in H3K9 methylation and decrease in H3K27 trimethylation have been identified (Fraga & Esteller, 2007; Han & Brunet, 2012). An essential view on the relation between epigenome and aging is represented through key points in Figure 1.7. In general, loss of heterochromatin and deregulation of euchromatin point out to an involvement of alterations in the chromatin remodeling systems. In fact, DNA- or histone-modifying enzymes and associated remodeling factors such as the Polycomb group of proteins or the NuRD complex show age-dependent alternation in both normal and pathological conditions (Pegoraro *et al.*, 2009). One of the first links between chromatin maintenance and aging came from *S. cerevisiae* and pionieristic studies on the NAD^+-dependent deacetylase Sir2 (Figure 1.8), the founding member of Sirtuins, an evolutionarily conserved family of aging regulators (Chang & Guarente, 2014). In yeast, Sir2 is mechanistically involved in maintaining heterochromatin at telomeres, ribosomal DNA (rDNA) and silent mating-type loci (Sinclair & Guarente, 1997). Experiments demonstrated a role in lifespan extension for Sir2 in yeast and for the orthologues of Sir2 in worms and flies (Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004). However, the overexpression of Sirtuin genes gathered perplexity in both yeast and mammals. In fact, the deletion of *SIR2* decreases RLS but increases CLS in yeast (Kaeberlein *et al.*, 1999; Fabrizio *et al.*, 2005), suggesting that two different cellular networks may manage these features of aging. In mammals, seven sirtuins can be found (SIRT1-7), localized in different cellular compartments and involved in regulating metabolism in different tissues in a non-redundant way (Chang & Guarente 2014). Initially,

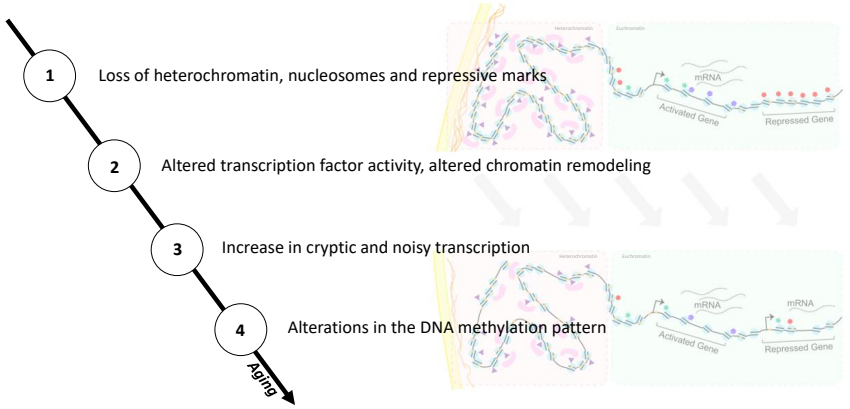

Figure 1.7 – The relation between epigenetic alterations and aging. The four key changes here presented are a strong evidence that epigenetics is involved in the regulation of aging (adapted from Both *et al.*, 2016).

overexpressing SIRT1 in mice failed to increase lifespan (Herranz *et al.*, 2010). Later, following experiments discovered that overexpressing SIRT1 specifically in the dorsomedial and lateral hypothalamic nuclei delayed aging and extended lifespan in both male and female mice (Satoh *et al.*, 2013). This underlines the fact that in mammals studies are complicated by the number of different tissues found, the way they are interconnected and communicate, as well as the different way they age, also in a gender-dependent manner (Herndon *et al.*, 2002; WHO, 2009; Horvath, 2013; Horvath *et al.*, 2015). This further highlights the importance of operating in model systems such as yeast before putting too effort in experimental procedures with more complex organism where so many variables have to be taken into account.

Sirtuin-activating compounds and NAD^+ metabolism

The fact that the activity of Sirtuins could have beneficial effects on lifespan pushed research towards the development of Sirtuin-Activating Compounds (STAC) as possible health-promoting and anti-aging drugs. Two compounds that received great attention are resveratrol and NAD^+. Resver-

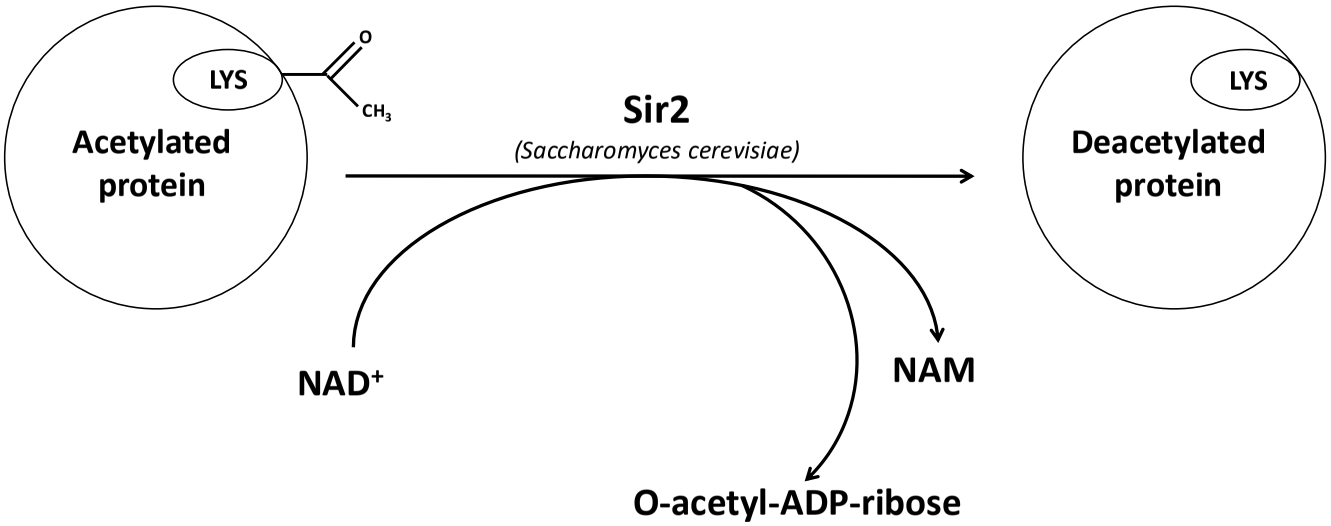

Figure 1.8 – Sirtuins: discovery and mechanism of action. Sirtuins are a highly conserved group of proteins that belongs to the class III NAD^+-dependent deacetylase family. Sirtuins are found in eukaryotes ranging from yeast to humans and are known to be evolutionarily conserved aging regulators. The founding member of the Sirtuin family is Sir2, identified in *S. cerevisiae*. Sir2, like other Sirtuins, uses NAD^+ as a (co)substrate, coupling the deacetylation of lysins on target proteins to the cleavage of NAD^+ into O-acetyl-ADP-ribose and nicotinamide (NAM). Sir2 was found to be involved in both replicative (Kaeberlein *et al.*, 1999) and chronological aging (Fabrizio *et al.*, 2005), favouring RLS and reducing CLS, respectively. Lysine, LYS.

atrol is a naturally available phenol found in the skin of grapes and as a consequence in wine, as well as in peanuts and cocoa powder (Burns *et al.*, 2002; Hurst *et al.*, 2008). Evidence from structural and enzymology studies indicated that resveratrol functions by binding a N-terminal STAC-binding domain (SBD), a key mediator of allosteric activation in Sirtuins (Dai *et al.*, 2015). Many studies have shown that resveratrol can extend lifespan in yeast, worms, flies, fishes and honeybees (Howitz *et al.*, 2003; Jarolim *et al.*, 2004; Wood *et al.*, 2004; Bauer *et al.*; Viswanathan *et al.*, 2005; Yu & Li, 2012; Rascón *et al.*, 2012; Liu *et al.*, 2015; Bonkowski & Sinclair, 2016). Clinical studies are now evaluating the effects of resveratrol in humans. Results were positive, finding that it improves metabolism, protects from cardiovascular dieases and delays cognitive decline (Timmers *et al.*, 2011; Wong *et al.*, 2011; Bhatt *et al.*, 2012; Crandall *et al.*, 2012; Magyar *et al.*, 2012; Poulsen *et al.*, 2013; Turner *et al.*, 2015). NAD^+, on the other hand, is a product of cellular metabolism: it can be generated *de novo* through the kynurenine pathway starting from tryptophan, or it can be obtained through salvage pathways from NA, NAM or nicotinic riboside (NR) (Kato & Lin, 2014). A description of NAD^+ biosynthetic pathways is reported in Figure 1.9. Sirtuins use NAD^+ as a (co)substrate, coupling the deacetylation of proteins with the cleavage of NAD^+ into O-acetyl-ADP-ribose and NAM, so NAD^+ precursors have gained attention with the purpose to modulate Sirtuin activity, hence influence cellular aging. In addition, in humans and mice NAD^+ level declines with age, so NAD^+-boosting molecules are also getting attention as a way to restore NAD^+ levels. For instance, precursors such as NR or nicotinamide mononucleotide (NMN) were found to improve glucose metabolism and insulin sensitivity, decrease inflammation, enhance mitochondrial functions and extende longevity (Ramsey *et al.*, 2008, Yoshino *et al.*, 2011, Cantó *et al.*, 2012, Gomes *et al.*, 2013, Karamanlidis *et al.*, 2013, Imai & Guarente, 2014, Zhang *et al.*, 2016). Further, several studies have demonstrated that NAM is an endogenous inhibitor of Sirtuins. In fact, NAM is able to modulate Sir2 activity in yeast cells in a way that recalls the deletion of *SIR2*, ultimately promoting a resemblance in RLS (Bitterman *et al.* 2002; Anderson *et al.* 2003a; Gallo *et al.* 2004; Sauve *et al.* 2005). On the other hand, effects of NAD^+ precursors on CLS have not been studied in detail. Therefore, on this basis, we set out to determine if NAM supplementation could cause any effects on CLS and whether these

effects could be Sir2-mediated. Results will be further discussed in *Chapter 3*.

In summary, while molecular mechanisms leading to chromatin alterations in aging are largely unknown, ways to enhance the quality of health can come from enzymes involved in post-translational modifications, as shown for Sirtuins. In fact, the incredible finding that naturally available compounds are potent epigenetic modulators upgrades dietetic strategies and nutritional approaches as promising ways to treat ageing and age-related diseases. Moreover, the above-mentioned dependency of Sirtuins from NAD^+ ascribes this class of deacetylases to the metabolic sensors. In fact, NAD^+ is both a metabolic cofactor and a transporter of electrons to the respiration chain, thus establishing a molecular link between carbon metabolism and energy status that can in turn be managed through the action of Sirtuins (Chang & Guarente, 2014). The impact of Sirtuins on metabolism is further underlined by the fact that these deacetylases also have non-histone targets. Many have been discovered in mammals (reviewed in Martínez-Redondo & Vaquero, 2015), while in yeast Sir2 was found to deacetylate and promote the inactivation of Pck1, the rate-limiting enzyme of gluconeogenesis (Lin *et al.*, 2009, Casatta *et al.*, 2013). This was shown to have a strong impact on longevity, with *sir2*Δ cells characterized by an increase in the rate of gluconeogenesis positively correlated with trehalose storage, ultimately promoting an extension of CLS (Casatta *et al.*, 2013, *Chapter 3*). This is in agreement with the fact that gluconeogenesis yields glucose-6-phosphate, known to support the production of trehalose, and with the increasing evidence that trehalose is a key carbohydrate involved in the survival during chronological aging. This feature will be further explained in detail in the following section, specifically dedicated to the relation between carbon metabolism and aging.

1.3.4 Deregulated Management of Nutrient Metabolism

As reported previously, a constitutively decreased activation of the nutrient-sensing pathways (TOR and IIS or Ras/cAMP in yeast) obtained through the control exerted by DR has anti-aging effects (Figure 1.5), whereas on

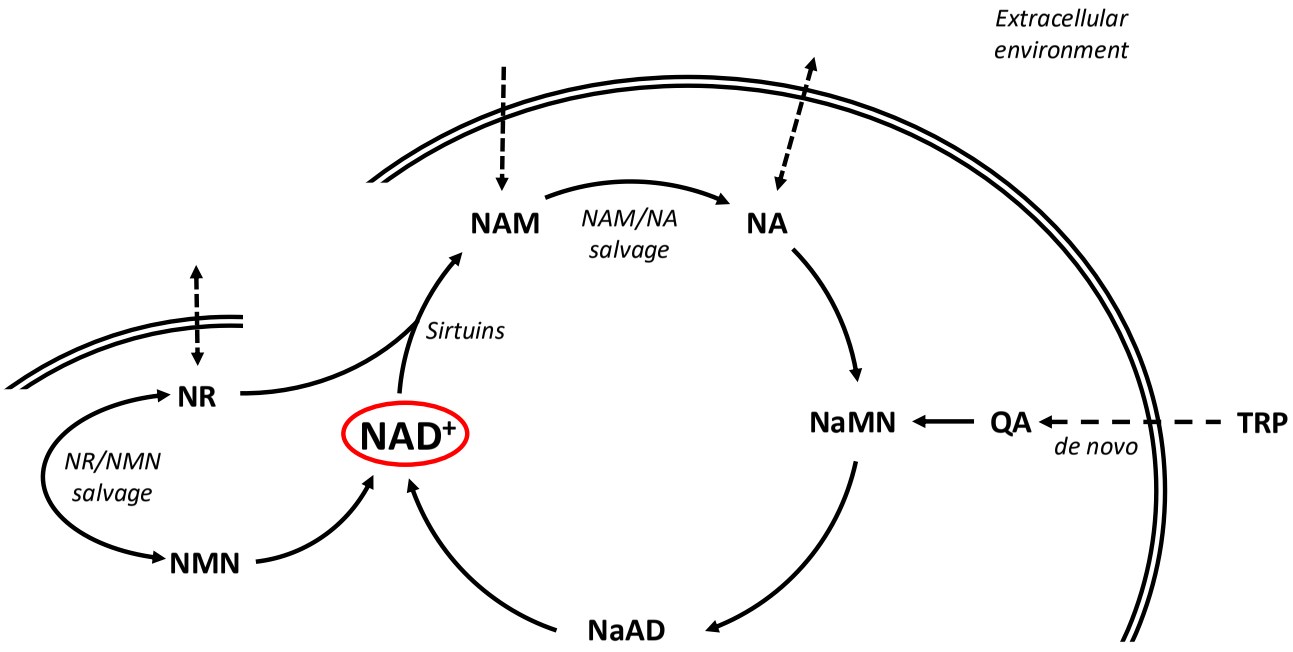

Figure 1.9 – Overview on NAD$^+$ metabolism. NAD$^+$ is a molecular junction between metabolism and energetic status, therefore many pathways participate in order to always maintain the NAD$^+$/NADH pool according to functional needs. The *de novo* or kynurenine pathway produces NAD$^+$ starting from the amino acid tryptophan. On the other hand, two different but interconnected salvage pathways are found, based on the precursors NAM/NA or NR/NMN. Evidence has accumulated regarding the positive effects of these compounds on both healthspan and lifespan. Tryptophan, TRP; quinolinic acid, QA; nicotinic acid, NA; nicotinamide, NAM; nicotinamide riboside, NR; nicotinamide mononucelotide, NMN; nicotinic acid mononucleotide, NaMN; deamido-NAD$^+$, NaAD.

the other hand anabolic signalings accelerate aging. In this scene, carbon sources represent a major factor as they participate not only in the activation of nutrient-sensing pathways but also in the furniture of strategic metabolic compounds involved in biosynthesis and energy metabolism. An imbalance in the usage of carbon metabolites represents a risk, and an impaired carbon metabolism is at the basis of not only many non-communicable metabolic diseases, such as diabetes, obesity and dyslipidemias, but also aging (Fontana *et al.*, 2010; López-Otín *et al.*, 2013). With respect to the complexity of multicellular organisms and despite evident differences, *S. cerevisiae* is being currently used to uncover new aspects of metabolic pathways in relation to aging, and by comparing different model systems that bear evolutionary conserved components, resemblance can be found on cellular scales in order to suggest innovative therapeutic interventions. Carbon metabolism in *S. cerevisiae* will now be introduced, followed by a deep focus on the key precursor acetyl-CoA as central carbon metabolite of this work.

Carbon metabolism and aging

In the microbial world, metabolic pathways are represented in all their diversity. In particular, the yeast *S. cerevisiae* has two principal metabolic routes, namely glycolytic and respiratory, which are also present in mammalian cells. Further, in yeast there is a strict preference hierarchy for metabolites, in agreement to their role in cellular maintenance and survival. In such a way, nutritional sources are able to regulate, through molecular inductive and repressive mechanisms, genetic expressions and enzymatic activities related to different metabolic pathways. This controlling system is extremely convenient when using model organisms, as it enables to selectively activate or represses pathways that are of interest, dissecting the system and ultimately determining the impact of particular nutrients and their related metabolism on aging and aging-related diseases. For instance, in *S. cerevisiae*, when glucose is available in high concentrations, it is preferentially used over other carbon sources. This phenomenon, defined as glucose repression, is achieved in a Mig1-dependent manner by the transcriptional repression of genes involved in the use of alternative

carbon sources such as those involved in gluconoegenesis, the glyoxylate cycle, the TCA cycle or the respiratory chain (Figure 1.10) (Gancedo, 1998; Carlson, 1999; Kayikci & Nielsen, 2015). Glycolysis can take place in both aerobic and anaerobic conditions, for the conversion of hexose phosphates in pyruvate. This latter is a strategic metabolite that can in turn have three different fates (Figure 1.11 and Pronk *et al.*, 1996). First, pyruvate can enter mitochondria though the mitochondrial pyruvate carrier (MPC) (Bricker *et al.*, 2012; Herzig *et al.*, 2012), a specific heteromeric carrier which is situated in the inner mitochondrial membrane. Once in the matrix, then the direct oxidative decarboxylation of pyruvate to acetyl-CoA takes place, catalysed by the pyruvate dehydrogenase complex. On the other hand, in the cytosol pyruvate can be decarboxylated to acetaldehyde by the pyruvate decarboxylase or carboxylated to oxaloacetate by the pyruvate carboxylase. Moreover, *S. cerevisiae* is a Crabtree-positive yeast, meaning that at high glucose concentrations fermentation occurs even in presence of oxygen (Barnett & Entian, 2005; Rodrigues *et al.*, 2006). As a consequence, alcoholic fermentation occurs, with pyruvate being massively decarboxylated to acethaldehyde, which in turn is reduced to ethanol by the alcohol dehydrogenase Adh1 (Figure 1.11). In this process, a small part of the produced acetahaldehyde is also converted to acetate by aldehyde dehydrogenases. As glucose is consumed, the non-fermentative metabolism is derepressed, and other carbon sources can be used, such as the ethanol and acetate previously produced as byproducts during fermentation. This phenomenon is called "diauxic shift", and can be viewed as a metabolic switch through which cells adapt to the new nutritional conditions by going through a massive transcriptional reprogramming thanks to the relief from the mechanism of glucose repression. Even if is true that a certain low level of respiration is always detectable in aerobic conditions during fermentation, it can however be observed that after the diauxic shift respiration noticeably increases (Ocampo *et al.*, 2012; *Chapter 2*). During the post-diauxic phase, the so called "reserve carbohydrates" are biosynthetized starting from glucose and are accumulated in yeast cells (Figure 1.12). This reserves are represented by trehalose, a non-reducing disaccharide composed of two α(1,1)-linked glucose molecules, and glycogen, a high molecular mass branched polysaccharide of linear α(1,4)-glucosyl chains with α(1,6)-linkages. During prolonged quiescence,

Figure 1.10 – Schematic description of glucose repression. The Snf1 heterotrimeric complex is highly conserved in eukaryotes and bears a kinase activity (due to the Snf1 subunit). When yeast cells become limited for glucose, the Snf1 kinase complex is active and phosphorylates Mig1, which in turn is exported from the nucleus, supporting the relief from glucose repression and allowing the expression of genes such as those involved in respiration and the utilization of alternative carbon sources. On the other hand, when glucose levels are high, Snf1 kinase complex is inactive due to autoinhibition promoted by the interaction of the N-terminal catalytic domain with the C-terminal regulatory domain of Snf1. In addition, dephoshorylation of Snf1 also occurs by Reg1/Glc7 activity, leading to inactivation of Snf1, fostering the Mig1-dependent glucose repression.

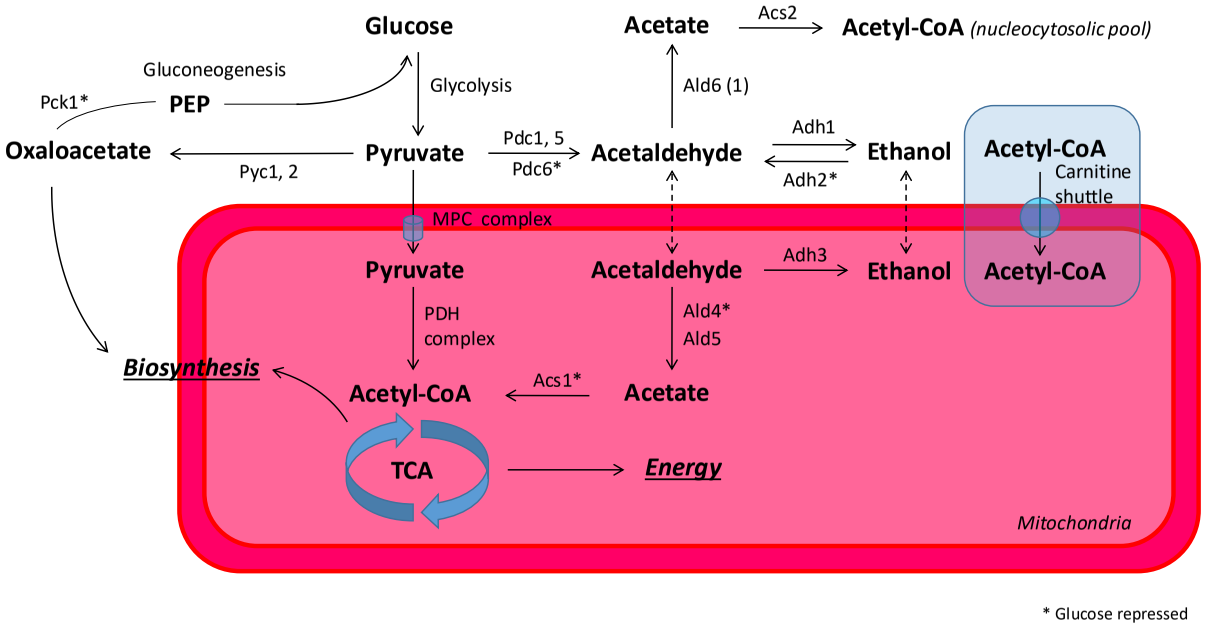

Figure 1.11 – Repartition of pyruvate. Pyruvate can be decarboxylated by pyruvate decarboxylase (Pdc1, 5, 6) and routed to a pathway where acetyl-CoA synthetase 2 (Acs2) replenishes the nucleocytosolic acetyl-CoA pool. This acetyl-CoA pool is used in post-translational modifications as a donor of acetyl groups that ultimately influence enzyme activity and chromatin structure. Noteworthy, however, pyruvate in the cytosol can also be carboxylated into oxaloacetate by pyruvate carboxylase (Pyc1, 2), supporting cellular biosynthetic needs. Further, pyruvate can enter the mitochondria through the MPC. Here, pyruvate is involved in the production of mitochondrial acetyl-CoA through oxidative decarboxylation catalysed by the pyruvate dehydrogenase complex (PDH). To a lasser extent, mitochondrial acetyl-CoA can also be produced from acetate by the CoA transferase activity of acetyl-CoA hydrolase (Ach1) or actively by acetyl-CoA synthetase 1 (Acs1). The mitochondrial acetyl-CoA pool is particularly important since it fuels the TCA cycle and consequently energetic metabolism and biosynthetic pathways. To make the carnitine shuttle functional, carnitine has to be administrated (see *Chapter 2* for details).

trehalose stores are preferentially maintained over glycogen ones, and evidence suggests that trehalose may have both stress-protectant functions and fueling properties to support the reentry in the cell cycle when resuming growth after quiescence (Shi *et al.*, 2010; Ocampo *et al.*, 2012; Kyryakov *et al.*, 2012; reviewed in Eleutherio *et al.*, 2015). The same was also found to be true for triacylglycerols (TAGs) (Kurat *et al.*, 2006, Zanghellini *et al.*, 2008, Petschnigg *et al.*, 2009): this neutral lipids are biosynthetized starting from the esterification of fatty acids with glycerol and stored in lipid droplets, evolutionarily conserved single-membrane organelles that function as lipid stores (Figure 1.12) (Mak, 2012; van Zutphen *et al.*, 2014). Herein, TAGs represent either a source to obtain raw material to build membranes or a source of acetyl-CoA units. Further, esterified fatty acids are removed by lipolysis and used in the process of resuming growth (Kurat *et al.*, 2006; Zanghellini *et al.*, 2008). In agreement, mutants lacking lipid droplets exhibit strong growth defects (Petschnigg *et al.*, 2009), confirming a fundamental role of TAGs as a resource for generating new cells. This is extremely important in the context of the yeast chronological aging, since as reported previously the experimental process to determine cellular lifespan is based on the capacity of yeast cells to resume growth when placed again in presence of nutrients, as synonym of the fitness to maintain molecular and cellular stability over time.

Carbon metabolism and acetyl-CoA - Scope of the thesis

Carbon metabolism intermediates are known to be fundamental precursors in the biosythesis of carbohydrates and lipids, whose metabolism is being increasingly acknowledged as a relevant lifespan regulator. Therefore, it is of interest to deepen the relations between metabolic pathways involved in the production of these compounds and how fluxes of single key metabolites impact on the cellular survival. In this context, a particularly interesting metabolite is acetyl-CoA, the activated form of acetate due to a thioester bond with coenzyme A. Acetyl-CoA is indeed important for many cellular events, depending on its cellular localization (Pietrocola et al., 2015). For instance, mitochondrial acetyl-CoA, generated from the uptake of pyruvate in the mitochondria as previously reported, enters

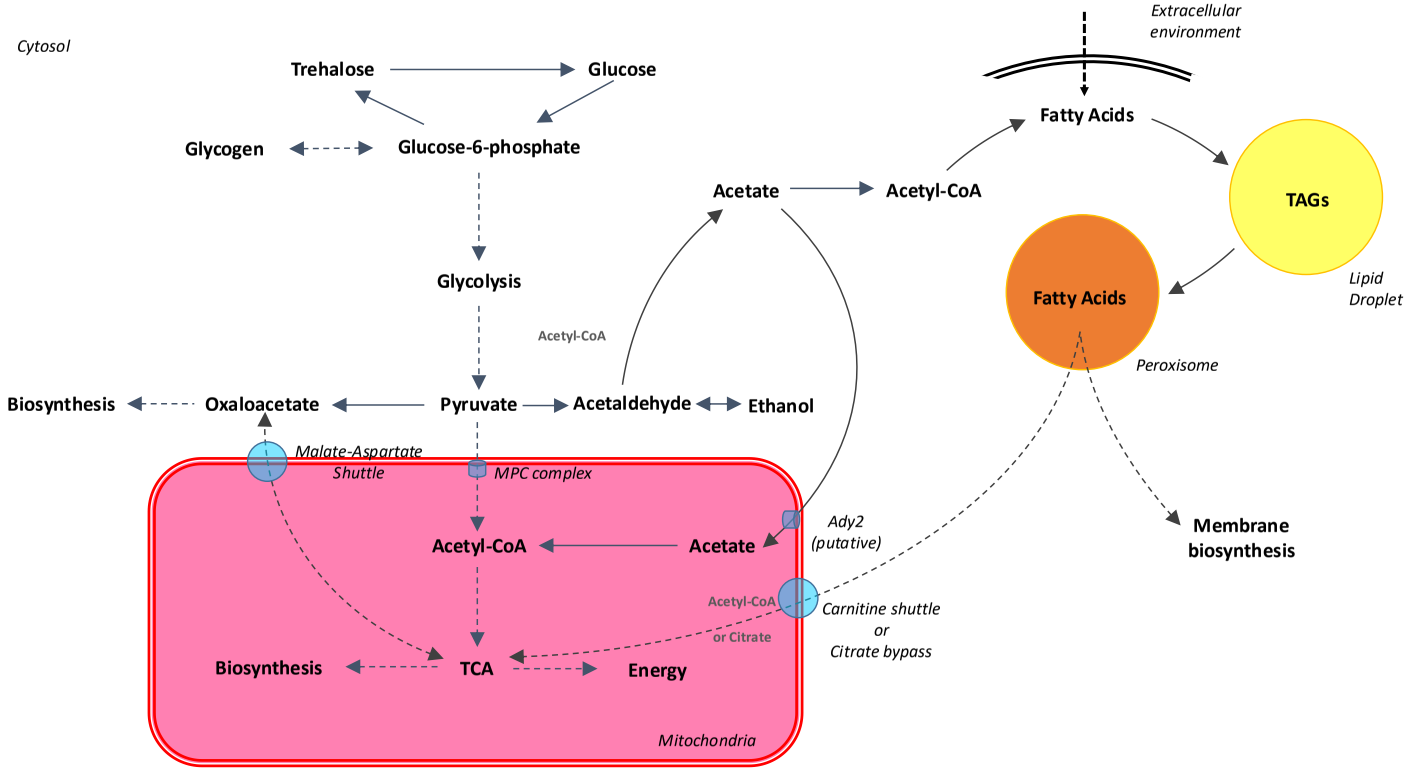

Figure 1.12 – Overview on storage metabolism. Reserve carbohydrates, namely glycogen and trehalose, are obtained starting from glucose and have a key role in quiescence and survival. While glycogen is consumed during fasting periods, trehalose has a main role in stress protection and resuming growth. TAGs are also storage molecules and through lipolysis release fatty acids, used in turn as raw material to build membranes or as a source of acetyl-CoA units. To make the carnitine shuttle functional, carnitine has to be administrated (see *Chapter 2* for details).

the TCA cycle and fosters both biosynthesis and energy metabolism. The other great distinct pool, represented by the nucleocytoplasmic acetyl-CoA, provides instead the fundamental bricks for the biosynthesis of macromolecules, including lipids such as fatty acids which in turn, if processed by β-oxidation, can provide again acetyl-CoA. In addition, the available nucleocytosolic pool is also used as a source of acetyl-groups for acetylation of target proteins. This post-translational modification is catalysed by acetyltransferases, such as those that acetylate the ϵ-$NH_3{}^+$ group of lysine residues on target proteins. This modification is reversible and extremely dynamic since it is indispensable to modulate enzyme activities and chromatin state, therefore the functional state of a cell, as well underlined by the example of Sirtuins reported above.

Proceeding through steps, three key points become relevant in the context of acetyl-CoA, metabolism and aging:

1. the regulation of the flux of acetyl-CoA precursors between cytosol and mitochondria;
2. the control of metabolism through (de)acetylation;
3. the role of fatty acid metabolism, intimately linked to acetyl-CoA.

A clear picture of acetyl-CoA and its destiny in yeast is depicted in Figure 1.13. The key points 1, 2 and 3 have been analysed employing the model organism *S. cerevisiae* and the results are reported throughout this thesis, with single chapters (respectively *Chapter 2*, *3* and *4*) specifically dedicated to every investigation. *S. cerevisiae* was chosen for two main reasons. First, as previously reported, to uncover quickly but efficiently novel cellular mechanism in an evolutionarily conserved context, in order to obtain, from this model, data that has the potential to be shifted to more complex eukaryote organisms. Second, yeast, as a single-celled eukaryote, offers a very broad and flexible cellular context, which in turn already resembles or can be manipulated in order to mimic specific traits of mammalian cells, including those acquired through cellular differentiation, thus further increasing the value of yeast as a model organism.

A brief outline of the chapters will now follow:

- *Chapter 1* presents an overview of the current findings and perspectives in matter of aging. In particular, four key *hallmarks of aging* (mitochondrial dysfunction, deregulation of autophagy, DNA and epigenetic alteration, metabolism deregulation) are identified as evolutionarily conserved and discussed according to recent data regarding the main model organisms and humans. Further, acetyl-CoA is presented as a key metabolite during aging and the thesis research topics are introduced.

- *Chapter 2* focuses on the flux of a strategic carbon metabolite, pyruvate, the end-product of glycolysis, as the main source through which mitochondria obtain acetyl-CoA. The characterization of the yeast *mpc1*Δ mutant for the MPC complex, essential for pyruvate uptake in the mitochondrial matrix, will be discussed. Yeast mutants lacking the Mpc1 subunit showed severe defects in pyruvate uptake, an impaired pyruvate metabolism, a strong rewiring of the acetate metabolism and a short-lived phenotype. Surprisingly yeast mutant cells recovered through the administration of carnitine, a transporter of acetyl groups between cellular compartments. The importance of maintaining this connection among compartments during aging is directly connected to the capacity of maintaining a good mitochondrial metabolic status and avoid pro-aging outcomes, such as the increase in the production and excretion of organic acids (Ross *et al.*, 2010) and mitochondrial dysfunctions, including respiration issues and ROS production.

- *Chapter 3* focuses on acetylation as a post-translational modification to modulate metabolism. The analysis will present the consequences of the disruption of *SIR2*, which codes for Sir2, the founding member of Sirtuins, acknowledged evolutionarily conserved aging regulators. In particular, the study will develop a metabolic perspective considered that Sir2 deacetylates Pck1, the enzyme that controls the rate-limiting step of gluconeogenesis. A strong relation between Sir2 and mitochondria will be underlined. Deleting *SIR2* enhanced the accumulation of storage nutrients and increased the efficiency of

mitochondrial metabolism and mitochondrial maintenance, overall resulting in a pro-survival effect.

- *Chapter 4* focuses on fatty acid metabolism, a process closely related to acetyl-CoA, how it is managed by the cell and how it is connected with aging, representing both a risk and a resource. The chapter will discuss the characterization of the mutant lacking the gene *OAF1*, which codes for a transcriptional factor that regulates the expression of genes involved in β-oxidation and the biogenesis and proliferation of peroxisomes. The importance of this study is also highlighted by the fact that Oaf1 was recently uncovered as a functional homologue of the mammalian PPARs (Phelps *et al.*, 2006). Data presented will show the impact of an impaired fatty acid metabolism, and the similarity between *oaf1*Δ mutants and white obese adipocytes will be underlined by numerous parallelism. In addition, results will further confirm the importance of mitochondrial metabolism during aging, in particular how mitochondria are sensitive to the cellular environment and how alterations in the mitochondrial status proceed cellular decay.

- In *Chapter 5*, conclusions will be presented as take-home messages, and future goals and strategies will be discussed.

Taken together, the presented data provides a unique view on acetyl-CoA by focusing on metabolic pathways and tackling different cellular and molecular aspects. In particular: (i) how not just acetyl-CoA itself, but its whole metabolism has a main role during cellular aging; (ii) how acetyl-CoA metabolism is linked to post-translational modifications that influence cellular aging; (iii) how nutritional sources and supplements are powerful modulators of cellular metabolism.

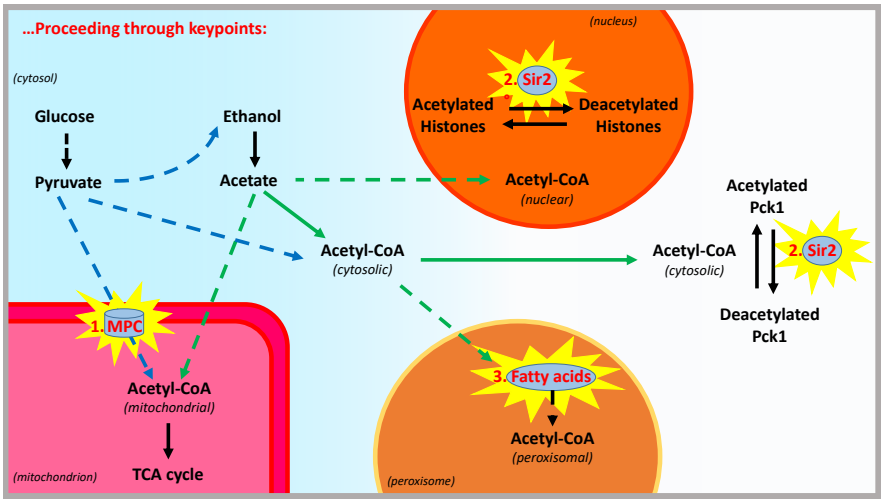

Figure 1.13 – Relation between acetyl-CoA and aging. The relation between acetyl-CoA and aging can be highlighted by three key points, regarding:
1. acetyl-CoA precursors flux regulation between cytosol and mitochondria;
2. acetylation of enzymes to regulate the functional state of a cell;
3. the role of fatty acids, intimately linked to acetyl-CoA.
An in-depth examination of every single key points will be performed in the *Chapters 2*, *3* and *4*, respectively.

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2 Acetyl-Coa Flux Regulation & Aging

Published as:

"Rewiring Yeast Acetate Metabolism Through MPC1 Loss of Function Leads to Mitochondrial Damage and Decreases Chronological Lifespan"

Orlandi Ivan, ***Damiano Pellegrino Coppola*** *& Marina Vai*

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2.1 Abstract

During growth on fermentable substrates, such as glucose, pyruvate, which is the end-product of glycolysis, can be used to generate acetyl-CoA in the cytosol via acetaldehyde and acetate, or in mitochondria by direct oxidative decarboxylation. In the latter case, the mitochondrial pyruvate carrier (MPC) is responsible for pyruvate transport into mitochondrial matrix space. During chronological aging, yeast cells which lack the major structural subunit Mpc1 display a reduced lifespan accompanied by an age-dependent loss of autophagy. Here, we show that the impairment of pyruvate import into mitochondria linked to Mpc1 loss is compensated by a flux redirection of TCA cycle intermediates through the malic enzyme-dependent alternative route. In such a way, the TCA cycle operates in a "branched" fashion to generate pyruvate and is depleted of intermediates. Mutant cells cope with this depletion by increasing the activity of glyoxylate cycle and of the pathway which provides the nucleocytosolic acetyl-CoA. Moreover, cellular respiration decreases and ROS accumulate in the mitochondria which, in turn, undergo severe damage. These acquired traits in concert with the reduced autophagy restrict cell survival of the *mpc1*Δ mutant during chronological aging. Conversely, the activation of the carnitine shuttle by supplying acetyl-CoA to the mitochondria is sufficient to abrogate the short-lived phenotype of the mutant.

2.2 Introduction

Aging of post-mitotic quiescent mammalian cells has been modelled in the yeast *Saccharomyces cerevisiae* by its chronological lifespan (CLS) (MacLean *et al.*, 2001; Longo & Kennedy, 2006). CLS represents the length of time a culture of non-dividing cells remains viable in stationary phase: viability is assessed by the ability to resume growth upon return to rich medium (Fabrizio & Longo, 2003). Evidence to date indicates that chronological aging is intimately regulated by signaling pathways which sense nutrient availability, namely TORC1-Sch9 and Ras-PKA, and carbon metabolism (Fontana *et al.*, 2010; Swinnen *et al.*, 2013). In this context, emerging data on some metabolites and nutrient manipulation/dietary regimens which proved to modulate aging not only in yeast but also in evolutionary diverse organisms have opened up new opportunities for therapeutic interventions promoting healthy aging in humans (Houtkooper *et al.* 2010; de Cabo *et al.* 2014). In particular, among the main metabolic intermediates, acetyl-CoA is increasingly being acknowledged as an important regulator of longevity (Eisenberg *et al.*, 2014a; Friis *et al.* 2014; Mariño *et al.* 2014). This metabolite is the activated form of acetate obtained via a thioester linkage with coenzyme A which cells use for macromolecule biosynthesis. Furthermore, in the mitochondria it is a crucial substrate for energy production since it fuels the TCA cycle and consequently the production of reducing equivalents which enter the electron transport chain and support the oxidative phosphorylation. In addition, acetyl-CoA also supplies the acetyl group for protein acetylation, a dynamic post-translational modification which occurs on a wide range of substrates, including histones and many metabolic enzymes, thus connecting metabolism, epigenetics and transcriptional regulation (Millar & Grunstein, 2006; Henriksen *et al.*, 2012; Lu & Thompson, 2010; Kaelin & McKnight, 2013). Recently, by manipulating the major routes of acetyl-CoA formation in yeast and mammalian cells, it has been shown that the nucleocytosolic pool of acetyl-CoA acts as a dominant suppressor of cytoprotective autophagy during aging (Eisenberg *et al.*, 2014a; Mariño *et al.* 2014). In line with this, during aging, histone hypoacetylation correlates with enhanced expression of *ATG* genes and induction of autophagy (Eisenberg *et al.*, 2009). This is a degradative process which is crucial for the maintenance of cellular homeostasis by remov-

ing misfolded/damaged or "obsolete" proteins and organelles, including mitochondria. It becomes fundamental in non-dividing cells where the intracellular damage cannot be "diluted" (Rubinsztein *et al.*, 2011). Nutrient depletion and inactivations of genes in the central nutrient signaling pathways are known inducers of autophagy (Cuervo, 2008). In *S. cerevisiae*, the nucleocytosolic pool of acetyl-CoA is synthesized by the acetyl-CoA synthetase 2 (Acs2) by activation of acetate in an ATP-dependent reaction. This enzyme is known as the glycolytic isoform (de Jong-Gubbels *et al.*, 1996) and besides its role in carbon metabolism it is required for histone acetylation (Takahashi *et al.*, 2006). The mitochondrial acetyl-CoA pool is generated by the Acs1 (the gluconeogenic isoform) and by the acetyl-CoA hydrolase 1 (Ach1) which catalyzes the transfer of the CoASH moiety from succinyl-CoA to acetate (Fleck & Brock, 2009). Moreover, according to culture conditions, acetyl-CoA can be formed and utilized in different ways. During growth on fermentable substrates, such as glucose, it is generated from pyruvate. This compound is the end-product of glycolysis and is a key node in the branching point between respiratory metabolism and alcoholic fermentation as well as assimilatory and dissimilatory metabolic reactions (Pronk *et al.*, 1996). At the branching point, it can follow three major fates (Figure 2.1): (i) decarboxylation to acetaldehyde which generates acetyl-CoA by the pyruvate dehydrogenase (PDH) bypass; (ii) anaplerotic carboxylation to oxaloacetate and (iii) the direct oxidative decarboxylation to acetyl-CoA by the PDH complex, which is located in the mitochondrial matrix. Pyruvate can cross the outer mitochondrial membrane while the passage across the inner mitochondrial membrane requires the mitochondrial pyruvate carrier (MPC) (Herzig *et al.*, 2010; Bricker *et al.*, 2012). This carrier effectively represents a link between cytosolic pyruvate metabolism and the TCA cycle. Loss of the major structural subunit Mpc1 results in defective mitochondrial pyruvate uptake (Bricker *et al.*, 2012) and, during chronological aging, in a short-lived phenotype accompanied by an age-dependent loss of autophagy (Eisenberg *et al.*, 2014a). In this work we investigated the metabolic changes underlying *MPC1* loss of function. We found that *mpc1*Δ cells make up for their impairment in mitochondrial pyruvate with a metabolic rewiring which involves several intermediates of the mitochondrially localized TCA cycle and the cytosolic glyoxylate shunt but ultimately results in a pro-aging process.

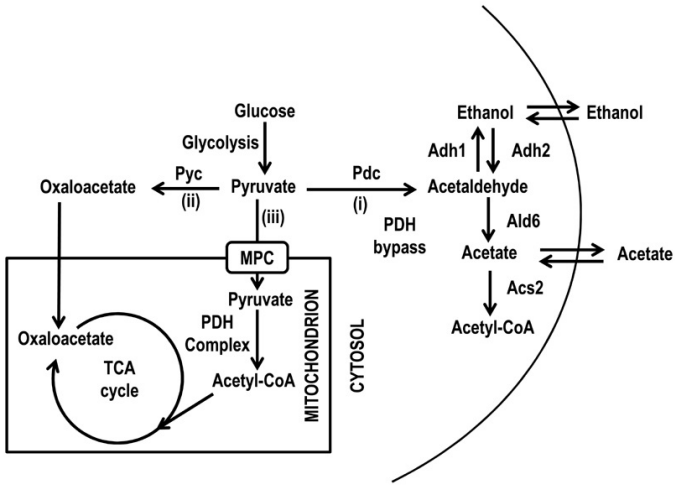


Figure 2.1 – Scheme of metabolic pathways allowing pyruvate utilization. The three pathways which originate from pyruvate after (i) decarboxylation to acetaldehyde, (ii) carboxylation to oxaloacetate and (iii) oxidative decarboxylation to acetyl-CoA are schematically shown. Acs, acetyl-CoA synthase; Adh, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; MPC, mitochondrial pyruvate carrier; Pdc, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; Pyc, pyruvate carboxylase.

2.3 Results & Discussion

Lack of Mpc1 is accompanied by an increase of Ald enzymatic activities. Since an impairment in the import of pyruvate into mitochondria linked to *MPC1* deletion significantly restricted CLS (Figure 2.2A) (Eisenberg *et al.*, 2014a), we decided to analyze in more detail the metabolic changes underlying this short-lived phenotype. Initially, in the context of a standard CLS experiment (Fabrizio & Longo, 2003), we measured the levels of some metabolites such as pyruvate, ethanol and acetate. These last two compounds are produced during glucose fermentation following decarboxylation of cytosolic pyruvate to acetaldehyde by pyruvate decarboxylase (Pdc) (Figure 2.1). Only upon glucose depletion, does the diauxic shift occurs and yeast cells switch to a respiration-based metabolism of the fermentation C2 by-products. Finally, when these carbon/energy sources are fully exhausted, cells enter a quiescent stationary phase. At the diauxic shift, in the *mpc1*Δ culture the amount of ethanol and acetate was similar to that in the wild type (wt) culture (Figure S1, 2.2B and C). Differently, during the post-diauxic phase, in the mutant the consumption of ethanol, which is re-introduced into the metabolism via its oxidation to acetate (Figure 2.1), was not affected significantly compared to that in the wt (specific consumption rate, qEtOH, of 1.43 ± 0.04 mmol•g•DW^{-1}•h^{-1} for the mutant and 1.12 ± 0.06 mmol•g•DW^{-1}•h^{-1} for the wt) (Fig. S1 and 2B), while the acetate continued to accumulate in the medium (Figure 2.2C). Such a prolonged secretion of acetate throughout the ethanol consumption phase suggests that in the *mpc1*Δ mutant there is an imbalance between acetate production rate from acetaldehyde and its conversion rate into acetyl-CoA. In fact, the acetate transport relies on an active transport for the dissociated form of the acid (subjected to glucose repression) accompanied by passive/facilitated diffusion of the undissociated acid (Casal *et al.*, 2008). During the post-diauxic phase the pH of the medium is far below the pK_a of acetic acid (4.75) (Orlandi *et al.*, 2013) and according to the Henderson-Hasselbalch equation, acetic acid is substantially undissociated: 98.6% at pH 2.9 (the value we measured at Day 3 after the diauxic shift). Consequently, in a condition where transmembrane diffusion strongly prevails over the active transport, the acetate export/import will take place according to the gradient between the intracellular and

extracellular concentrations of acetate.

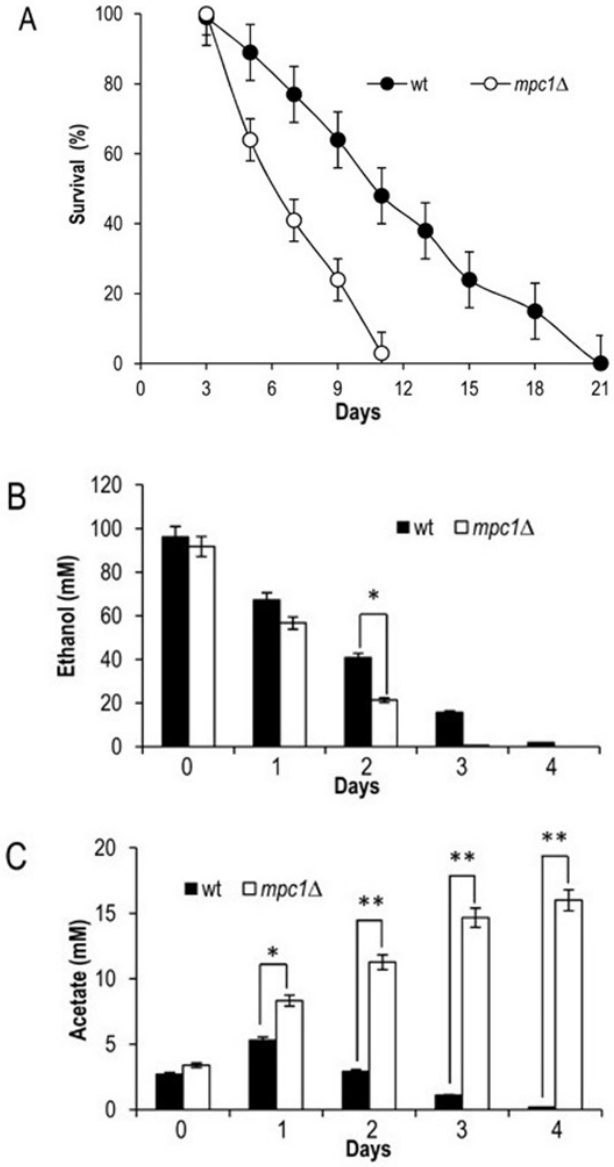


Figure 2.2 – *(Caption next page.)*

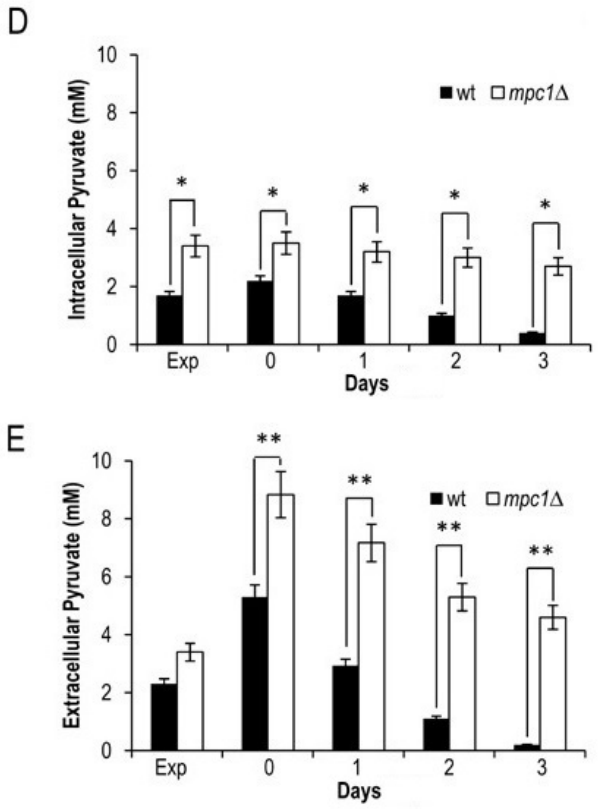


Figure 2.2 – *MPC1* inactivation shortens CLS in concert with increased extracellular acetate and pyruvate. Wild type (wt) and *mpc1*Δ mutant cells were grown in minimal medium/2% glucose and the required supplements in excess (see *Materials & Methods*) and followed up to stationary phase. (A) CLS of wt and *mpc1*Δ mutant cells. At each time-point, survival was determined by colony-forming capacity. 72 h after the diauxic shift (Day 3) was considered the first age-point (see *Materials & Methods*). Day 0, diauxic shift. Data refer to mean values of three independent experiments. Standard deviations (SD) are indicated. Bar charts of extracellular ethanol (B) and acetate (C) concentrations at different time points after the diauxic shift (Day 0). In parallel, intracellular (D) and extracellular (E) pyruvate concentrations were measured. Exp, exponential growth phase. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessedby one-way ANOVA test is indicated (* $P \leq 0.05$ and ** $P \leq 0.01$).

Concerning intracellular pyruvate, its concentration was higher in the *mpc1*Δ mutant compared to that in the wt not only in exponential phase, as already observed by (Bricker *et al.*, 2012), but also at/after the diauxic shift (Figure 2.2D). This was also associated with an increase in the extracellular pyruvate (Figure 2.2E) which reflects an overflow of pyruvate within the cytosol. Similar results were obtained when the experiments were also performed by growing the histidine-prototroph *mpc1*Δ mutant (*mcp1::HIS3*) in a histidine-supplemented medium as previously carried out for the wt (Figure S2) indicating that the different composition of amino acids in the medium does not affect the results. Afterwards, we measured the enzymatic activities of alcohol dehydrogenases (Adhs) catalysing the interconversion of acetaldehyde and ethanol (de Smidt *et al.*, 2008) and of acetaldehyde dehydrogenases (Alds) which produce acetate by oxidizing the acetaldehyde generated from pyruvate during fermentation and that obtained during ethanol oxidation (Figure 2.1). No significant difference was found between the wt and the *mpc1*Δ strain in the Adh activity levels in exponential phase (Figure 2.3A), where the Adh1 isoenzyme is chiefly responsible for ethanol formation from acetaldehyde, consistent with the similar amounts of ethanol detected in both cultures (Figure 2.2B). Similarly, at/after the diauxic-shift where the cytosolic Adh2 is the major ethanol oxidizer, Adh activities displayed no significant difference (Figure 2.3A). On the contrary, during all the growth phases analyzed, Ald activity levels were higher in the mutant compared with the wt (Figure 2.3B). In particular, a great increase was observed for Ald6 which is the major cytosolic isoform and is not glucose-repressed (Saint-Prix *et al.*, 2004) compared with that of the mitochondrial counterparts Ald5 and Ald4 (Figure 2.3C and D) indicating that the mutant exhibits an increased ability to generate acetate, especially the cytosolic one, which can be used as substrate to produce acetyl-CoA. Accordingly, in the mutant, the nucleocytosolic Acs2-mediated pathway is upregulated during chronological aging (Eisenberg *et al.*, 2014a). Moreover, an increased cytosolic acetate pool can also account for the extracellular acetate detected in the *mpc1*Δ culture (Figure 2.2C) whose prolonged accumulation, however, indicates that the flux towards its formation exceeds its utilization. This takes place despite the upregulation of Acs2 enzymatic activity (Eisenberg *et al.*, 2014a) suggesting that the enzyme and/or the flux downstream is/are working at maximum capacity

in line with data which show that increase in Acs activity does not result in enhanced acetate utilization (Jong-Gubbels *et al.*, 1998; Remize *et al.*, 2000).

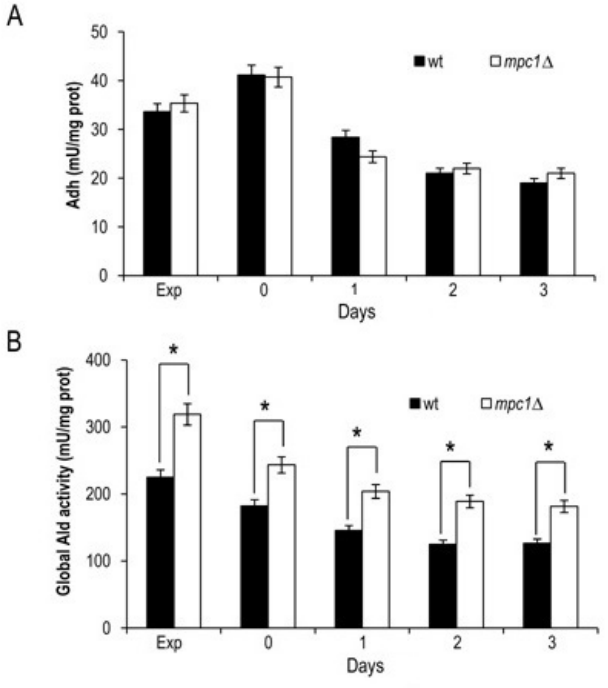


Figure 2.3 – *(Caption next page.)*

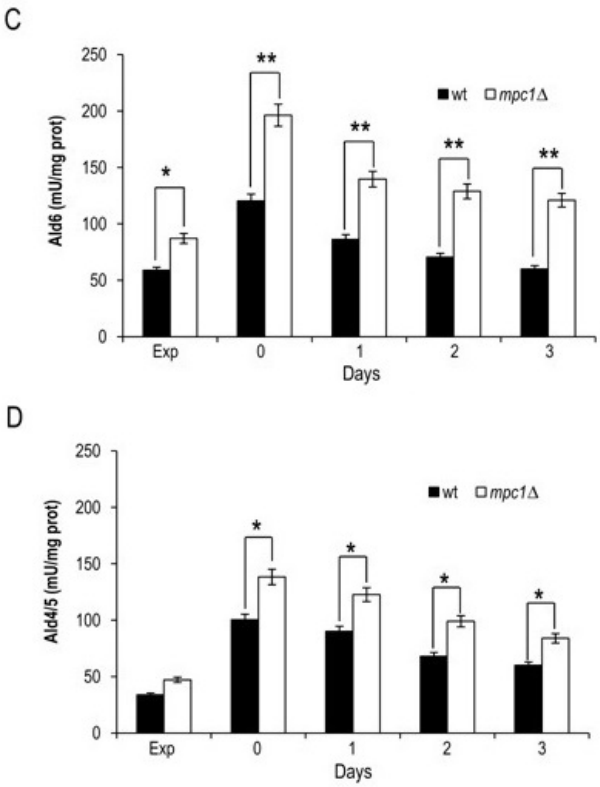
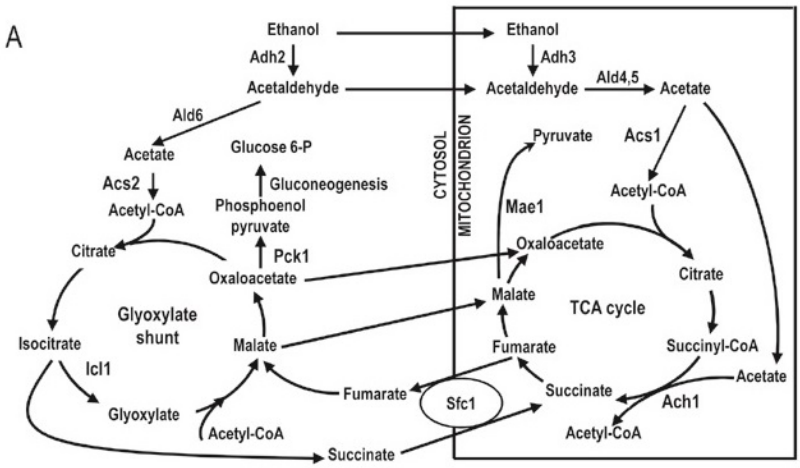


Figure 2.3 – In *mpc1*Δ cells the extracellular abundance of acetate correlates with enhanced Ald enzymatic activity. Bar charts of total Adh (A), total Ald (B), Ald6 (C) and Ald4/5 (D) enzymatic activities measured at the indicated time points for wt and *mpc1*Δ mutant cells grown as in Figure 2.2. Exp, exponential growth phase. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

Lack of Mpc1 is accompanied by an increase of malic enzyme activity and a decrease in respiration. Starting from these results, we focused on the mitochondrially localized TCA cycle which can be fed with acetyl-CoA generated either from acetate or following oxidation of mitochondrial pyruvate. We measured the levels of citrate, succinate and malate which are intermediates of this cycle but also metabolic connections with the glyoxylate shunt. This is an anaplerotic device of the TCA cycle which allows the formation of C4 units from C2 units (acetate) by bypassing oxidative decarboxylation (Figure 2.4A) (Lee *et al.*, 2010). At/after the diauxic shift, a clear global decrease was observed for all three intermediates in the *mpc1*Δ cells compared with the wt counterparts (Figure 2.4B-D). This decrease was particularly marked for malate which can be used to generate pyruvate in the mitochondria for biosynthetic purposes. This reaction of oxidative decarboxylation is catalyzed by the mitochondrial malic enzyme encoded by *MAE1* (Boles *et al.*, 1998). As shown in Figure 2.4E, in the *mpc1*Δ mutant during the post-diauxic phase, the malic enzyme activity was doubled in comparison with the wt, suggesting that the impairment of pyruvate import into mitochondria linked to Mpc1 loss is compensated by a flux redirection through the Mae1-dependent alternative route. This can explain the severe growth defect observed by (Bricker *et al.*, 2012) when the *mpc1*Δ allele was combined with *MAE1* deletion.



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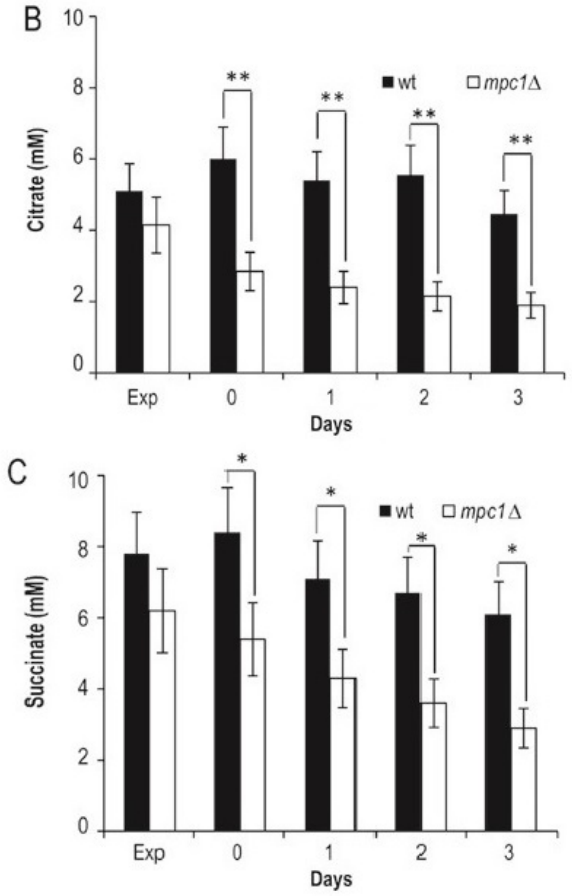

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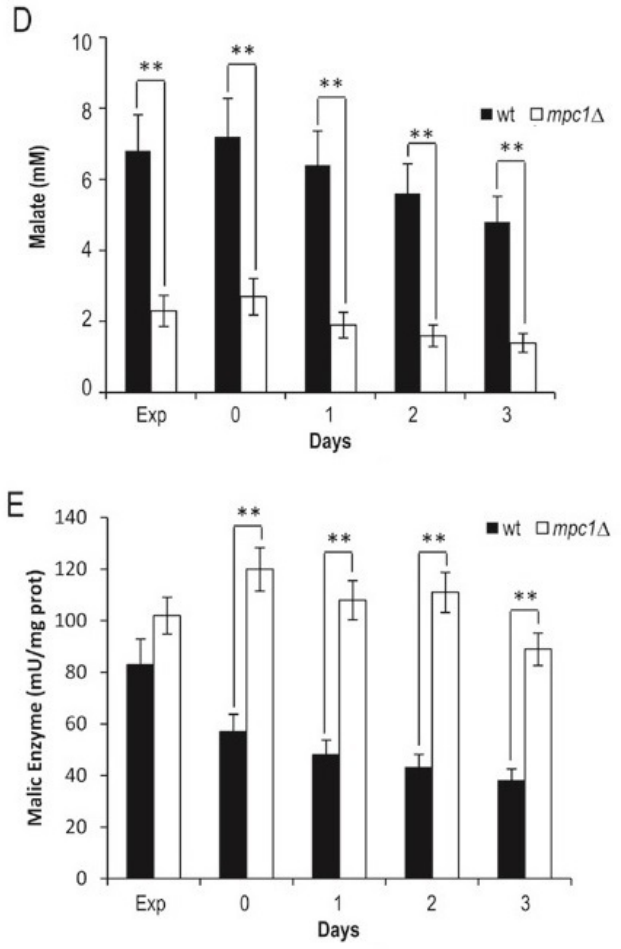


Figure 2.4 – Lack of Mpc1 results in low levels of TCA cycle intermediates and enhanced malic enzyme activity. (A) Scheme of the TCA cycle and of the glyoxylate shunt. Ach1, acetyl-CoA hydrolase 1; Acs, acetyl-CoA synthase; Adh, alcohol dehydrogenase; Ald, aldehyde dehydrogenase; Icl1, isocitrate lyase 1; Mae1, malic enzyme; Pck1, phosphoenolpyruvate carboxykinase 1; Sfc1, succinate-fumarate carrier. Wt and *mpc1*Δ cells were grown as in Figure 2.2 and at the indicated time points the concentrations of citrate (B), succinate (C) and malate (D) were measured. The bar chart of malic enzyme activity (E) is also reported. Exp, exponential growth phase. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

Moreover, when cells switch to a respiration-based metabolism by using ethanol and acetate, the glyoxylate shunt becomes operative and begins replenishing the TCA cycle intermediates. In addition, during growth on C2 compounds, this shunt is the exclusive source of oxaloacetate which is the substrate of phosphoenolpyruvate carboxykinase (Pck1), the key enzyme of gluconeogenesis (dos Santos *et al.*, 2003). Measurements of the enzymatic activities of isocitrate lyase (Icl1), which is one of the unique enzymes of the glyoxylate shunt, and Pck1 indicated that these activities were higher in *mpc1*Δ cells compared with wt ones (Figure 2.5A and B). Concomitantly, in *mpc1*Δ cells cellular respiration decreased (Figure 2.5C). Icl1 is localized in the cytosol and from isocitrate it generates succinate and the name-giving metabolite glyoxylate which condenses with acetyl-CoA yielding malate. The last one can return to the mitochondria (Figure 2.4A). Similarly, the major fate of cytosolic succinate is assumed to be its transfer into mitochondria (Lee *et al.*, 2010). Moreover, its transport by the Sfc1 carrier provides cytosolic fumarate for conversion to malate which can be used for gluconeogenesis (Palmieri *et al.*, 1997). Thus, taken together, these data indicate that in the *mpc1*Δ mutant, an increase in the glyoxylate shunt might represent an increase in metabolite feeding from the cytosol to support a mitochondrial impaired TCA cycle. In this context, the cytosol of the mutant can provide the metabolic environment required to fulfill the increased requirement of substrates for the glyoxylate shunt. In fact, the end-product of the Acs2 synthetase, which is increased in the mutant (Eisenberg *et al.*, 2014a), is the nucleocytosolic acetyl-CoA. In the cytosol, this metabolite, following condensation with oxaloacetate, produces citrate which is then isomerized to isocitrate (the substrate of Icl1). In addition, the cytosol of the mutant might also be a suitable environment which can "promote" Pck1 enzymatic activity. In fact, Pck1 is acetylated by Esa1 and this acetylation is required for its enzymatic activity: an increase of Pck1 enzymatic activity is associated with an increase of the acetylated form of the enzyme (Lin *et al.*, 2009; Casatta *et al.*, 2013). Accumulating evidence indicates that the availability of acetyl-CoA, the donor substrate for acetylation, can be a metabolic input for the acetylation itself (Cai *et al*, 2011; Guan & Xiong, 2011; Choudhary *et al.*, 2014) , so it is reasonable to hypothesize that changes of acetyl-CoA levels may also influence Esa1 activity.

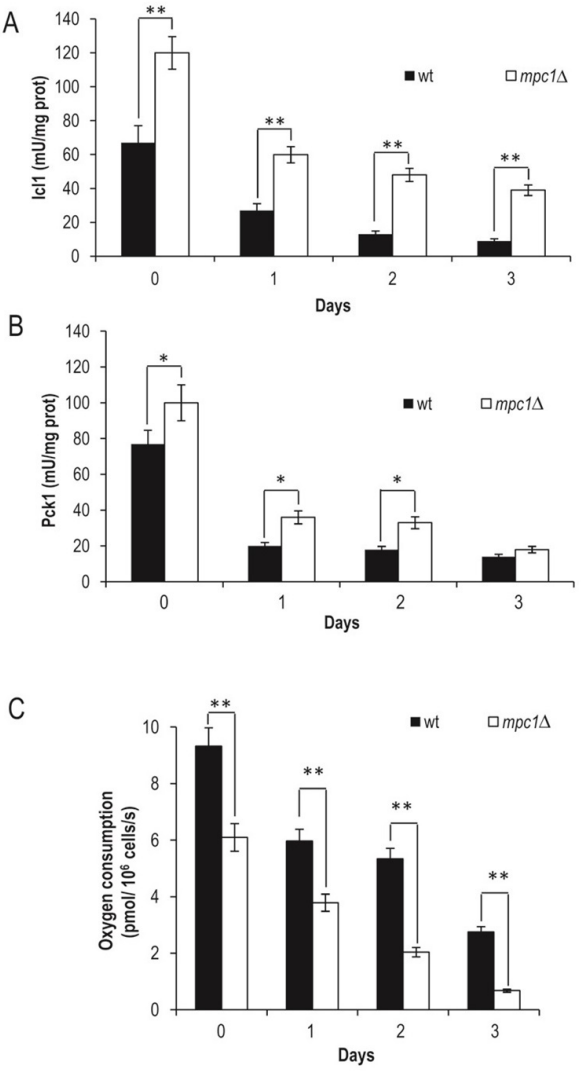
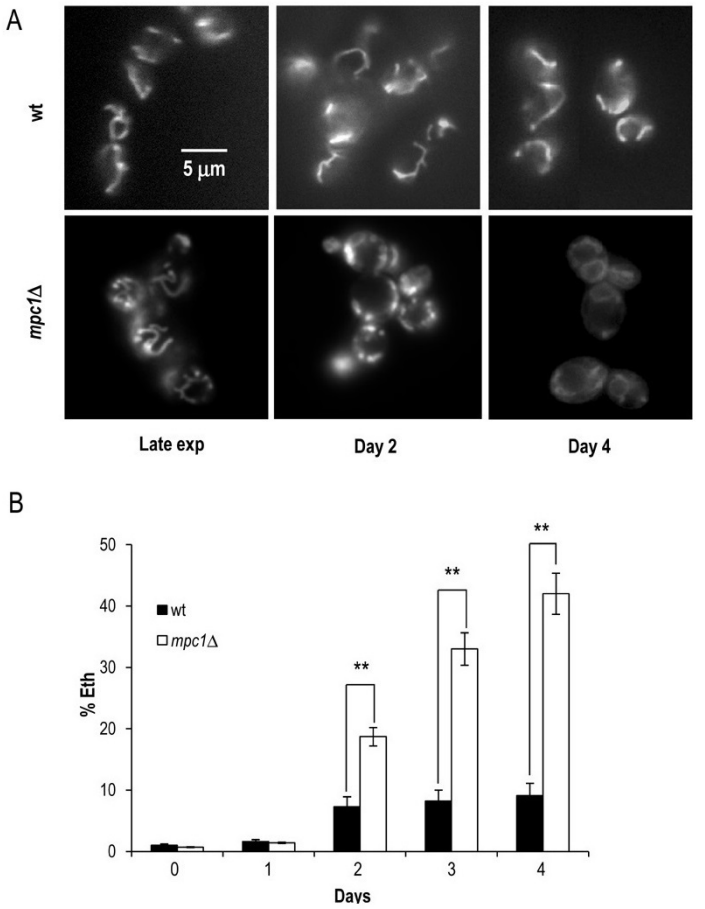


Figure 2.5 – *MPC1* inactivation increases glyoxylate/gluconeogenesis and reduces respiration during chronological aging. At the indicated time points Icl1 (A) and Pck1 (B) enzymatic activities of wt and *mpc1*Δ mutant cells were measured. In parallel, cellular respiration (C) was also monitored. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

Carnitine restores chronological longevity of the mpc1Δ mutant. After the diauxic shift, a metabolic change from fermentation to respiration takes place implying that energy metabolism relies on mitochondrial functionality. Since in the *mpc1*Δ cells we observed a decrease in respiration, we decided to analyze mitochondrial membrane potential and morphology by using the fluorescent dye, 3,3"-dihexyloxacarbocyanine iodide ($DiOC_6$) (Koning *et al.*, 1993). In fact, mitochondrial morphology reflects the functional status of mitochondria and is regulated by the orchestrated balance of two opposing events: fission and fusion of mitochondria (Knorre *et al.*, 2013). As shown in Figure 2.6A, a typical tubular network was observed for wt cells whereas for the mutant fluorescent punctiform structures appeared at Day 2 after the diauxic shift. These structures are indicative of mitochondrial fragmentation and are linked to an elevated activity of the mitochondrial fission machinery (Merz *et al.*, 2007). In addition to an altered morphology, the mitochondria of the mutant displayed a time-dependent reduction in membrane potential and, at Day 4, did not accumulate $DiOC_6$ (Figure 2.6A). Mitochondrial dysfunctions are intrinsically related to reactive oxygen species (ROS) of which superoxide anion is one of the most potentially harmful. This radical derives mainly from leakage of electrons from the respiratory chain and, among others, can target mitochondria with detrimental effects (Breitenbach *et al.*, 2014; Demir *et al.*, 2010). Chronologically aging *mpc1*Δ cells had a higher ROS content, measured as the superoxide-driven conversion of non-fluorescent dihydroethidium (DHE) into fluorescent ethidium (Eth), compared with that of the wt cells (Figure 2.6B). Notably, culturing *mpc1*Δ cells in a carnitine-supplemented medium was sufficient to avoid this phenomenon (Figure 2.6C). Moreover, oxygen consumption measurements indicated that in these cells cellular respiration increased (Figure 2.6D).



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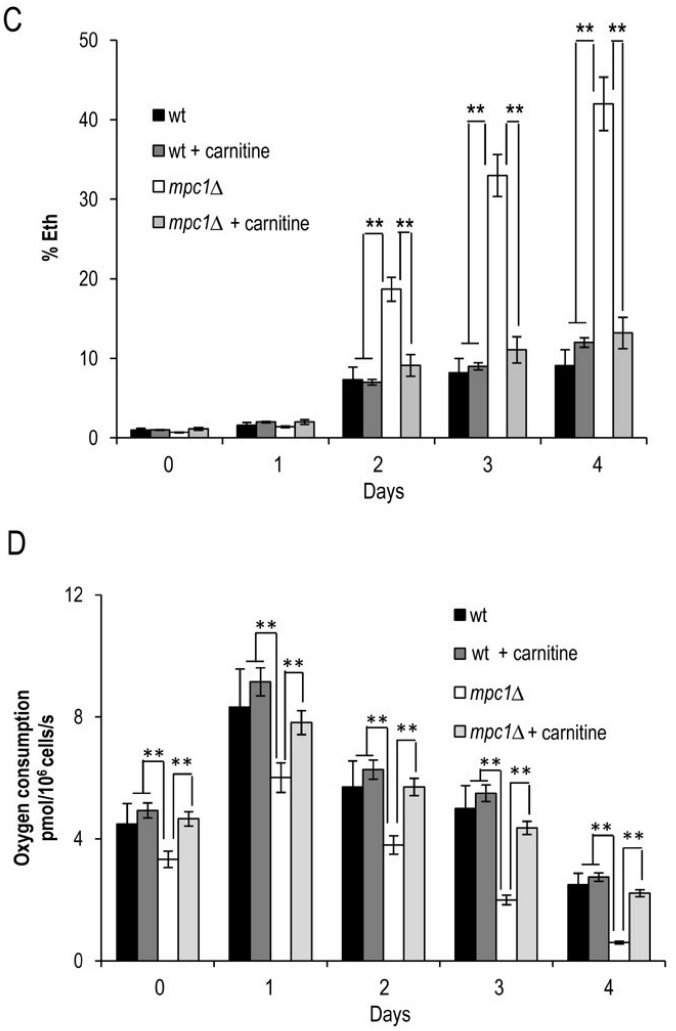
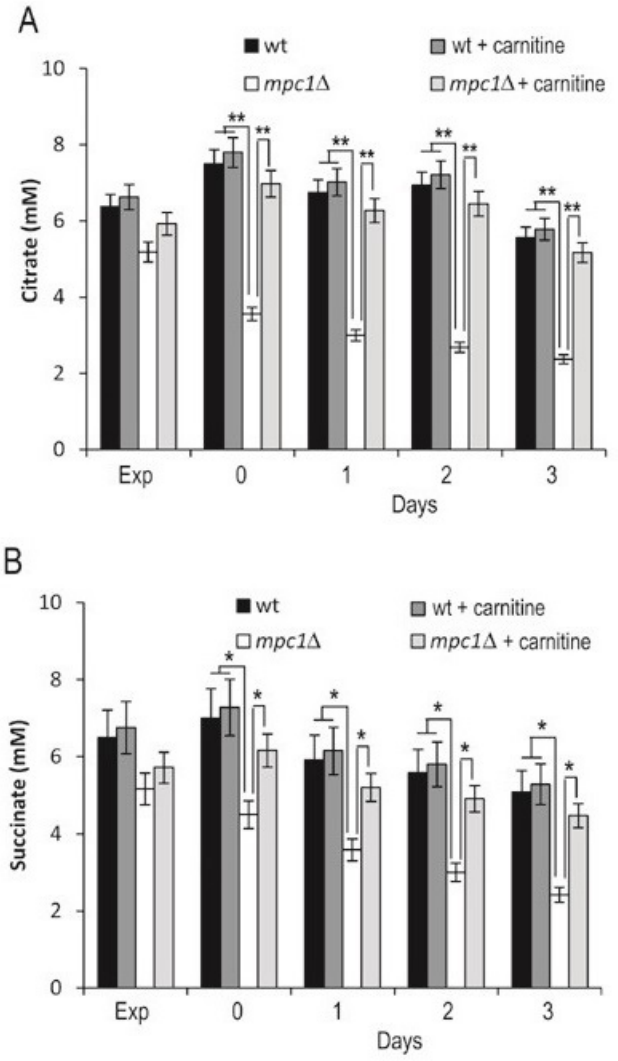


Figure 2.6 – Chronologically aging *mpc1*Δ cells display damaged mitochondria. A) Representative images of wt and *mpc1*Δ cultures of Figure 2.2 stained with $DiOC_6$ to visualize mitochondrial membranes. Morphologies of the mitochondria in late exponential phase (Late exp) are also shown. The same cultures were assessed for the presence of intracellular superoxide by conversion of non-fluorescent dihydroethidium into fluorescent ethidium (Eth). Summary graphs of the percentage of fluorescent/superoxide positive cells (% Eth) are reported (B). (C) Summary graphs of % Eth cells and (D) cellular respiration determined in wt and *mpc1*Δ cultures grown in minimal medium/2% glucose supplemented with carnitine (10 mg/L). Day 0, diauxic shift. For the determination of Eth cells, evaluation of about 1000 cells for each sample (three technical replicates) in three independent experiments was performed. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

In *S. cerevisiae*, carnitine is involved in a process referred to as the carnitine shuttle which allows the transport of acetyl-CoA to the mitochondria. This transport system which is non-functional unless carnitine is supplied with the medium (van Roermund *et al.*, 1999; Swiegers *et al.*, 2001), involves the transfer of the acetyl moiety of acetyl-CoA to carnitine and the subsequent transport of the acetylcarnitine to the mitochondria. Here, a mitochondrial carnitine acetyltransferase catalyses the reverse reaction generating carnitine and acetyl-CoA which enters the TCA cycle (Palmieri *et al.*, 1999; van Roermund *et al.*, 1999). As shown in Figure 2.7A-C, the supplemental carnitine did not significantly affect the levels of citrate, succinate and malate in the wt whilst this was not the case for the *mpc1*Δ mutant where the levels of all three intermediates increased and were restored to wt-like ones. No effect was observed on the malic enzyme activity which at/after the post diauxic shift in the *mpc1*Δ cells was still the double of that of the wt (Figure 2.7D) suggesting that the presence of carnitine does not abolish the Mae1-dependent flux towards mitochondrial pyruvate generation. Concomitantly, in the *mpc1*Δ cells the enzymatic activities of Icl1 and Pck1 were reduced to the physiological levels measured in the wt (Figure 2.7D and E). Thus, all this suggests that in the *mpc1*Δ mutant the activation of the carnitine shuttle can properly feed the TCA cycle by supplying acetyl-CoA to the mitochondria. Hence, the compensative metabolite feeding from the cytosol provided by the glyoxylate shunt seems to be no longer required.



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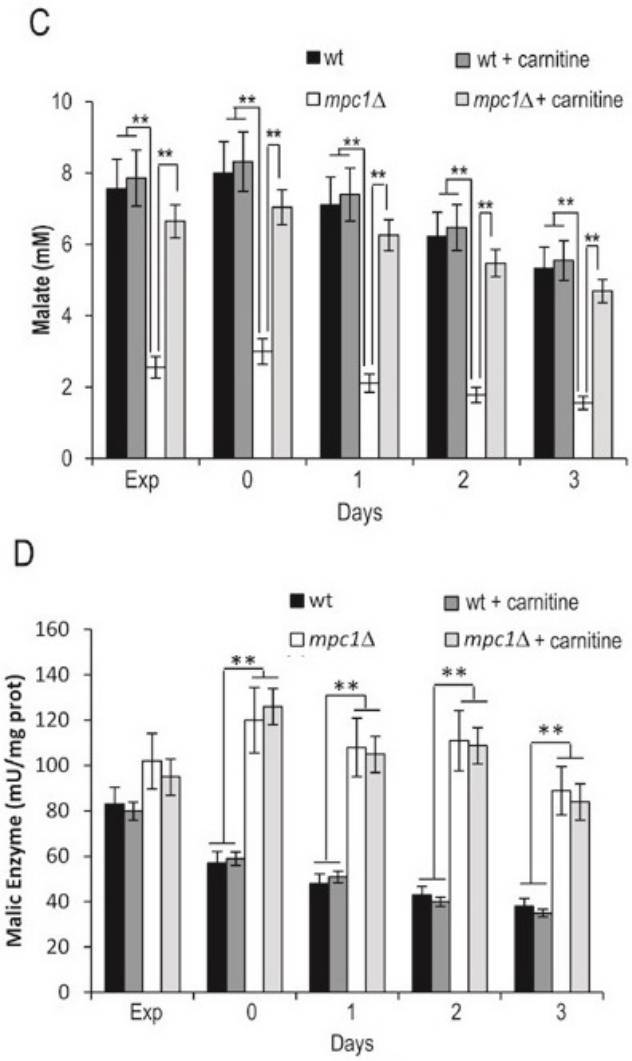

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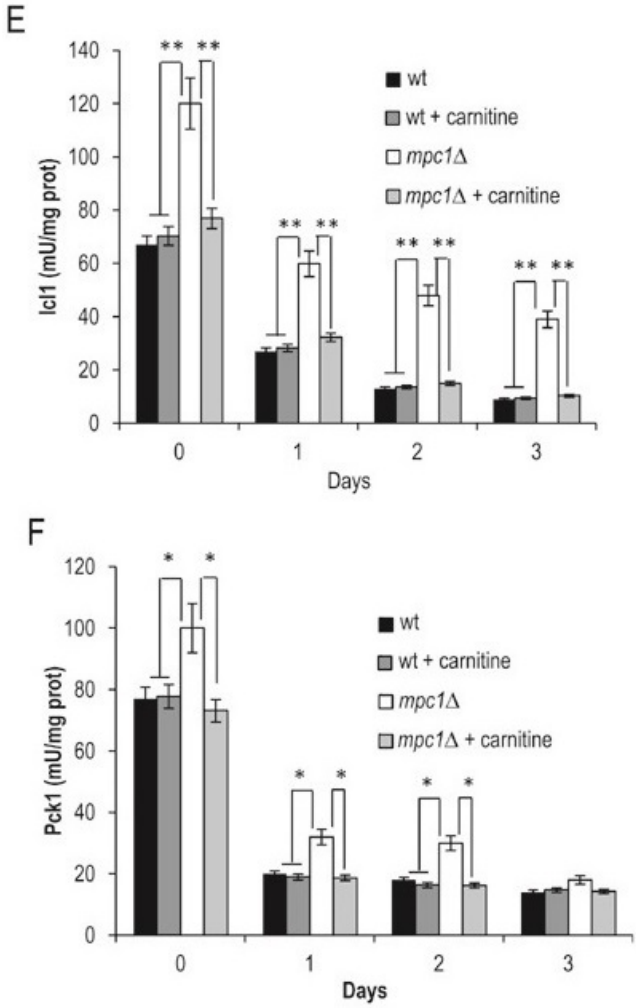
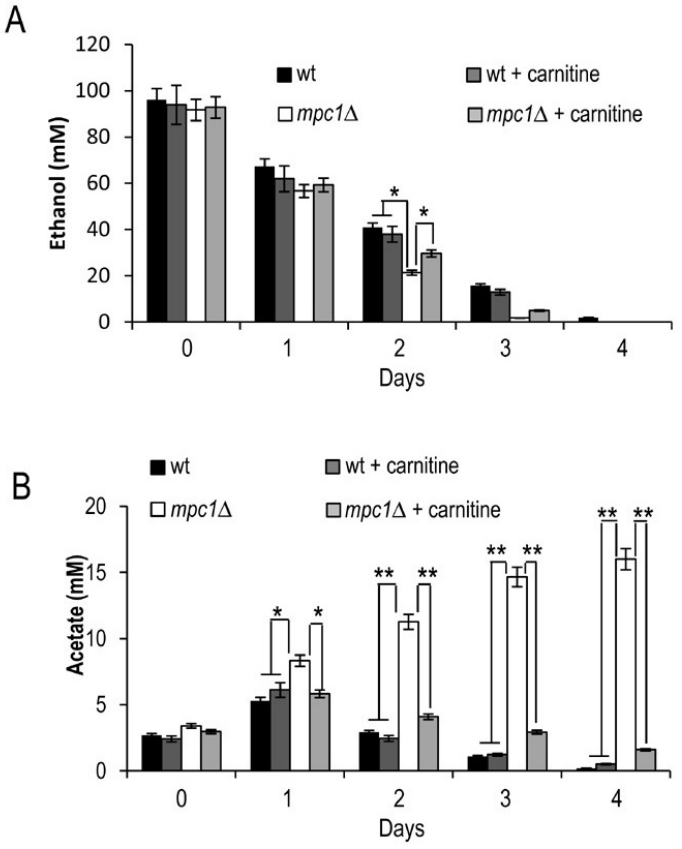


Figure 2.7 – Carnitine increases the levels of the TCA cycle intermediates in the *mpc1*Δ mutant. Wt and *mpc1*Δ cells were grown in minimal medium/2% glucose supplemented with carnitine (10 mg/L) and, at the indicated time points, the concentrations of citrate (A), succinate (B) and malate (C) were measured together with Mae1 (D), Icl1 (E) and Pck1 (F) enzymatic activities. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

Moreover, following carnitine supplementation, during the post-diauxic phase no effect was observed on the ethanol consumption in both the wt and the mutant strains (Figure 2.8A). Similarly, the acetate utilization in the wt was not affected, while in the *mpc1*Δ mutant its utilization was promoted (Figure 2.8B). This indicates that in the latter the activation of the carnitine shuttle and the consequent acetyl-CoA transport to the mitochondria can result in an enhancement in the flux downstream from the acetate activation allowing acetate utilization. Finally, in the *mpc1*Δ mutant these metabolic changes matched the almost completely restored chronological longevity (Figure 2.8C).



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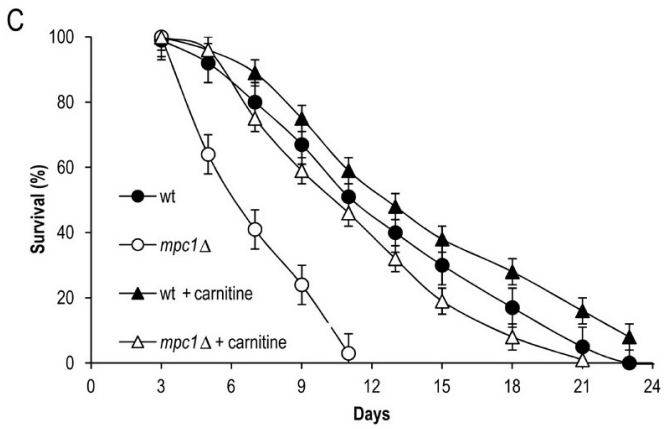

Figure 2.8 – Carnitine promotes acetate utilization in the *mpc1*Δ mutant in concert with increased CLS. Bar charts of extracellular ethanol (A) and acetate (B) concentrations at different time points after the diauxic shift (Day 0) measured for cells grown as in Figure 2.7. (C) CLS of the same cells determined as in Figure 2.2. Data refer to mean values of three independent experiments. SD is indicated. * $P \leq 0.05$ and ** $P \leq 0.01$.

In conclusion, these data collectively indicate that the lack of the Mpc1 transporter brings about a chain of metabolic events which, in order to counteract the decrease of the pyruvate supply in the mitochondria, by influencing the global acetyl-CoA metabolism ultimately restrict cell survival during chronological aging. In particular, after the diauxic shift when cells utilize the earlier produced ethanol/acetate and increase their respiration demand, one of the metabolic traits of the *mpc1*Δ mutant is a TCA cycle operating in a "branched" fashion with a propensity to shunt intermediates towards pyruvate generation via the malic enzyme. This kind of not-complete cyclic functioning of the TCA cycle by depleting it of intermediates influences not only the respiration, which is reduced in the mutant, but also might reduce mitochondrial acetyl-CoA pool. In fact, a TCA cycle characterized by low levels of intermediates (Figure 2.4B-D) generates less succinyl-CoA. This is the substrate for the CoA-transferase reaction from succinyl-CoA to acetate, catalyzed by Ach1 in cells released from glucose repression (Fleck & Brock, 2009). In the mitochondria, this reaction allows the production of acetyl-CoA (Eisenberg *et al.*, 2014a). Moreover, the CoA-transferase reaction is using acetate as acceptor, which implies that Ach1 is also important for acetate detoxification and mitochondrial functionality

during chronological aging (Orlandi *et al.*, 2012). Consequently, in a condition where acetate-generating activities of Ald4/Ald5 are increased, as it is the case of the mutant, a reduction in CoA-transferase enzymatic activity could play a causative role in promoting/enhancing the mitochondrial damage observed in the mutant. Furthermore, since during the utilization of ethanol and acetate, the sole possible route for the net synthesis of C4 dicarboxylic acids for replenishing the TCA cycle of intermediates is the glyoxylate shunt, it follows that in the *mpc1*Δ cells this anaplerotic shunt is enhanced in order to keep the "branched" TCA cycle functioning. In turn, it follows that the pathway providing the cytosolic acetyl-CoA must be increased to support an enhanced glyoxylate demand. In line with this, the cytosolic Ald6 enzymatic activity is increased (Figure 2.3C) and hyperactivation of the Acs2 activity has been detected (Eisenberg *et al.*, 2014a). Interestingly, this synthetase is responsible not only for supplying acetyl-CoA for carbon metabolism, but also for protein acetylation, particularly of histones (Takahashi *et al.*, 2006). In addition, it has been shown that during chronological aging, upregulation of Acs2 activity culminates in histone H3 hyperacetylation associated with transcriptional downregulation of several autophagy-essential *ATG* genes (Eisenberg *et al.*, 2014b). In this context, *mpc1*Δ cells display an age-dependent loss of autophagy (Eisenberg *et al.*, 2014a); this feature, given the reciprocal cross-talk between autophagy and mitochondria, can negatively affect the removal of the damaged mitochondria of the mutant and consequently contribute to its inability to maintain proper cellular homeostasis during the aging process. Notably, Ald6, which is responsible of generating cytosolic acetate, is degraded preferentially by autophagy (Suzuki *et al.*, 2014) and the persistence of its enzymatic activity seems to be disadvantageous for the survival during nitrogen starvation (Onodera *et al.*, 2004). Thus, *mpc1*Δ cells make up for their impairment in mitochondrial pyruvate with a metabolic rewiring in which the pro-aging outcome prevails.

2.4 Materials & Methods

Yeast strains and growth conditions. The *mcp1*Δ mutant (*mcp1::HIS3*) was generated by PCR-based methods in a BY4741 background (*MATa his3*Δ*-1 leu2*Δ*-0 met15*Δ*-0 ura3*Δ*-0*) and the accuracy of gene replacement was verified by PCR with flanking and internal primers. At least two different clones were tested for any experiment. Yeast cells were grown in batches at 30 °C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/L) with 2% glucose andthe required supplements added in excess to a final concentration of 200 mg/L, except for leucine at 500 mg/L to avoid auxotrophy starvation (Fabrizio *et al.*, 2005; Boer *et al.*, 2008). L-carnitine (Sigma) was supplemented to a concentration of 10 mg/L. 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 (Vanoni *et al.*, 1983). Duplication times (Td) were obtained by linear regression of the cell number increase over time on a semilogarithmic plot.

CLS determination. Survival experiments in expired medium were performed on cells grown in minimal medium/2% glucose and the required supplements as described above. 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 (Figure S1). Cell survival was monitored by harvesting aliquots of cells starting with 72 h (Day 3, first age-point) after the diauxic shift (Day 0). CLS was measured according to (Fabrizio *et al.*, 2005) by counting colony-forming units (CFUs) every 2-3 days. The number of CFUs on Day 3 was considered the initial survival (100%).

Metabolite measurements and enzymatic assays. At designated time points, aliquots of the yeast cultures were centrifuged and both pellets (washed twice) and supernatants were frozen at -80 °C until used. Rapid sampling for intracellular metabolite measurements was performed according to the leakage-free cold methanol quenching method developed by (Canelas *et al.*, 2008) in which pure methanol at ≤ -40 °C and a ratio of cell culture to quenching solvent of 1:5 (final methanol concentration ≥ 83%) were

used. Metabolites from the cell pellets were extracted in 5 ml of a solution of 75% (v/v) boiling absolute ethanol containing 0.25 M Hepes, pH 7.5, as described in (Canelas *et al.*, 2009). The concentrations of glucose, ethanol, acetate, pyruvate, citrate, succinate and malate were determined using enzymatic assays (K-HKGLU, K-ETOH, K-ACET, K-PYRUV, K-SUCC, K-CITR and K-LMALR kits from Megazyme). Ethanol specific consumption rate (qEtOH), expressed in mmol•g•DW^{-1}•h^{-1}, was calculated from measured cell dry weights (DWs) and extracellular ethanol concentrations. DW was measured as described (Agrimi *et al.*, 2011). All the enzymatic activities were assayed immediately after preparation of cell-free extracts. Cells were resuspended in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM dithiothreitol and broken with acid-washed glass beads by shaking on a vortex for several cycles interspersed with cooling on ice. The activities of cytosolic and mitochondrial aldehyde dehydrogenase (Ald) were measured as described by (Aranda *et al.*, 2003), of alcohol dehydrogenase (Adh) according to (Postma *et al.*, 1989), of phosphoenolpyruvate carboxykinase (Pck1) and isocitrate lyase (Icl1) as in (Casatta *et al.*, 2013). Malic enzyme activities were determined according to (Boles *et al.*, 1998) with either 0.4 mM NAD^+ or $NADP^+$ as the redox cofactor. The enzymatic activity was measured in the decarboxylation direction to avoid interference with pyruvate decarboxylase and Adh. Total protein concentration was estimated using the BCA™ Protein Assay Kit (Pierce).

Oxygen consumption and fluorescence microscopy. The basal oxygen consumption of intact cells was measured at 30 °C using a "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System, Hansatech Instruments, Norfolk, UK) as previously reported (Orlandi *et al.*, 2013). Data were recorded at sampling intervals of 1 s (Oxygraph Plus software, Hansatech Instruments, Norfolk, UK). All assays were conducted in biological triplicate. ROS were detected with dihydroethidium (DHE, Sigma) according to (Madeo *et al.*, 1999). The mitochondrial membrane potential was assessed by staining with $DiOC_6$ (Molecular Probes, Invitrogen), according to (Koning *et al.*, 1993); cells were also counterstained with propidium iodide to discriminate between alive and dead cells. A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350F ccd

camera was used. Digital images were acquired using FW4000 software (Leica).

Statistical analysis of data. All values are presented as the mean of three independent experiments with the corresponding standard deviation (SD). Three technical replicates were analyzed in each independent experiments. Statistical significance was assessed by one-way ANOVA test. P value of $\leq$ 0.05 was considered statistically significant.

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Supplementary Material

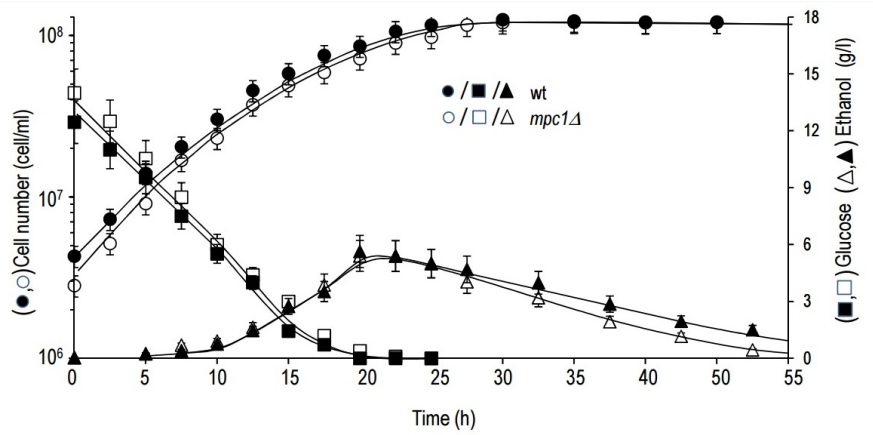

Fig. S1

FIGURE S1. Wild type (wt) and *mpc1*Δ mutant cells were grown in minimal medium/2% glucose and the required supplements in excess (see *Materials & Methods*). Cell growth was monitored by counting cell number over time and, in parallel, the extracellular concentrations of glucose and ethanol were measured in medium samples collected at different time-points in order to define the growth profile (exponential phase, diauxic shift, post-diauxic phase and stationary phase) of the cultures. One representative experiment is shown.

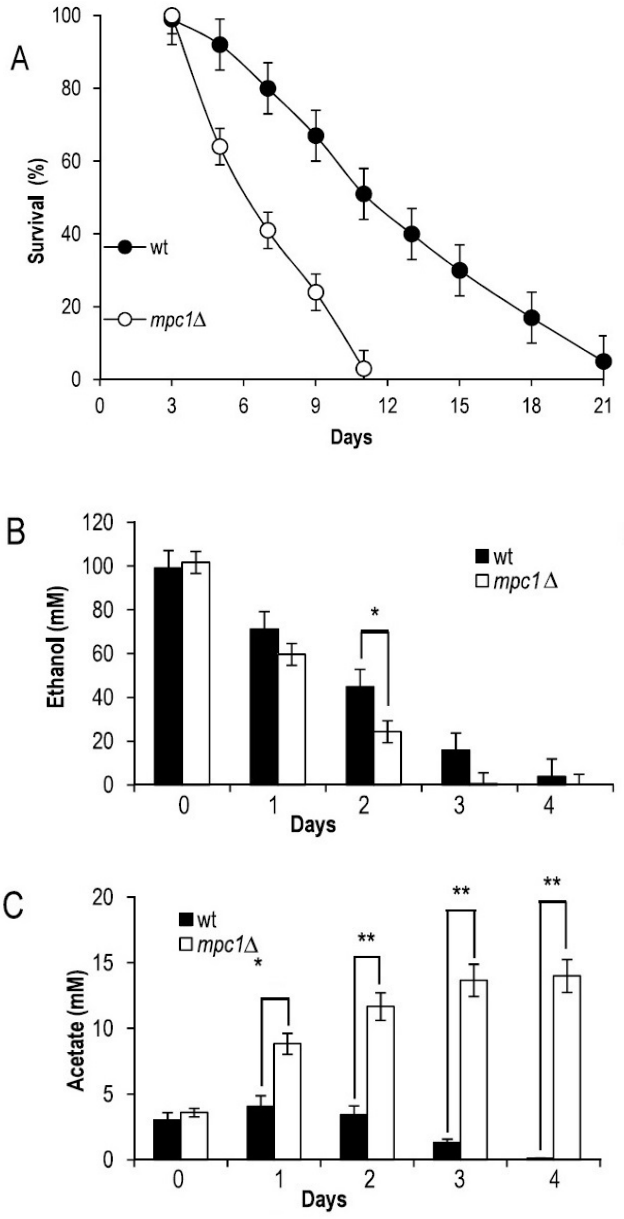

FIGURE S2. *(Caption next page.)*

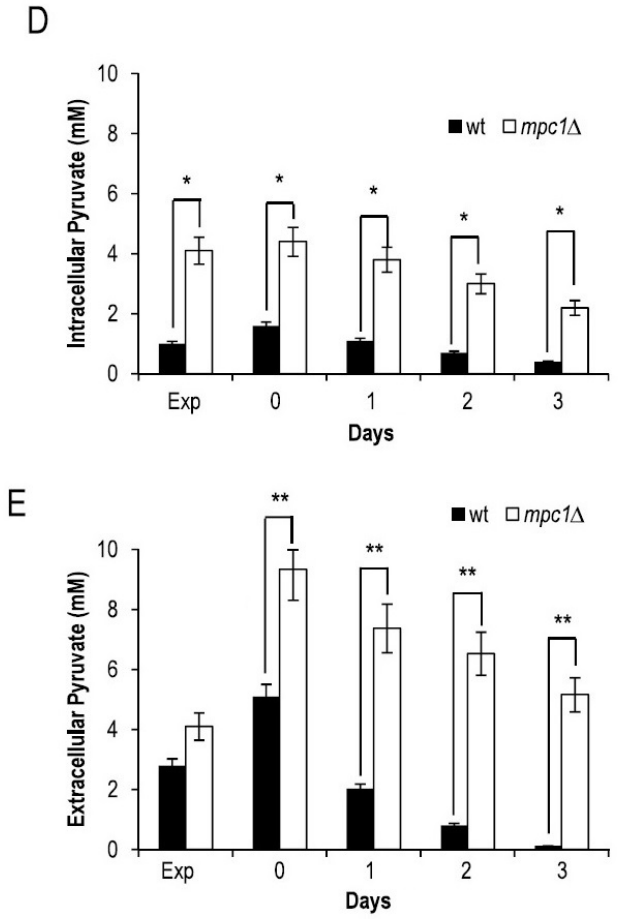

FIGURE S2. The wt BY4741 strain (*MATa his3*Δ*-1 leu2*Δ*-0 met15*Δ*-0 ura3*Δ*-0*) and its derivative *mpc1*Δ mutant (*mcp1*Δ*::HIS3*) were grown in minimal medium/2% glucose containing the same supplements (His, Leu, Met and Ura) in excess and followed up to stationary phase. (A) CLS of wt and *mpc1*Δ mutant cells. At each time-point, survival was determined by colony-forming capacity. 72 h after the diauxic shift (Day 3) was considered the first age-point. Day 0, diauxic shift. Data refer to mean values of three independent experiments. Standard deviations (SD) are indicated. Bar charts of extracellular ethanol (B) and acetate (C) concentrations at different time-points after the diauxic shift (Day 0). In parallel, intracellular (D) and extracellular (E) pyruvate concentrations were measured. Exp, exponential growth phase. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance, assessed by one-way ANOVA test, is indicated. (* $P \leq 0.05$ and ** $P \leq 0.01$).

3 Acetylation of Enzymes as a Modification to Regulate Longevity

Published as:

"Nicotinamide Supplementation Phenocopies SIR2 Inactivation by Modulating Carbon Metabolism and Respiration During Yeast Chronological Aging"

Orlandi Ivan, ***Damiano Pellegrino Coppola****, Maurizio Strippoli, Rossella Ronzulli & Marina Vai*

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3.1 Abstract

Nicotinamide (NAM), a form of vitamin B_3, is a byproduct and non-competitive inhibitor of the deacetylation reaction catalyzed by Sirtuins. These represent a family of evolutionarily conserved NAD^+-dependent deacetylases that are well-known critical regulators of metabolism and aging and whose founding member is Sir2 of *Saccharomyces cerevisiae*. Here, we investigated the effects of NAM supplementation in the context of yeast chronological aging, the established model for studying aging of post-mitotic quiescent mammalian cells. Our data show that NAM supplementation at the diauxic shift results in a phenocopy of chronologically aging *sir2*Δ cells. In fact, NAM-supplemented cells display the same chronological lifespan extension both in expired medium and extreme Calorie Restriction. Furthermore, NAM allows the cells to push their metabolism toward the same outcomes of *sir2*Δ cells by elevating the level of the acetylated Pck1. Both these cells have the same metabolic changes that concern not only anabolic pathways such as an increased gluconeogenesis but also respiratory activity in terms both of respiratory rate and state of respiration. In particular, they have a higher respiratory reserve capacity and a lower non-phosphorylating respiration that in concert with a low burden of superoxide anions can affect positively chronological aging.

3.2 Introduction

Nicotinic acid, and its amide form, nicotinamide (NAM) are the two major forms of vitamin B_3, collectively called niacin, used for nicotinamide adenine dinucleotide (NAD^+) biosynthesis (Bogan & Brenner, 2008; Canto *et al.*, 2015). This essential coenzyme is also synthesized from L-tryptophan taken up from the diet, through the kynurenine pathway, which is often referred to as *de novo* synthesis (Bogan & Brenner, 2008). For this, niacin supplementation prevents diseases due to a dietary deficiency of tryptophan, such as pellagra (Sauve, 2008). NAD^+, however, has other roles beyond that of coenzyme for oxidoreductases: it is also an obligate co-substrate for some families of NAD^+-consuming enzymes that cleave the NAD^+ molecule at its glycosidic bond. One of these families is represented by Sirtuins, which are evolutionarily conserved NAD^+-dependent deacetylases comprising seven members in mammals (SIRT1-SIRT7) and whose founding member is Sir2 of the single-celled yeast *Saccharomyces cerevisiae* (Imai *et al.*, 2000; Michan & Sinclair, 2007; Schwer & Verdin, 2008). Sirtuins play crucial roles in the regulation of health and lifespan in many different organisms in response also to various nutritional and environmental stimuli (Houtkooper *et al.*, 2012; Morris, 2013; Imai & Guarente, 2014; Poulose & Raju, 2015; Winnik *et al.*, 2015). Consequently, a strong consensus exists regarding the possibility that the modulation of Sirtuin activity can be a great promise for the development of pharmacological therapies with healthspan-extending effects (Imai, 2010; Sinclair & Guarente, 2014; Bhullar & Hubbard, 2015; Gertz & Steegborn, 2016). Sirtuins consume one molecule of NAD^+ for every acetyl residue removed from specific lysines on histone and non-histone substrates, generating O-acetyl-ADP-ribose and a salvageable NAM that can be recycled as a substrate for NAD^+ synthesis (Tanny & Moazed, 2001; Sauve *et al.*, 2006). Moreover, NAM is also an endogenous non-competitive inhibitor of Sirtuins: it reacts with an intermediate of the deacetylation reaction shifting it towards reformation of substrates (NAD^+ and acetylated target), (Jackson *et al.*, 2003; Avalos *et al.*, 2005; Sauve *et al.*, 2005; Sauve *et al.*, 2006; Sanders *et al.*, 2007). Thus, NAM can affect the enzymatic activity of Sirtuins both as NAD^+ precursor and as inhibitor of the deacetylation reaction. In the field of aging-related research, the effects of NAM supplementation have been

investigated in *S. cerevisiae* in the context of replicative aging (Bitterman *et al.*, 2002; Anderson *et al.*, 2003). In fact, in this budding yeast, two lifespan models have been well established: the replicative and chronological models that provide the ability to simulate the aging process of mitotically active and post-mitotic quiescent mammalian cells, respectively (MacLean *et al.*, 2001; Longo & Kennedy, 2006). The replicative lifespan (RLS) refers to the number of daughter cells (buds) generated by a mother, which undergoes asymmetrical cell division, in the presence of nutrients before death (Steinkraus *et al.*, 2008), whilst the chronological lifespan (CLS) is the mean and maximum length of time a culture of non-dividing cells remains viable in stationary phase. In a typical CLS experiment, starting 72 h after the diauxic shift, viability is measured as the ability to resume growth upon return to rich medium (Fabrizio & Longo, 2007; Longo *et al.*, 2012). Sir2 is involved in both replicative and chronological aging. In the former, Sir2 activity promotes RLS (Kaeberlein *et al.*, 1999; Imai *et al.*, 2000). Cells grown in the presence of NAM phenocopy *sir2*Δ ones: they show a similar shortening of RLS and other features associated with *SIR2* loss of function such as reduction in silencing and increased recombination at the rDNA locus due to the inhibition of Sir2 enzymatic activity (Bitterman *et al.*, 2002; Sauve *et al.*, 2005). In chronological aging, on the other hand, most evidence so far supports a pro-aging role for Sir2 (Fabrizio *et al.*, 2005; Smith *et al.*, 2007; Casatta *et al.*, 2013). In particular, its deletion further extends the CLS caused by a severe form of Calorie Restriction (CR) obtained by incubation in water (Fabrizio *et al.*, 2005; Casatta *et al.*, 2013). In addition, in chronologically aging cells, lack of Sir2 strongly influences carbon metabolism which turned out to be characterized by an enhanced glyoxylate/gluconeogenic flux. This, on the one hand, affects positively acetate metabolism and, on the other, increases the intracellular trehalose stores that are useful for long-term survival of non-dividing cells (Casatta *et al.*, 2013).

Here, we present results showing that NAM supplementation at the onset of chronological aging inhibits Sir2 activity, in particular Sir2-mediated deacetylation of Pck1. Due to this inhibition, NAM-supplemented cells phenocopy a *sir2*Δ strain displaying the same metabolic changes such as an enhanced glyoxylate/gluconeogenesis and increased trehalose. Inter-

estingly, we found that lack of the Sir2 activity also affects the respiratory activity in terms both of respiratory rate and state of respiration. In particular, it correlates with a lower non-phosphorylating respiration that leads to a lower superoxide anion (O_2^-) content. Finally, NAM-supplemented cells also phenocopy the CLS of *sir2*Δ cells both in expired medium and in extreme CR condition.

3.3 Results & Discussion

During chronological aging NAM supplementation like SIR2 inactivation promotes Pck1 enzymatic activity and gluconeogenesis. Several studies have demonstrated that NAM is a strong endogenous non-competitive inhibitor of Sir2 activity, which also modulates Sir2-mediated replicative longevity (Anderson *et al.*, 2002; Bitterman *et al.*, 2002; Anderson *et al.*, 2003; Gallo *et al.*, 2004; Sauve and Schramm, 2003; Sauve *et al.*, 2005). In this context, NAM supplementation to yeast growth media shortens RLS in a similar way to that of a *sir2*Δ mutant (Bitterman *et al.*, 2002). Since, in the chronological aging paradigm, a pro-aging role has emerged for Sir2 (Fabrizio *et al.*, 2005; Longo *et al.*, 2012; Casatta *et al.*, 2013), we set out initially to determine whether NAM supplementation would cause any effect on CLS and whether these effects were Sir2-mediated. Since NAM concentrations as low as 5 mM are predicted to inhibit Sir2 activity (Anderson *et al.*, 2003; Gallo *et al.*, 2004) we examined the effects of two concentrations (1 mM and 5 mM) within the proposed physiological range. As shown in Figure 3.1A and Table S2, exogenously supplied NAM at the diauxic shift (Day 0) extended the CLS of the wild type (wt) while that of the *sir2*Δ mutant was unaffected even with the highest concentration.

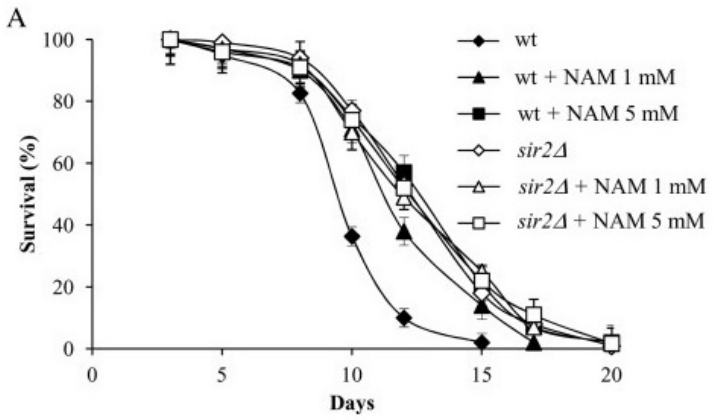


Figure 3.1 – *(Caption next page.)*

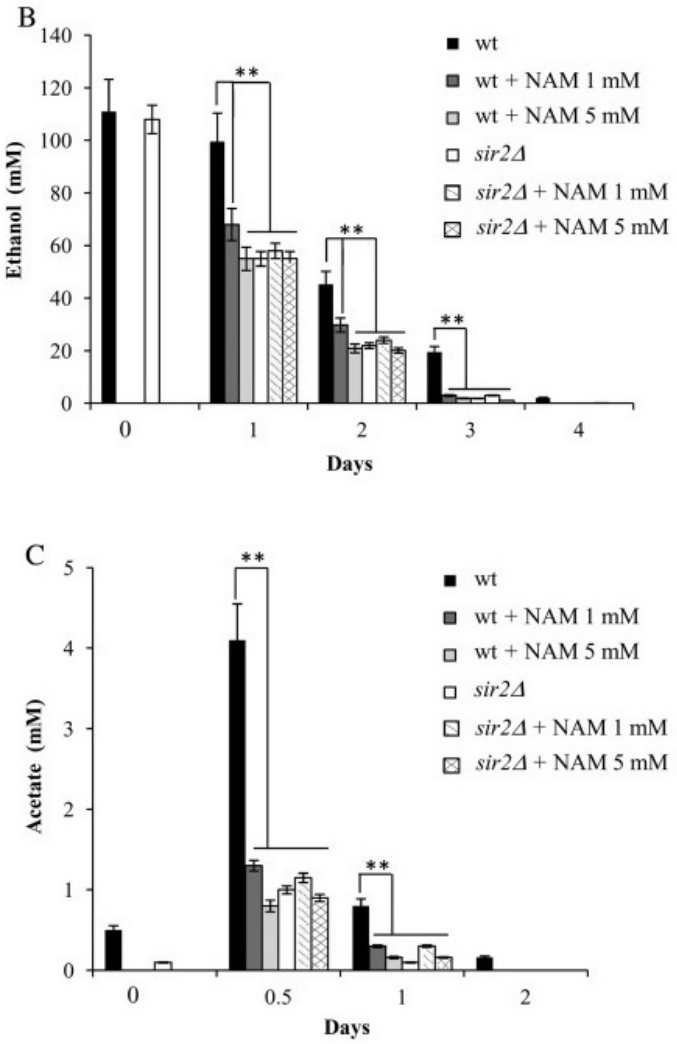
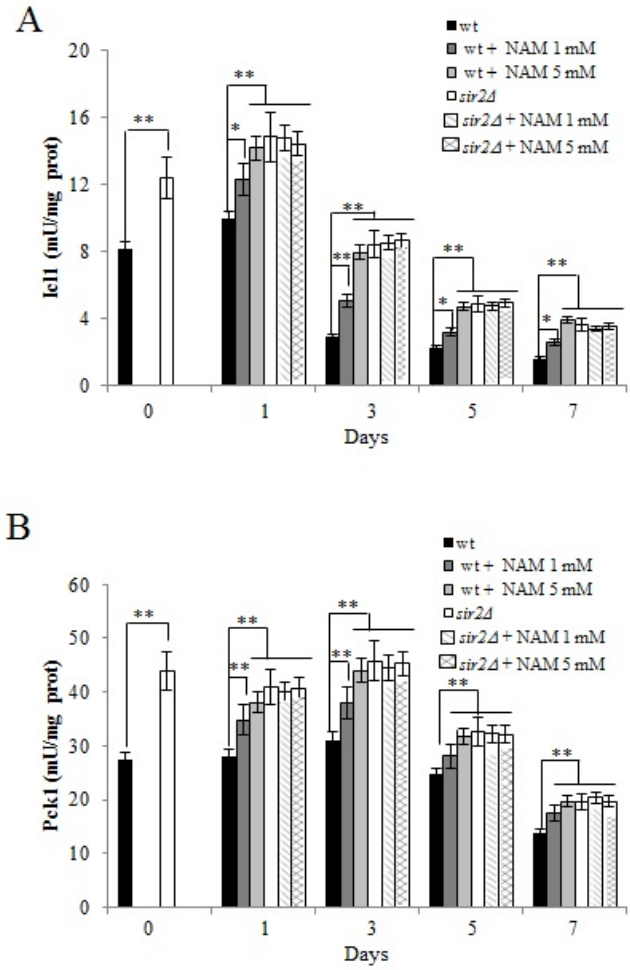


Figure 3.1 – NAM supplementation at the diauxic shift affects CLS and extracellular ethanol/acetate similarly to *SIR2* inactivation. Wild type (wt) and *sir2*Δ cells were grown in minimal medium/2% glucose and the required supplements in excess (see *Materials & Methods*). At the diauxic shift (Day 0), nicotinamide (NAM) was added to the expired media at the indicated concentrations. (A) At each time point, survival for both treated and untreated cultures was determined by colony-forming capacity on YEPD plates. 72 h after the diauxic shift (Day 3) was considered the first age-point. Bar charts of extracellular ethanol (B) and acetate (C) concentrations at different time-points after Day 0. All data refer to mean values of three independent experiments with three technical replicates each. Standard deviations (SD) are indicated. Statistical significance as assessed by one-way ANOVA test is indicated (* $P \leq 0.05$ and ** $P \leq 0.01$).

At Day 0, glucose is exhausted and cells begin to consume the fermentation C2 byproducts (ethanol/acetate) via the TCA cycle and the glyoxylate shunt. Afterwards, when these byproducts are exhausted, cells enter the stationary phase. *SIR2* inactivation is associated with a fast depletion of ethanol during the post-diauxic phase and with very low levels of extracellular acetate (Fig. 1B and C) (Fabrizio *et al.*, 2005; Casatta *et al.*, 2013). Interestingly, NAM added to wt cultures led to an increase in ethanol consumption, depleted by Day 3, and to a dramatically limited accumulation of extracellular acetate (Figure 3.1B and C). No effect was observed for the *sir2*Δ mutant: both treated and untreated cultures displayed the same fast exhaustion of ethanol and reduced accumulation of acetate in the medium (Figure 3.1B and C). Since ethanol/acetate metabolism strongly influences chronological aging and changes in the utilization of these C2 compounds differently impact CLS (Longo *et al.*, 2012; Orlandi *et al.*, 2013; Hu *et al.*, 2014), we investigated whether the supplemental NAM could have any effect on the metabolic pathways required for the utilization of ethanol/acetate as carbon/energy sources. Initially, we examined the glyoxylate shunt, which becomes operative during the post-diauxic phase, and the gluconeogenesis. This pathway yielding glucose-6-phosphate from the oxaloacetate provided by the glyoxylate shunt, also supports the production of trehalose stores. Trehalose has been proposed as a key carbohydrate for surviving starvation during chronological aging and, in addition, extends CLS (Shi *et al.*, 2010; Ocampo *et al.*, 2012). Thus, starting from Day 0, we measured the enzymatic activities of isocitrate lyase (Icl1), one of the unique enzymes of the glyoxylate shunt, and of phosphoenolpyruvate carboxykinase (Pck1) which catalyzes the main flux-controlling step of the gluconeogenesis. At the same time, we also evaluated the acetylation level of Pck1 since this post-translational modification is, on the one hand, crucial for Pck1 enzymatic activity and, on the other, regulated by the deacetylase activity of Sir2 (Lin *et al.*, 2009). With regard to the wt culture, following NAM addition, the levels of both the enzymatic activities increased and remained constantly higher than those detected in the absence of NAM (Figure 3.2A and B); concomitantly intracellular trehalose content also increased (Figure 3.2C). This indicates that NAM has a stimulatory effect on the glyoxylate/gluconeogenic flux. It follows that in the medium of NAM-treated wt cultures, ethanol depletion increases and acetate levels decrease (Figure 3.1B and

C). The increase of Icl1 and Pck1 enzymatic activities and of the trehalose amount was concentration-dependent, reaching for 5 mM NAM values comparable with the ones of the *sir2*Δ mutant (Figure 3.2A-C). In fact, in line with an enhanced glyoxylate/gluconeogenic flux that characterizes the mutant (Casatta *et al.*, 2013), the latter showed high Icl1 and Pck1 enzymatic activities and trehalose levels. No significant effect was observed when *sir2*Δ cultures were supplied with NAM (Figure 3.2A-C).



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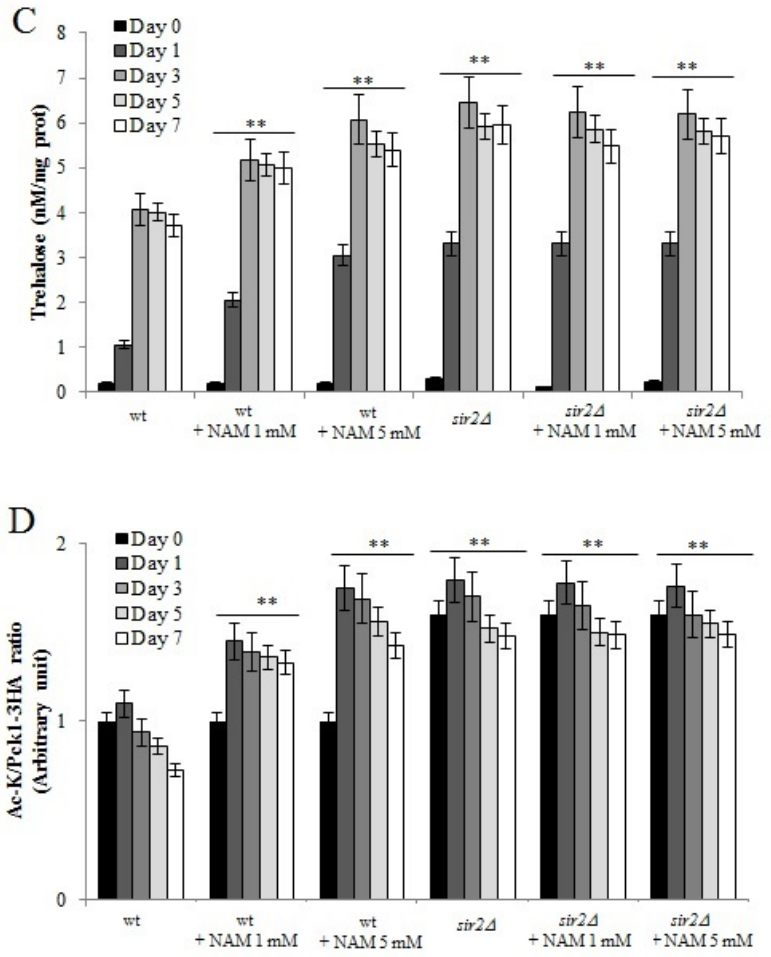

Figure 3.2 – NAM supplementation at the diauxic shift increases gluconeogenesis similarly to *SIR2* inactivation. Wt and *sir2*Δ cells were grown and supplied with NAM at the diauxic shift (Day 0) as in Fig. 3.1. At the indicated time points, Icl1 (A) and Pck1 (B) enzymatic activities and intracellular trehalose content (C) were measured for both treated and untreated cultures. (D) Bar charts of the ratio of Ac-K to Pck1-3HA values obtained by densitometric quantification of signal intensity of the corresponding bands on Western blots. At different time points from wt and *sir2*Δ cells expressing 3HA-tagged Pck1 (supplied with NAM at Day 0), total protein extracts were prepared and subjected to immunoprecipitation with anti-HA antibody followed by Western analysis. Immunodecoration was performed with anti-Ac-K and anti-HA antibodies. All data refer to mean values determined in three independent experiments with three technical replicates each. SD is indicated. (* $P \leq 0.05$ and ** $P \leq 0.01$).

Moreover, in the NAM-treated wt culture, the increase of the Pck1 enzymatic activity (Figure 3.2B) was associated with an increase in the amount of the acetylated active form of the enzyme compared with that without NAM (Figure 3.2D). In the *sir2*Δ culture the level of the acetylated Pck1 was higher than the wt one (Figure 3.2D), in agreement with previous data (Casatta *et al.*, 2013) and with the lack of the Sir2-targeted deacetylation (Lin *et al.*, 2009), and was not affected by NAM (Figure 3.2D). No significant difference in the amount of total Pck1 was detected between treated and untreated cultures for both strains (data not shown). Taken together these data suggest that NAM, in the context of a standard CLS experiment, inhibits Sir2 activity, in particular Sir2-mediated deacetylation of Pck1. Due to this inhibition, NAM-treated wt cells mimic a *sir2*Δ strain displaying metabolic features such as an enhanced glyoxylate/gluconeogenesis and increased trehalose stores which can be advantageous for chronological survivability. Notably, NAM supplementation also phenocopies the CLS of *sir2*Δ cells in a condition of extreme CR. In fact, it is well known that *SIR2* deletion exacerbates the CLS extension linked to the transfer of post-diauxic cells from their expired medium to water (Figure 3.3A) (Fabrizio *et al.*, 2005; Casatta *et al.*, 2013) . Supplementing water with NAM did not further extend the long-lived phenotype of the *sir2*Δ cells, whereas 5 mM NAM was sufficient for extending CLS of the wt to the same extent as the *sir2*Δ one (Figure 3.3A). This reinforces the notion that Sir2 activity blocks extreme CLS extension (Fabrizio *et al.*, 2005). Furthermore, the extended CLS achieved for the wt following NAM supplementation required the presence of Pck1 as in the case of *SIR2* deletion (Lin *et al.*, 2009; and Figure 3.3B). In fact, the long-lived phenotype in water of NAM-supplemented cells was completely abolished by deleting *PCK1* (Figure 3.3B) further supporting a connection among gluconeogenesis, Sir2 activity and chronological aging.

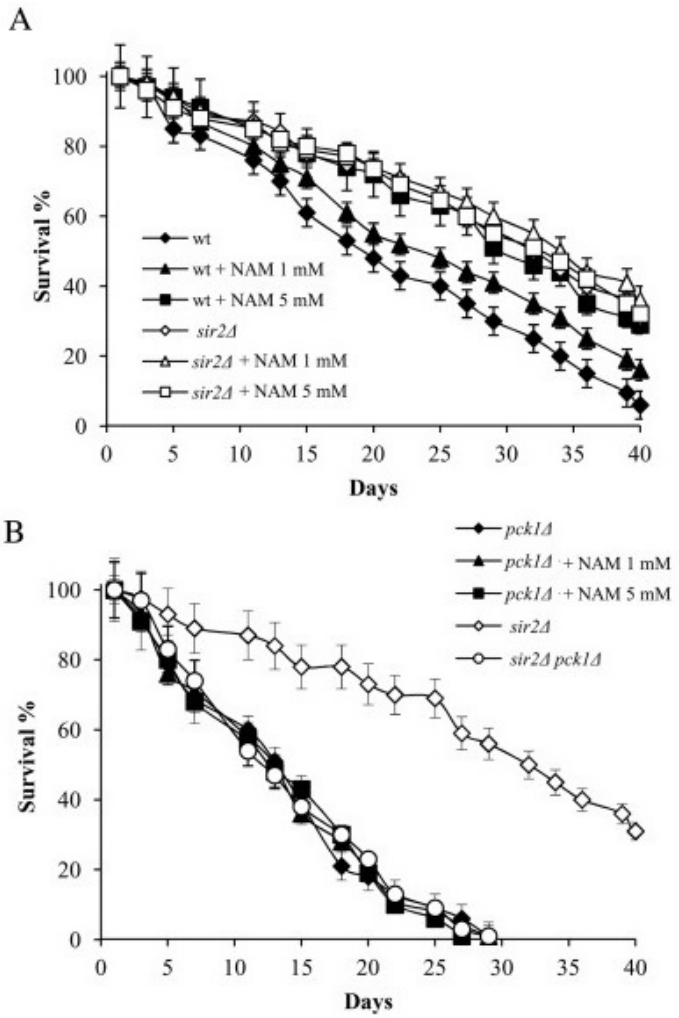

Figure 3.3 – NAM supplementation promotes extreme CLS extension. At Day 1 after the diauxic shift, the indicated strains (grown as in Fig. 3.1) were switched to water and challenged with NAM. Every 48 h, cultures were resuspended in fresh water and each time, NAM was added when reported. At the indicated time points, viability (A and B) was measured. Data refer to mean values determined in three independent experiments with three technical replicates each. SD is indicated.

Lack of Sir2 and NAM supplementation affect both the respiratory rate and the state of respiration during chronological aging. Bearing in mind that following the diauxic shift cells switched to a respiration-based metabolism and considering also the influence of respiration on CLS (Bonawitz *et al.*, 2006; Ocampo *et al.*, 2012), we next assessed several respiratory parameters in both NAM-treated and untreated cultures of the wt and *sir2*Δ strains. NAM supplementation at Day 0 to wt cells resulted in a decrease in their basal oxygen consumption (Table 3.1). This matched well the effects of *SIR2* inactivation. In fact, in the *sir2*Δ mutant, basal oxygen consumption was lower than the wt one displaying values close to those measured for wt cells treated with 5 mM NAM (Table 3.1). No significant effect was observed when *sir2*Δ cultures were supplied with NAM (Table 3.1). In the *sir2*Δ and NAM-treated cultures the lower level of respiration compared with the wt one was not due to a limitation of reducing equivalents since in the presence of the uncoupler CCCP (*Materials & Methods*) they increased respiration to the same extent as the wt (Table 3.1). This indicates that the maximal respiratory capacity is similar and that the membrane potential is retained. Moreover, in these cells the ratio of uncoupled respiration to basal respiration (J_{MAX}/J_R) was higher than that of the wt (Table 3.2) indicating that NAM supplementation as well as *SIR2* inactivation is accompanied by a higher respiratory reserve capacity. Notably, during the post-diauxic phase, the *sir2*Δ and NAM-treated cultures seem to modulate their respiratory efficiency by coupling ATP generation to electron transport better than the wt. In fact, the non-phosphorylating respiration was lower than that measured for the wt, the levels of which significantly increased as a function of time in culture (Table 3.1). In line with this, the net respiration (*Materials & Methods*), which is an estimate of the coupled respiration, was significantly higher in the *sir2*Δ and NAM-treated cultures (Table 3.2). Additionally, in the wt the ratio between the net respiration and the uncoupled respiration ($netR/J_{MAX}$), which expresses the fraction of the electron transfer system utilized to drive ATP synthesis (Gnaiger, 2014), severely decreased after 3 days from the diauxic shift and at Day 5 it was reduced to values close to zero (Table 3.2), indicative of a non-phosphorylating respiration state. In the *sir2*Δ and NAM-treated cultures, at the same time-points, the values of this ratio were higher than the wt ones (Table 3.2) suggesting that these cells can retain a higher respiratory

activity coupled with ATP production for a longer period. Consistently, in the same time-frame, whilst cellular ATP levels in the wt decreased, in the *sir2*Δ and NAM-treated cultures, they remained high and higher ATP/J_R ratios were also observed (Table 3.3). Thus, wt cells supplemented with NAM at the diauxic shift and *sir2*Δ cells display the same metabolic changes that concern not only anabolic pathways such as gluconeogenesis but also the respiratory activity in terms both of respiratory rate and state of respiration.

		J_R		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	9.36 ± 0.28	14.05 ± 0.32	7.30 ± 0.27	4.27 ± 0.29
wt 1 mM NAM		12.55* ± 0.27	6.80* ± 0.17	3.30** ± 0.37
wt 5 mM NAM		10.93** ± 0.39	5.95** ± 0.31	3.25** ± 0.24
*sir2*Δ NT	7.85** ± 0.33	10.86** ± 0.15	5.85** ± 0.15	3.11** ± 0.19
*sir2*Δ 1 mM NAM		10.74** ± 0.32	5.77** ± 0.33	3.20** ± 0.31
*sir2*Δ 5 mM NAM		10.71** ± 0.28	5.71** ± 0.36	3.14** ± 0.27

		J_{MAX}		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	19.26 ± 0.47	26.53 ± 0.16	25.31 ± 0.13	11.39 ± 0.21
wt 1 mM NAM		26.47 ± 0.35	25.53 ± 0.21	11.55 ± 0.15
wt 5 mM NAM		27.05 ± 0.29	25.91 ± 0.25	10.56 ± 0.22
*sir2*Δ NT	18.97 ± 0.43	26.19 ± 0.24	25.33 ± 0.17	11.26 ± 0.19
*sir2*Δ 1 mM NAM		26.55 ± 0.31	25.62 ± 0.22	11.33 ± 0.21
*sir2*Δ 5 mM NAM		26.32 ± 0.29	25.13 ± 0.31	10.98 ± 0.18

		J_{TET}		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	1.61 ± 0.28	3.75 ± 0.32	3.89 ± 0.27	4.12 ± 0.29
wt 1 mM NAM		2.18* ± 0.27	2.68* ± 0.17	2.73** ± 0.37
wt 5 mM NAM		1.61** ± 0.39	1.70** ± 0.31	1.82** ± 0.24
*sir2*Δ NT	1.15** ± 0.33	1.24** ± 0.15	1.51** ± 0.15	1.70** ± 0.19
*sir2*Δ 1 mM NAM		1.29** ± 0.32	1.48** ± 0.33	1.80** ± 0.31
*sir2*Δ 5 mM NAM		1.21** ± 0.28	1.49** ± 0.36	1.77** ± 0.27

Table 3.1. Effect on respiration rates of NAM supplementation at the diauxic shift. Oxygen uptake rates (J) are expressed as pmol/10^6 cells/s. Basal respiration rate (J_R), uncoupled respiration rate (J_{MAX}) and non-phosphorylating respiration rate (J_{TET}). Substrates and inhibitors used in the measurements of the respiratory parameters are detailed in the text. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments with three technical replicates each. SD is indicated. Values obtained for wt were used as reference for comparisons with the corresponding ones determined for NAM-supplied and *sir2*Δ cells. (* $P \leq 0.05$ and ** $P \leq 0.01$, one-way ANOVA test).

		J_{MAX}/J_R		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	2.11 ± 0.28	1.80 ± 0.26	3.40 ± 0.18	3.17 ± 0.13
wt 1 mM NAM		2.11** ± 0.14	3.82** ± 0.21	4.04** ± 0.17
wt 5 mM NAM		2.46** ± 0.27	4.35** ± 0.37	4.46** ± 0.33
*sir2*Δ NT	2.31 ± 0.21	2.41** ± 0.36	4.38** ± 0.26	4.48** ± 0.32
*sir2*Δ 1 mM NAM		2.47** ± 0.11	4.43** ± 0.15	4.49** ± 0.32
*sir2*Δ 5 mM NAM		2.46** ± 0.21	4.41** ± 0.16	4.45** ± 0.23

		netR		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	7.2 ± 0.22	10.26 ± 0.30	3.41 ± 0.23	0.17 ± 0.12
wt 1 mM NAM		10.11 ± 0.21	4.12* ± 0.36	0.57** ± 0.17
wt 5 mM NAM		9.94 ± 0.29	4.25* ± 0.27	1.4** ± 0.21
*sir2*Δ NT	6.94 ± 0.31	9.85 ± 0.33	4.34* ± 0.32	1.41** ± 0.23
*sir2*Δ 1 mM NAM		9.87 ± 0.40	4.26* ± 0.28	1.4** ± 0.29
*sir2*Δ 5 mM NAM		9.91 ± 0.38	4.19* ± 0.25	1.37** ± 0.24

		$netR/J_{MAX}$		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	0.36 ± 0.09	0.37 ± 0.07	0.11 ± 0.06	0.011 ± 0.02
wt 1 mM NAM		0.38 ± 0.05	0.16** ± 0.03	0.05** ± 0.03
wt 5 mM NAM		0.38 ± 0.04	0.18** ± 0.02	0.13** ± 0.05
*sir2*Δ NT	0.36 ± 0.05	0.38 ± 0.06	0.18** ± 0.05	0.12** ± 0.03
*sir2*Δ 1 mM NAM		0.37 ± 0.04	0.18** ± 0.04	0.13** ± 0.05
*sir2*Δ 5 mM NAM		0.36 ± 0.07	0.17** ± 0.05	0.13** ± 0.03

Table 3.2. Effect on respiratory parameters of NAM supplementation at the diauxic shift. Oxygen uptake rates (J) are expressed as pmol/10^6 cells/s. Respiratory reserve capacity (J_{MAX}/J_R), net respiration ($netR = J_R - J_{TET}$) and fraction of the electron transfer system utilized for ATP synthesis ($netR/J_{MAX}$). Substrates and inhibitors used in the measurements of the respiratory parameters are detailed in the text. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments with three technical replicates each. SD is indicated. Values obtained for wt were used as reference for comparisons with the corresponding ones determined for NAM-supplied and *sir2*Δ cells. (* $P \leq 0.05$ and ** $P \leq 0.01$, one-way ANOVA test).

		ATP[a]		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	6.63 ± 0.42	6.32 ± 0.21	5.91 ± 0.37	3.73 ± 0.41
wt 1 mM NAM		6.44 ± 0.19	6.11* ± 0.17	6.36** ± 0.37
wt 5 mM NAM		6.24 ± 0.55	6.37* ± 0.43	6.05** ± 0.29
*sir2*Δ NT	6.42 ± 0.46	6.50 ± 0.58	6.44* ± 0.19	6.56** ± 0.34
*sir2*Δ 1 mM NAM		6.45 ± 0.51	6.40* ± 0.42	6.47** ± 0.31
*sir2*Δ 5 mM NAM		6.42 ± 0.37	6.38* ± 0.22	6.57** ± 0.49

		ATP[b]		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	3.81 ± 0.39	3.63 ± 0.26	3.21 ± 0.32	2.12 ± 0.31
wt 1 mM NAM		3.69 ± 0.47	3.52* ± 0.38	3.63** ± 0.28
wt 5 mM NAM		3.58 ± 0.37	3.63* ± 0.25	3.69** ± 0.19
*sir2*Δ NT	3.70 ± 0.16	3.75 ± 0.23	3.72* ± 0.09	3.78** ± 0.24
*sir2*Δ 1 mM NAM		3.72 ± 0.18	3.69* ± 0.27	3.74** ± 0.33
*sir2*Δ 5 mM NAM		3.61 ± 0.09	3.71* ± 0.21	3.75** ± 0.36

		ATP^a/J_R		
Strain	Day 0	Day 1	Day 3	Day 5
wt NT	1.16 ± 0.08	0.98 ± 0.14	1.13 ± 0.07	1.32 ± 0.09
wt 1 mM NAM		1.02 ± 0.15	1.48* ± 0.09	1.69* ± 0.11
wt 5 mM NAM		1.06 ± 0.16	1.68** ± 0.08	1.92** ± 0.15
*sir2*Δ NT	1.04 ± 0.11	1.04 ± 0.06	1.71** ± 0.13	1.96** ± 0.20
*sir2*Δ 1 mM NAM		1.03 ± 0.15	1.73** ± 0.09	1.92** ± 0.13
*sir2*Δ 5 mM NAM		1.07 ± 0.07	1.72** ± 0.16	1.94** ± 0.15

Table 3.3. Cellular ATP measurements starting from the diauxic shift. ATP content expressed as [a] μmol/g (dry weight) of cells and [b] pmol/10^6 cells. J_R, basal respiration rate expressed as μmol/g (dry weight)/s. Day 0, diauxic shift. Data refer to mean values determined in three independent experiments with three technical replicates each. SD is indicated. Values obtained for wt were used as reference for comparisons with the corresponding ones determined for NAM-supplied and *sir2*Δ cells. (* $P \leq 0.05$ and ** $P \leq 0.01$, one-way ANOVA test).

Lack of Sir2 and NAM supplementation preserve functional mitochondria during chronological aging. Starting from the aforementioned results regarding respiratory activity, for the purpose of investigating mitochondrial functionality we determined the Index of Respiratory Competence (IRC), namely the percentage of cells competent to respire (Parrella & Longo, 2008) for both NAM-treated and untreated cultures. At Day 1, all the cultures had an IRC of about 100% (Figure 3.4A) which indicates that all the cells are still respiration-competent. Afterwards, this value for the wt decreased progressively as expected (Orlandi *et al.*, 2013) reaching about 20% by Day 21. For the *sir2*Δ mutant, the time-dependent loss of mitochondrial functionality was lower and by Day 21 IRC was still about 43% (Figure 3.4B). No effect was observed following NAM supplementation to this culture. On the contrary, NAM supplementation to the wt increased the IRC and NAM effect, in the same way as for the other parameters so far analyzed, was concentration-dependent showing for 5 mM NAM values similar to those of the *sir2*Δ culture (Figure 3.4A and B). This indicates, on the one hand, that the lower level of basal respiration displayed by the *sir2*Δ and NAM-treated wt cultures (Table 3.1) is not due to some impairment in mitochondrial functionality and, on the other, that the lack of Sir2 enzymatic activity elicits somehow a "protective" effect on mitochondria. Consistently, in the *sir2*Δ and NAM-treated wt cells, $DiOC_6$ staining revealed the presence at Day 7 of mitochondria organized in a tubular network reflecting a proper functional state (Knorre *et al.*, 2013; Mishra & Chan, 2014) (Figure 3.4C). Moreover, since $DiOC_6$ accumulates at the mitochondrial membrane depending on their membrane potential (Koning *et al.*, 1993), the bright fluorescence shown by these cells indicates that the mitochondrial membrane potential is retained in agreement with the results of the maximal/uncoupled respiration (Table 3.1). Uncoupling mitochondria with the addition of CCCP resulted in loss of fluorescence due to the dissipation of the mitochondrial membrane potential (Figure S1).

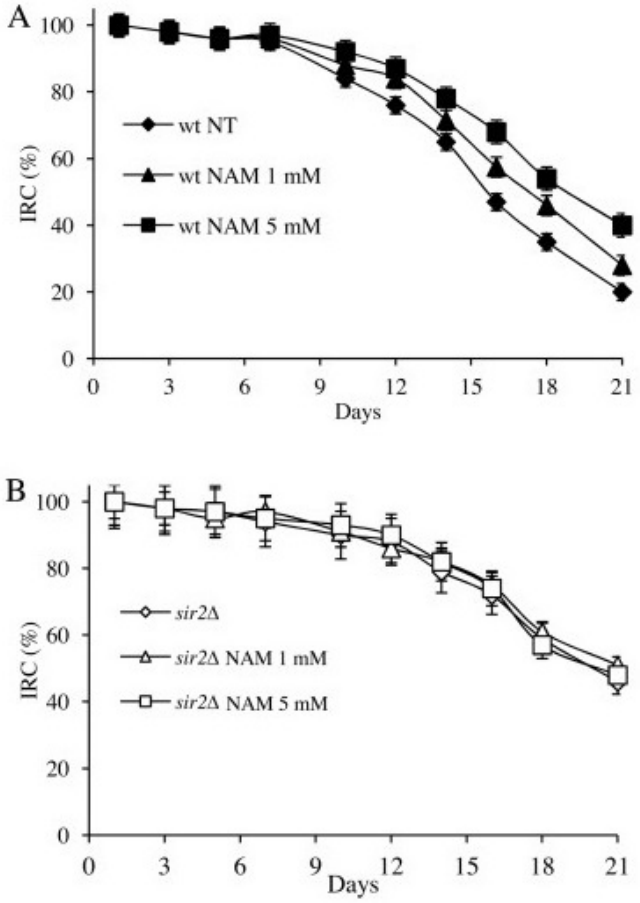

Figure 3.4 – *(Caption ahead.)*

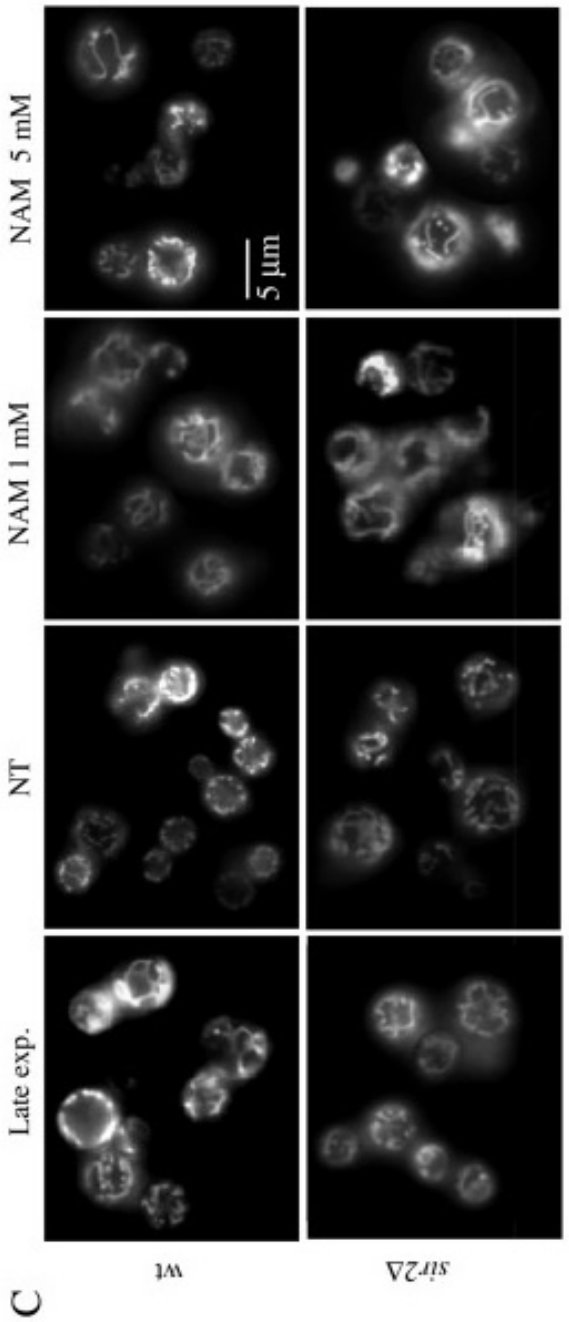

Figure 3.4 – *(Caption next page.)*

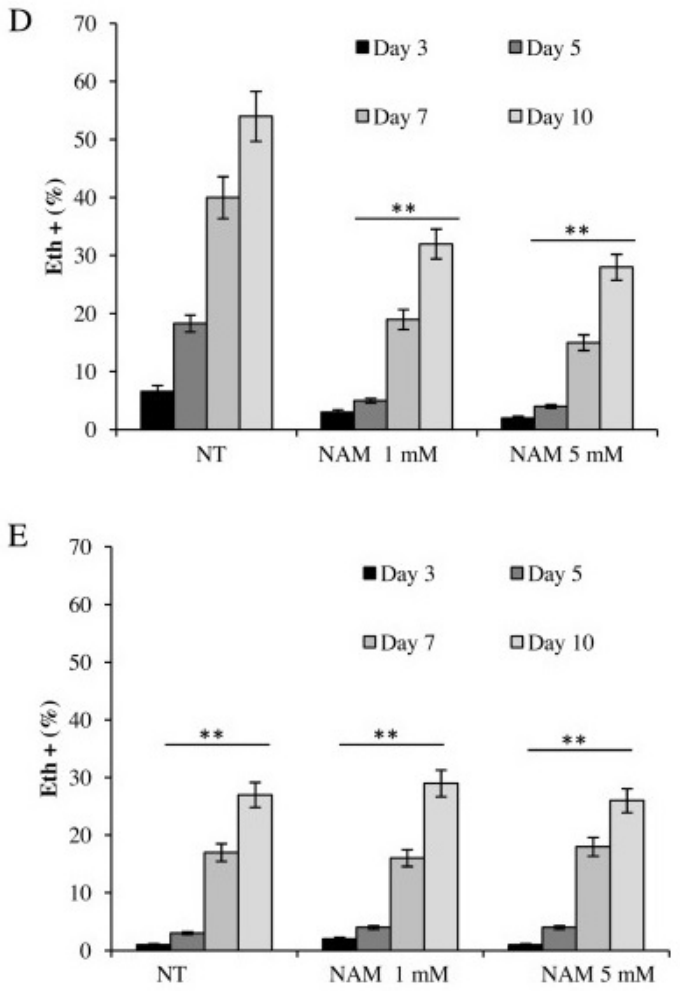

Figure 3.4 – Lack of Sir2 enzymatic activity has a positive effect on mitochondrial functionality. (A and B) After the diauxic shift (Day 0), at indicated time-points aliquots of wt and *sir2*Δ cultures, supplied with NAM as in Fig. 3.1, were serially diluted and plated onto YEPD and YEPG plates in order to determine the index of respiratory competence (IRC). SD of three independent experiments with three technical replicates each is indicated. (C) Representative images of cells from the same cultures at Day 7 stained with $DiOC_6$ to visualize mitochondrial membranes. Morphologies of the mitochondria in late exponential phase are also shown. (D and E) Summary graphs of the percentage of O_2^--accumulating cells assessed by the superoxide-driven conversion of non-fluorescent dihydroethidium into fluorescent ethidium (Eth). For the determination of Eth^+ cells, evaluation of about 1000 cells was performed in three independent experiments with three technical replicates. SD is indicated.(* $P \leq 0.05$ and ** $P \leq 0.01$).

Mitochondria, besides their primary role of generating energy and metabolic intermediates, are also the major intracellular site of reactive oxygen species (ROS) production, whose harmful effects are involved in promoting and/or accelerating chronological aging (Barros *et al.*, 2010; Pan, 2011; Breitenbach *et al.*, 2014). Thus, we next quantified ROS accumulation by using the non-fluorescent dihydroethidium (DHE) which is oxidized to fluorescent ethidium (Eth) in the presence of O_2^- which derives mainly from leakage of electrons from the respiratory chain. All cultures accumulated O_2^- as a function of time but in the *sir2*Δ and NAM-treated cells O_2^- content was significantly lower compared with that of the wt for all time points (Figure 3.4D and E). Since it is known that non-phosphorylating respiration is prone to generate O_2^- (Hlavata *et al.*, 2003; Guerrero-Castillo *et al.*, 2011) these data correlate well with the respiratory state observed after *SIR2* inactivation or NAM supplementation further suggesting that, in a population of chronologically aging cells, the state of respiration rather than its rate dictates the degree of O_2^- production. In conclusion, NAM supplementation to wt cells at the diauxic shift results in a phenocopy of chronologically aging *sir2*Δ cells. In fact, both display the same metabolic traits, O_2^-content and CLS extension both in expired medium and in extreme CR condition (water). It has been already shown that after the diauxic shift when cells utilize ethanol/acetate, lack of Sir2 correlates with an increase of the acetylated active form of Pck1, with an enhanced glyoxylate-requiring gluconeogenesis and increased trehalose stores (Casatta *et al.*, 2013). NAM supplementation at the onset of chronological aging allows the cells to push their metabolism toward the same outcomes by elevating the level of the acetylated Pck1. Furthermore, in the *sir2*Δ mutant, an enhanced glyoxylate-requiring gluconeogenesis favors growth in the presence of ethanol as carbon/energy source (Table S3; Casatta *et al.*, 2013). The same growth behavior is observed by providing NAM to wt cells during exponential phase, whilst no effect is detected for *sir2*Δ cultures (Table S3) providing further evidence that the metabolic trait of NAM-supplied cells are Sir2-mediated and that lack of Sir2 deacetylase activity affects gluconeogenesis. A significant number of metabolic enzymes involved in glycolysis/gluconeogenesis, the TCA cycle, as well as fatty acid synthesis/degradation are acetylated and their acetylation status

can directly influence their enzymatic activity or stability regulating the rate or direction of a metabolic flux (Wang *et al.*, 2010; Zhao *et al.*, 2010; Guan & Xiong, 2011; Wellen & Thompson, 2012; Xiong & Guan, 2012). This is also the case of human Pck1,whose SIRT2-mediated deacetylation plays an important role in maintaining glucose homeostasis in response to the availability of nutrients (Chakravarty *et al.*, 2005; Zhao *et al.*, 2010; Jiang *et al.*, 2011). Notably, our data reveal new other metabolic traits linked to the absence of the enzymatic activity of Sir2. In fact, during chronological aging, both NAM-supplemented and *sir2*Δ cells compared with wt ones have: i) decreased mitochondrial oxygen consumption, ii) a higher respiratory reserve capacity, and iii) a lower non-phosphorylating respiration. The respiratory activity is essential for chronological longevity (Bonawitz *et al.*, 2006; Aerts *et al.*, 2009; Ocampo *et al.*, 2012); however, yeast cells have a large respiratory capacity to sustain CLS and only mutations/deficiencies affecting respiration below a threshold (about 40% of wt) significantly limit CLS (Ocampo *et al.*, 2012). In addition, it is of fundamental importance that the respiratory activity is properly adjusted to sustain the metabolic remodeling at the diauxic shift and the changes that go along with the entry into the stationary phase (Bonawitz *et al.*, 2007; Ocampo *et al.*, 2012). These changes include slowing down energy consumption, enhancement of cell stress resistance and accumulation of storage carbohydrates. Lack of Sir2 activity has no effects on the maximal respiratory capacity of the cells that, although having a reduction of total oxygen consumption, are not respiring below the critical threshold. In other words, these cells are not respiratory deficient and retain the ability to reprogram efficiently their metabolism at the diauxic shift. Thus, the respiration was lower than that of the wt but not limiting and is enough to sustain anabolic pathways such as gluconeogenesis and the synthesis of trehalose. It is reasonable to hypothesize that the reduced respiration can result from the enhanced gluconeogenic flux occurring in the *sir2*Δ mutant as well as in NAM-supplied cells. During the post-diauxic phase, the cytosolic glyoxylate shunt plays an important role in the metabolic cross-feeding between the cytoplasm and the mitochondria. In fact, it is the exclusive source of both oxaloacetate, the substrate of the Pck1, which fuels the gluconeogenesis and C4 dicarboxylic acids that are required to replenish with intermediates the mitochondrially localized TCA cycle. These C4

compounds include oxaloacetate, malate and succinate. In particular, the last one is transported into the mitochondria by a counter-exchange with fumarate (Palmieri *et al.*, 1997). Moreover, in the mitochondria, succinate is oxidized to fumarate thus generating reduced flavin cofactors ($FADH_2$). This reaction is catalyzed by succinate dehydrogenase, a heterotetrameric enzyme complex (also known as Complex II), which is a physical link between the TCA cycle and the electron transport chain. In fact, the fumarate produced is utilized in the TCA cycle and $FADH_2$ enters the electron transport chain (Baile & Claypool, 2013). As discussed above, the lack of Sir2 activity favors the gluconeogenic route by increasing the acetylated active form of Pck1. In this context, there is an increased requirement of cytosolic oxaloacetate, that can be only supplied by the glyoxylate shunt. Consequently, an increase in the cytosolic oxaloacetate feeding might be required in order to fulfill the needs for driving an enhanced gluconeogenesis resulting in an attenuation of the metabolite fuel toward the TCA cycle which might lead to a reduced respiration. Gene expression profiles of 3-day-old *sir2*Δ cells show downregulation of genes encoding components of the Complex III (Cyt1, Cor1, Cyc1, Qcr6 and Qcr9) and Complex IV (Cox1, Cox4, Cox5b and Cox6) (Fabrizio *et al.*, 2005). Interestingly, both *sir2*Δ cells and NAM-supplied ones appear to modulate their respiratory efficiency by coupling ATP generation to electron transport better than the wt allowing them to obtain energy and, at the same time, to cope with a lower burden of O_2^-. In fact, despite a reduced respiration, these cells display a net respiration close to or even higher than that of the wt due to a reduced non-phosphorylating respiration. To the best of our knowledge, to date, there is no evidence that Sir2 directly controls the activity of the components of the electron transport chain. In addition, we observed no changes in the maximal respiratory capacity. In this context, we can only speculate that a lower charge of electron transport distributed over a not limiting respiratory capacity might reduce electron leakage from the respiratory chain resulting in a lower non-phosphorylating respiration that leads to a lower O_2^- production. Taken together, all these metabolic changes can contribute to the establishment of a more efficient quiescent program.

3.4 Materials & Methods

Yeast strains, growth conditions and CLS determination. All strains used in this work are listed in Table S1. The 3HA-tagged strains were undistinguishable from the congenic untagged ones with respect to overall morphology, cellular volumes, duplication times (Td), metabolite levels and CLS. Cells were grown in batches at 30 °C in minimal medium (Difco Yeast Nitrogen Base without amino acids, 6.7 g/L) with 2% w/v glucose and the required supplements added in excess as described (Orlandi *et al.*, 2014). Experiments were also performed by growing the histidine/leucine/uracil prototroph mutants in media containing the same supplements required by W303-1A obtaining similar results. Cell growth was monitored by counting cell number using a Coulter Counter-Particle Count and Size Analyser (Vanoni *et al.*, 1983) and, in parallel, the extracellular concentration of glucose and ethanol was measured in medium samples collected at different time points in order to define the growth profile (exponential phase, diauxic shift (Day 0), post-diauxic phase and stationary phase) of the cultures (Orlandi *et al.*, 2014). Td were obtained by linear regression of the cell number increase over time on a semilogarithmic plot. Treatments were performed at Day 0 by adding nicotinamide (NAM, Sigma) at the final concentrations of 1 mM and 5 mM. Cell survival in expired medium was monitored by harvesting aliquots of cells starting with 72 h (Day 3, first age-point) after the diauxic shift (Day 0). CLS was measured according to (Fabrizio *et al.*, 2005) by counting colony-forming units (CFU) every 2-3 days. The number of CFU on Day 3 was considered the initial survival (100%). Survival experiments in water (pH adjusted to 3.2) were performed as described (Orlandi *et al.*, 2013). Every 48 h, NAM (1 mM and 5 mM) was added to the culture after washing. Viability was assessed by CFU.

Metabolite measurements and enzymatic assays. At designated time points, aliquots of the yeast cultures were centrifuged, and both pellets (washed twice) and supernatants were collected and frozen at -80 °C until used. Glucose, ethanol and acetic acid concentrations in the growth medium were determined using enzymatic assays (K-HKGLU, K-ETOH, and K-ACET kits from Megazyme). Intracellular trehalose was extracted and measured as described in (Lee & Goldberg, 1998). The released glucose was quan-

tified using the K-HKGLU kit. ATP was extracted following the boiling buffered ethanol procedure (Gonzalez *et al.*, 1997) and quantified using the luciferin-luciferase assay (ATP determination kit, Molecular Probes) and a Cary Eclipse luminometer (Varian), according to the manufacturers' instructions. Cell dry weight was measured as described (Agrimi *et al.*, 2011). Immediately after preparation of cell-free extracts (Orlandi *et al.*, 2014), phosphoenolpyruvate carboxykinase (Pck1) and isocitrate lyase (Icl1) activities were assayed according to (de Jong-Gubbels *et al.*, 1995). Total protein concentration was estimated using the BCA^{TM} Protein Assay Kit (Pierce).

Immunoprecipitation and Western analysis. Total protein extracts were prepared and immunoprecipitated with anti-HA mAb (12CA5, Roche) according to (Vai *et al.*, 1990; Casatta *et al.*, 2013). Immunoprecipitated proteins were resolved by SDS-PAGE on 8% polyacrylamide slab gels and subjected to Western analysis. Immunodecoration was carried out using anti-HA (12CA5, Roche) and anti-acetylated-lysine (Ac-K-103, Cell Signaling) monoclonal primary antibodies. Secondary antibodies were purchased from Amersham. Binding was visualized with the ECL Western Blotting Detection Reagent (Amersham). After ECL detection, films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer and quantified with Scion Image software.

Respiration assays and fluorescence microscopy. The basal/routine oxygen consumption of intact cells was measured at 30 °C using a "Clark-type" oxygen electrode in a thermostatically controlled chamber (Oxygraph System, Hansatech Instruments, Nortfolk, UK) as previously reported (Orlandi *et al.*, 2013). Data were recorded at sampling intervals of 1 s (Oxigraph Plus software, Hansatech Instruments, Norfolk, UK). Triethyltin bromide (TET, Sigma) at 37.5 mM and carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) at 10 μM were added sequentially to the oxygraph chamber and oxygen consumption was followed online (Hlavata *et al.*, 2003; Agrimi *et al.*, 2011). The former allows the assessment of the non-phosphorylating respiration due to proton leakage and the latter to measure the maximal/uncoupled respiratory capacity by dissipating the proton gradient across the mitochondrial membrane. In this case, saturating amounts of ethanol

(100 mM) were provided as respiratory substrate (Dejean *et al.*, 2000). The addition of 2 M antimycin A (Sigma) accounted for non-mitochondrial oxygen consumption. Respiratory rates for the basal/routine oxygen consumption (J_R), the maximal/uncoupled oxygen consumption (J_{MAX}) and the non-phosphorylating oxygen consumption (J_{TET}) were determined from the slope of a plot of O_2 concentration against time, divided by the cellular concentration. The net respiration (netR) was estimated by subtracting J_{TET} from J_R and used to calculate the net routine control ratio as in (Gnaiger, 2014): netR/J_{MAX}.
Index of respiratory competence (IRC) was measured according to (Parrella & Longo, 2008) by plating identical samples on YEP/2% glucose (YEPD) agar plates and on YEP/3% v/v glycerol (YEPG) plates. IRC was calculated as colonies on YEPG divided by colonies on YEPD times 100%.
O_2^- were detected with dihydroethidium (DHE, Sigma) according to (Madeo *et al.*, 1999). Staining with 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$, Molecular Probes, Invitrogen) was performed as described in (Koning *et al.*, 1993) to analyze mitochondrial membrane potential and morphology. $DiOC_6$ staining was also performed after incubation with 10 μM CCCP for 15 min to induce mitochondrial membrane depolarization. Cells were counterstained with propidium iodide to discriminate between live and dead cells. A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350 F ccd camera was used. Digital images were acquired using FW4000 software (Leica).

Statistical analysis of data. All values are presented as the mean of three independent experiments ± standard deviation (SD). Three technical replicates were analyzed in each independent experiment. Statistical significance was assessed by one-way ANOVA test. The level of statistical significance was set at a P value of ≤ 0.05.

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Supplementary Material

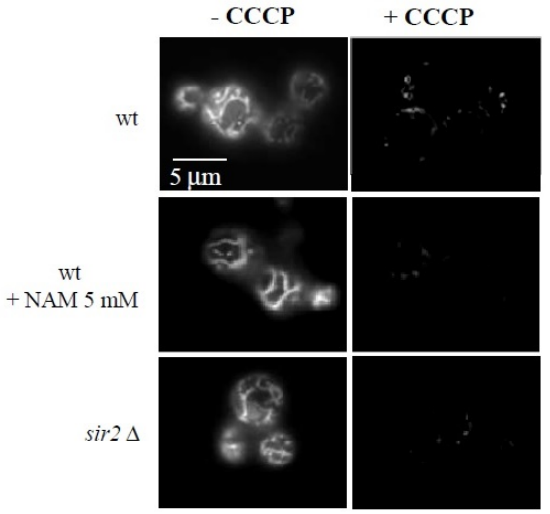

FIGURE S1. Effect of the uncoupler CCCP on membrane potential. Cells of the cultures of Fig. 3.4 at Day 7 were incubated with 10 μM CCCP for 15 minutes and then examined with $DiOC_6$ by fluorescence microscopy. Representative images are shown.

Strain	Genotype	Source
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	P.P. Slominski
YVU21	W303-1A *sir2Δ::URA3*	Calzari *et al.*, 2006
YVU83	W303-1A *pck1Δ::KlLEU2*	Casatta *et al.*, 2013
YVU84	W303-1A *sir2Δ::URA3 pck1Δ::KlLEU2*	Casatta *et al.*, 2013
YVU90	W303-1A *PCK1-3HA::KlURA3*	Casatta *et al.*, 2013
YVU91	W303-1A *sir2Δ::HIS3 PCK1-3HA::KlURA3*	Casatta *et al.*, 2013

Table S1. Strains used in this study.

Strain	Mean CLS	Maximum CLS
wt NT	8.9 ± 0.53	11.6 ± 0.31
wt 1 mM NAM	10.7 ± 0.40*	13.9 ± 0.47*
wt 5 mM NAM	12.1 ± 0.60**	17.4 ± 0.35**
*sir2*Δ NT	12.4 ± 0.42**	18.3 ± 0.47**
*sir2*Δ 1 mM NAM	12.1 ± 0.51**	18.4 ± 0.31**
*sir2*Δ 5 mM NAM	12.4 ± 0.26**	18.5 ± 0.21**

Table S2. NAM supplementation increases CLS similarly to *SIR2* inactivation. Data determined from CLS experiments of Fig. 3.1A which refer to the time-point where stationary cultures showed 50% (Mean CLS) and 10% (Maximum CLS) of survival. SD of three independent experiments with three technical replicates each is indicated. Values obtained for wt were used as reference for comparisons with the corresponding ones determined for NAM-supplied and *sir2*Δ cells. (* $P \leq 0.05$ and ** $P \leq 0.01$, oneway ANOVA test).

Strain	Td (h)
wt NT	4.42 ± 0.23
wt 5 mM NAM	3.34 ± 0.19**
*sir2*Δ NT	3.31 ± 0.36**
*sir2*Δ 5 mM NAM	3.35 ± 0.26**

Table S3. NAM supplementation favors growth on ethanol. Duplication time (Td) of the indicated strains growing on YEP medium with 2% ethanol as carbon source. *Td was calculated as ln2/k, where k is the constant rate of exponential growth. Data represent the average of three independent experiments. SD is indicated. Values obtained for wt were used as reference for comparisons with the corresponding ones determined for NAM-supplied and *sir2*Δ cells. (** $P \leq 0.01$, oneway ANOVA test).

4 Compounds related to Acetyl-CoA - Fatty acids and Survival during Aging

Manuscript in Preparation

4.1 Abstract

Among its fates, acetyl-CoA has a main role in lipid metabolism. For instance, from an anabolic perspective, it represents the source of carbon units to build lipid macromolecules, such as fatty acids, that if esterified with glycerol give triacylglycerols, storage molecules that are conserved inside lipid droplets. From a catabolic perspective, when required, triacylglycerols are used as a source of fatty acids, which if processed through β-oxidation inside peroxisomes, give back acetyl-CoA units to satisfy cellular carbon or energy needs. In the model organism *Saccharomyces cerevisiae*, the expression of genes involved in the catabolism of fatty acids relies on the presence of Oaf1, a member of the Zn_2Cys_6 protein family of transcriptional activators. Oaf1 is regarded as a fatty acid sensor: in fact, it is activated by fatty acids, such as the unsaturated oleate (C18:1), and together with its sole partner Pip2, forms a functional heterodimer which induces the expression of genes involved in β-oxidation and peroxisome biogenesis and proliferation. Here, we show that the lack of *OAF1* impedes on one side to generate acetyl-CoA from fatty acids, and on the other side hinders their canalization in the biosynthetic pathways. The capacity to still store but not use fatty acids generates “obese” cells, with massive lipid droplets and an increased level of free fatty acids, which in turn promotes the activation of stress defences and lipid-induced cell death. This situation strikingly resembles that of mammalian white obese adipocytes, further increasing the relevance of yeast as a powerful model organism. In conclusion, the importance to efficiently obtain and use fatty acids, for instance to produce acetyl-CoA, demonstrates how not just the molecule itself, but the whole acetyl-CoA metabolism can be viewed as a major determinant of cellular growth and aging.

4.2 Introduction

During the last decades, the relation between nutrients and cellular aging has been uncovered, and the investigation of the molecular events behind the effects of diet on cellular longevity is actually an intense research field. Fatty acids, already known for their structural role in cellular membranes and as functional groups for post-translational modifications, also represent a valuable nutritional source: in fact, when catabolised through β-oxidation, fatty acids provide acetyl-CoA, an acknowledged key intermediate linking carbon metabolism to cellular aging in a multifactorial way. To explore the relation between fatty acid metabolism and cellular aging, the budding yeast *Saccharomyces cerevisiae* was employed as a model organism. In the yeast *S. cerevisiae*, *OAF1* encodes a member of the Zn_2Cys_6 protein family of transcriptional activators that is essential for the induction of genes involved in the catabolism of fatty acids (Rottensteiner *et al.*, 1997). Oaf1 is activated by fatty acids, such as the unsaturated oleate (C18:1) or the saturated palmitate (C16:0), and in turn binds its partner Pip2, forming a functional heterodimer which is responsible for the regulation of transcription (Rottensteiner *et al.*, 1997). The Oaf1:Pip2 heterodimer binds to a specific upstream activating sequence found in target genes, the oleate-response element (ORE) (Rottensteiner *et al.*, 1997). Among the genes that present the ORE there are those involved in β-oxidation of fatty acids, peroxisome biogenesis and peroxisome organization. In particular, in *S. cerevisiae* peroxisomes are the exclusive site for β-oxidation (Kunau *et al.*, 1995; Trotter, 2001; van Roermund *et al.*, 2003). The number and size of operative peroxisomes is largely determined by nutritional conditions, in particular by the available carbon sources (Veenhius *et al.*, 1987; Rottensteiner *et al.*, 1997). While in presence of a fermentable sugar like glucose, acknowledged as the yeast preferred carbon source, the transcription of genes involved in the metabolism of fatty acids is repressed, in presence of non-fermentable carbon sources such as ethanol or glycerol transcription is derepressed, and with oleate or palmitate is induced (Baumgartner *et al.*, 1999; Phelps *et al.*, 2006; Karpichev *et al.*, 2008), causing a proliferation of peroxisomes coupled to the induction of β-oxidation (Veenhuis *et al.*, 1987). When not supplied in the culture medium, yeast cells produce fatty acids by endogenous synthesis

(Tehlivets *et al.,* 2007; Petchnigg *et al.,* 2009). Even so, uptake of extracellular fatty acids appears to be not regulated according to cellular lipid requirements (Petchnigg *et al.,* 2009), underlyning the adaptability of the control of lipid homeostasis. In presence of an excess of fatty acids, yeast cells resort to their esterification using glycerol, forming storage triacylglycerols (TAGs) which are accumulated in lipid droplets (LDs) (Kohlwein, 2010). LDs are ubiquitous intracellular organelles found in prokaryotes as well as in eukaryotes ranging from fungi to humans, and are used as lipid depots (Hapala *et al.,* 2011). In particular, LDs are composed by a bulk of TAGs and steryl esters surrounded by a phospholipid monolayer (Leber *et al.*, 1994). This system allows cells to create an organized pool of esterified fatty acids, which are in turn released and used in a controlled manner as cells grow and divide or enter the post-mitotic stationary phase (Petchnigg *et al.,* 2009; Kohlwein, 2010). Further, this system is also a powerful buffering tool as an overload of fatty acids has been shown to be toxic (*lipotoxicity*) and may induce a type of programmed cell death termed "liponecrosis" (Sheibani *et al.,* 2014; Richard *et al.,* 2014). Fatty-acid induced cell death is evolutionarily conserved, and adipose and non-adipose human cells such as liver, kidney and pancreatic β cells are sensible to lipotoxicity (Cinti *et al.,* 2005; Wei *et al.,* 2006; Laybutt *et al.,* 2007; Cunha *et al.,* 2008; Murano et al., 2008; Katsoulier *et al.,* 2009). Therefore, liponecrosis may represent an important new process involved in the incidence of pathologies such as non-alcoholic fatty liver disease, renal diseases and type 2 diabetes, all strongly related to obesity (Wu *et al.,* 2016; Gross *et al.,* 2016). Moreover, it was discovered that the transcription factor Oaf1 has ligand-binding domain, function and regulation resembling those of mammalian PPARs (Phelps *et al.,* 2006), well-known for their role in the aforementioned non-communicable diseases (Gross *et al.,* 2016; Polvani *et al.,* 2016).

Here, we describe the behaviour of *oaf1*Δ cells in a panel of different media conditions both during growth and when growth stops. Mutant cells were analysed from a cellular and molecular perspective, which well couples with the transcriptional and biochemical data available in literature (Baumgartner *et al.,* 1999; Rottenstainer *et al.,* 1996, 1997, 2003; Karpichev *et al.,* 2008). Our purpose was to investigate in a shor-time period the effects of the fatty acid oleate in a cellular context where lipid metabolism

is strongly impaired. The results obtained stress the point on how nutrients, in this case carbon sources, may represent a strong driving factor, ultimately regulating life and death of a cell.

4.3 Results & Discussion

Oaf1 is indispensable for growth in presence of oleate, ensuring the correct usage and storage of fatty acids. Yeast cells can use different organic compounds as carbon sources, which can be divided in fermentable and non-fermentable. This division is based on the capability of a carbon source to favour different types of metabolism, namely fermentation or respiration. When *S. cerevisiae* is grown in media containing high concentrations of glucose as sole carbon source, fermentation occurs (Gancedo, 1998; Carlson, 1999). This fermentative metabolism is defined by a high glycolytic rate, a negligible aerobic respiration and in general by the down-regulation of the expression of genes related to the metabolism of other carbon sources by means of a glucose-mediated repression mechanism (Gancedo, 1998; Carlson 1999; Schüller, 2003). On the contrary, ethanol, a non-fermentable carbon source, involves a massive expression of genes related to gluconeogenesis, the TCA cycle and mitochondrial biogenesis (DeRisi *et al.*, 1997; Carlson, 1998; Orlandi *et al.*, 2013; Turcotte *et al.*, 2009). Other non-fermentable carbon sources, such as fatty acids, for their usage require also the biogenesis of dedicated compartments, the peroxisomes (Veenhius *et al.*, 1987). It follows that every carbon source influences yeast growth in a peculiar manner. Therefore, we decided to examine how differently carbon sources could influence the growth of *oaf1*Δ mutant cells. At a first analysis, the spot assay (Figure 4.1) showed that wild type (wt) and *oaf1*Δ cells were both able to grow on solid media containing glucose, ethanol or glycerol. On the contrary, on sole oleate, wt cells were markedly slow-growing while *oaf1*Δ cells were clearly incompetent (Figure 4.1), in agreement with literature (Luo *et al.*, 1996). A deeper analysis revealed a differential pattern also in growth kinetics (Figure 4.2), underlyning that Oaf1 is a transcriptional factor specific for the metabolism of fatty acids and essential for growth in presence of fatty acids as sole carbon source, as *oaf1*Δ cells were not able to grow on oleate alone. Moreover, the growth of the *oaf1*Δ strain in presence of the mix ethanol/glycerol/oleate compared to the mix ethanol/glycerol highlighted that the lack of Oaf1 goes further the incapacity to metabolise fatty acids and has a broader effect since it reduces the growth rate even in a non-repressed condition where other carbon sources, such as ethanol and glycerol, are available and usable

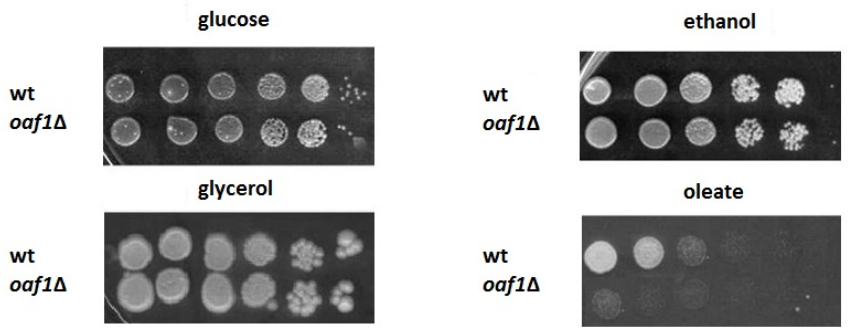

Figure 4.1 – Oleate is not used by *oaf1*Δ as a carbon source. A representative experiment is shown. Images were obtained taking into account the different growth conditions and relative incubation times. Starting from an amount of 10^8 total exponential-growth phase cells, six ten-fold dilutions were performed.

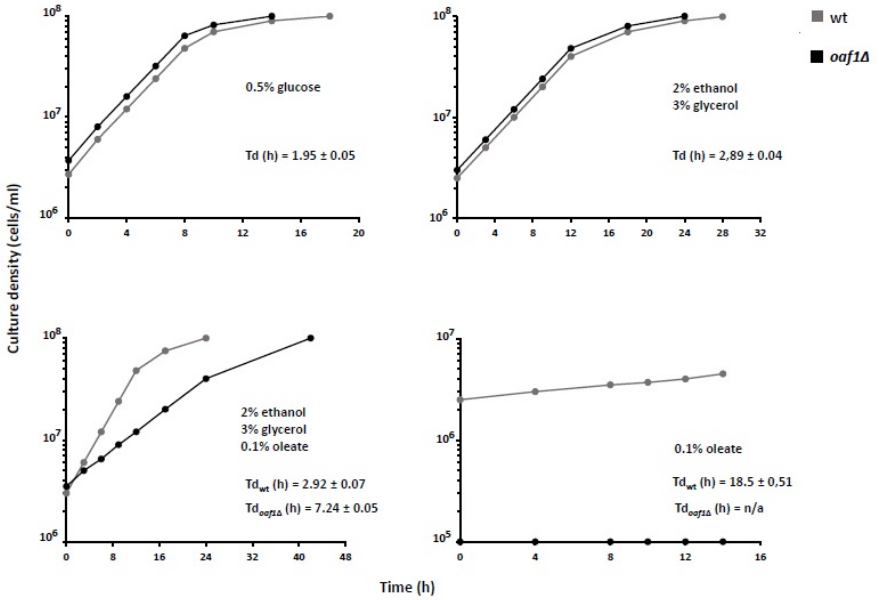

Figure 4.2 – Oleate has a deleterious effect on the growth of *oaf1*Δ cells. A representative experiment is shown. All strains were inoculated at the same cellular density (culture volume no more than 20% of the flask volume). Growth was monitored by determining cell number using a Coulter Counter-Particle Count and Size Analyser, as described (Vanoni *et al.*, 1983). Doubling time (Td) from different growth curves, obtained by linear regression of the cell number increase over time on a semilogarithmic plot, is reported as mean ± standard deviation (SD).

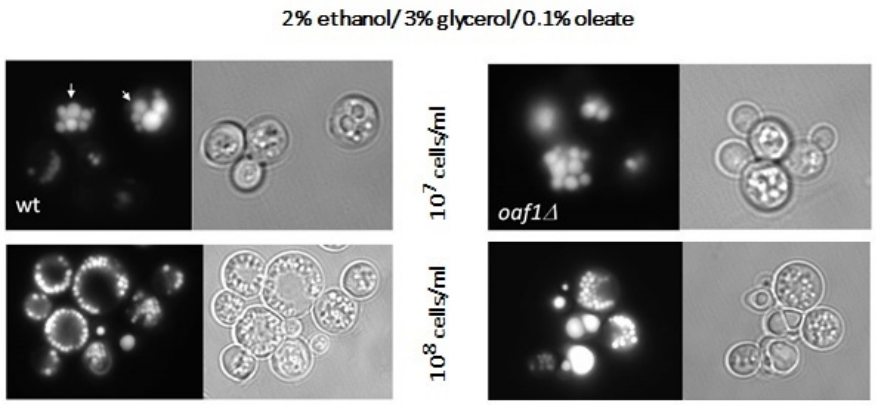

Figure 4.3 – The mutant *oaf1*Δ is prone towards the accumulation of big LDs. Representative image of wt and *oaf1*Δ cells stained with Nile Red to visualize LDs at key cellular densities (during growth and after growth stopped).

(Figure 4.2). This means that the sole presence of oleate is able to induce a manifestation of the *oaf1*Δ phenotype. Nevertheless, all growing strains were able to reach a final culture density of approximately 10^8 cells/ml independently from the mix of carbon sources. A noteworthy difference was also noticed in cell volumes during growth and became evident when growth stopped. While in the mix ethanol/glycerol, wt and *oaf1*Δ cells had comparable volumes (45.7 ± 0.4 μm^3 and 46.7 ± 0.5 μm^3, respectively), in presence of oleate, compared to wt cells, *oaf1*Δ cells had a clearly higher volume (46.5 ± 0.5 μm^3 and 49.5 ± 0.5 μm^3, respectively). Among the hypotheses, this difference in volumes, coupled to the observation of big vacuolar-like structures (Figure 4.3, greyscale) suggested the presence of a metabolic incompetence in *oaf1*Δ cells to be found in the capacity to uptake and store but not consume fatty acids due to the lack of *OAF1*.

Therefore, we analysed the LDs content with Nile Red staining (Greenspan *et al.*, 1985). In wt cells, the accumulation of LDs was characterized by an initial generation of these stores, increasing in both size and number, followed by usage in time (Figure 4.3). In contrast, *oaf1*Δ cells overaccumulated LDs, thus building up massive intracellular lipid pools which lasted in time (Figures 4.3 and 4.4). For their shape, these latter LDs were found to be reminiscent of the ones found in mammalian white adipocytes. In this sense, the capacity of the cell to buffer an excess of fatty acids can be

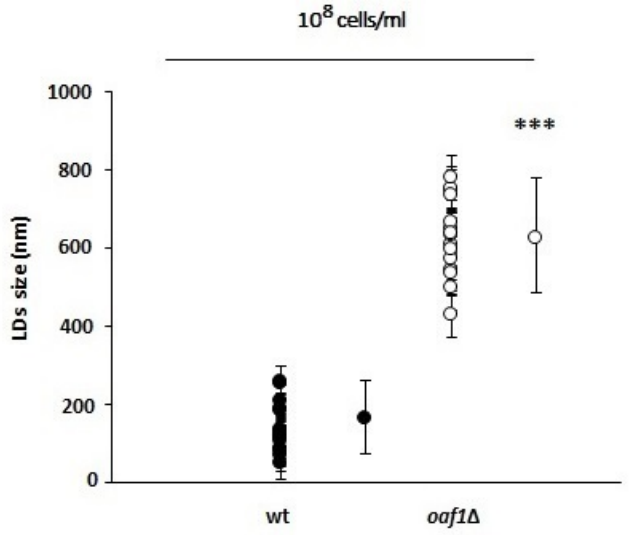

Figure 4.4 – LDs size increases in *oaf1*Δ cells in presence of oleate. Wt and *oaf1*Δ mutant cells were grown in presence of 2% ethanol/ 3% glycerol/ 0.1% oleate and the required supplements (see *Materials & Methods*) and analysed after growth stopped. For the determination of LDs size, evaluation of about 1000 cells for each sample in three independent experiments was performed. SD is indicated. Mean size are reported as single dots. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

overcome, bringing to a high level of free fatty acids (FFA), which has been shown to be toxic (Garbarino *et al.*, 2009; Petschnigg *et al.*, 2009; Connerth *et al.*, 2010; Rockenfeller *et al.*, 2010; Ruggles *et al.*, 2014). On this basis, we decided to analyse the lipid composition of cells by thin-layer chromatography (Thomson III *et al.*, 1965; Folch *et al.*, 1975). Compared to wt cells at the same culture density, *oaf1*Δ cells showed a higher amount of FFA (Figure 4.5). The absence of *OAF1* has a direct impact on the catabolism of fatty acids, while the anabolic pathways remain functional, meaning that *oaf1*Δ cells are able only to accumulate and store oleate. These findings in yeast have striking similarities with obesity in humans. In fact, an overaccumulation of LDs is a distinct trait of obesity. The adipose tissue is critical in regulating lipid metabolism and exceeding the storage capacity of adipocytes saturates them with a big, massive intracellular LDs (Harms & Seale, 2013; Cedikova *et al.*, 2016), consequently rising the amount of FFA (van Herpen & Schrauwen-Hinderling ,2008; Brookheart *et al.*, 2009; Virtue & Vidal-Puig ,2010; Krahmer *et al.*, 2013). In this sense, the mutant *oaf1*Δ phenocopies adipocytes during obesity.

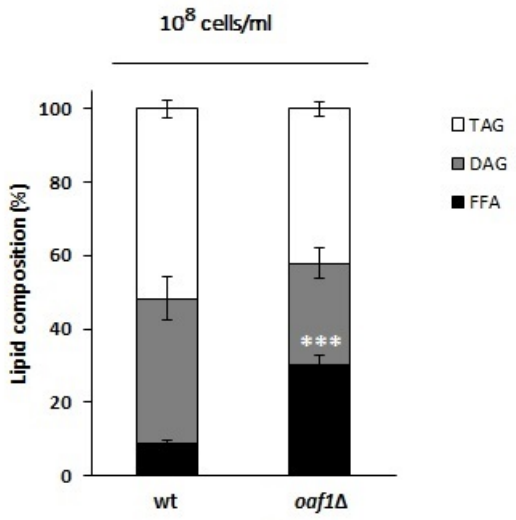

Figure 4.5 – The lipidic pattern is altered in *oaf1*Δ cells in presence of oleate and free fatty acids markedly increase. Wt and *oaf1*Δ cells were grown in presence of 2% ethanol/ 3% glycerol/ 0.1% oleate and the required supplements (see *Materials & Methods*) and analysed after growth stopped. Lipid categories are represented as percentages. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

Oaf1 is indispensable for protection against lipotoxicity and liponecrosis. The lipotoxic effect of FFA was found in both yeasts and mammals (Kohlwein, 2010). Both chain length and degree of unsaturation of a FFA are correlated to the level of toxicity (Eisenberg & Büttner, 2014). In yeast, a shorter chain length as well as a higher degree of unsaturation contribute to a higher toxicity, and efforts are being made to classify lipid-induced cell death (Garbarino *et al.*, 2009; Petschnigg *et al.*, 2009; Rockenfeller *et al.*, 2010; ; Richard *et al.*, 2014; Sheibani *et al.*, 2014). On this basis, we analysed *oaf1*Δ mutants performing an annexin V and propidium iodide (PI) staining to assess the involvement of apoptosis and necrosis. Cultures were grown using the carbon source mix ethanol/glycerol/oleate, that is, the previously identified derepressed condition in which cells can respond to the presence of oleate. As shown in Figure 4.6, at an equivalent culture density, the percentage of *oaf1*Δ cells positive to PI was around 5-fold that of wt cells. Overall, this analysis shows that, when in presence of non-

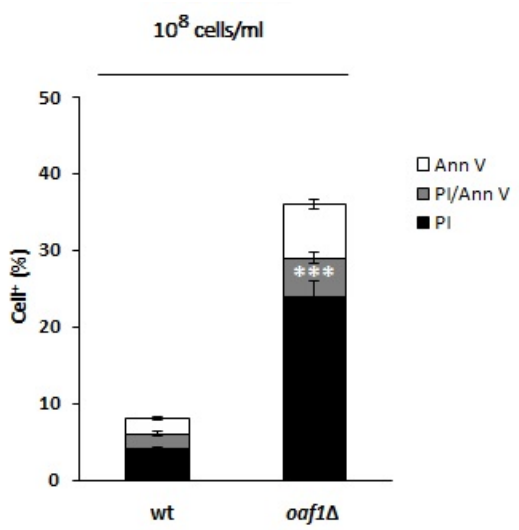

Figure 4.6 – The lipidic pattern of *oaf1*Δ cells can be related to an increase in cell death. Wt and *oaf1*Δ cells were grown in presence of 2% ethanol/ 3% glycerol/ 0.1% oleate and the required supplements (see *Materials & Methods*) and analysed after growth stopped. Spheroplasts were prepared and the exposed phosphatidylserine was visualized with the FITC-labelled Annexin V binding assay. Annexinv V (Ann V), Propidium iodide (PI). Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

esterfied oleate, *oaf1*Δ cells undergo an early cell death with a necrotic-like pattern, as suggested by the high percentage of PI^+ *oaf1*Δ cells. This cell death pattern is in agreement with literature (Rockenfeller *et al.*, 2010; Richard *et al.*, 2014; Sheibani *et al.*, 2014) and mostly important, is reminiscent of the necrosis which affects white obese adipocytes in mammals, including humans (Cinti *et al.*, 2005; Strissel *et al.*, 2007; Murano *et al.*, 2008; West, 2009; Giordano *et al.*, 2013).
Further, in the latest years, lipid homeostasis has been linked to the endoplasmic reticulum stress and lipid perturbations emerged as a novel, evolutionarily conserved trigger of the unfolded protein response (UPR) in organisms ranging from yeast to mammals (Deguil *et al.*, 2011; Austriaco, 2012; Volmer & Ron, 2015). In particular, the same cause-effect nexus was confirmed in different mammalian cells, including obese adipocytes (Kawasaki *et al.*, 2012; Zha & Zhou, 2012; Han & Kaufman, 2016). Therefore, we evaluated the UPR activation in wt and *oaf1*Δ cells in presence and absence of oleate using an appropriate UPRE-*CYC*-*lacZ* reporter system. In this reporter, the UPR response element regulates the expression of β-galactosidase, of which the activity can be assayed (see *Materials & Methods*). As shown in Figure 4.7, *oaf1*Δ cells, compared to wt cells, present an early difference in the UPR induction and, as population grows, the UPR induction gets higher, indicating a lipid perturbation related to oleate. Since one of the UPR downstream effects is to promote autophagy (Chakraborty *et al.*, 2016), we investigated whether autophagy could be induced or not in *oaf1*Δ cells in presence of oleate. For this purpose, the well-known GFP-Atg8 reporter system was used (Nair *et al.*, 2011; *Materials & Methods*). This chimeric protein is encoded by a sequence under the control of the native *ATG8* promoter in the proper locus. GFP-Atg8 can be used to build a functional autophagosome, and after the fusion of this latter with a vacuole, the autophagic body breaks down and as soon as proteins are exposed to vacuolar hydrolases the GFP moiety is released. Both GFP-Atg8 and free GFP can be then quantified through western blot analysis and correlate with the autophagic rate. As shown in Figure 4.8, *oaf1*Δ cells, compared to wt cells, present an increase in the activation of autophagy and indeed this can be ascribed to the activation of the UPR promoted by oleate.

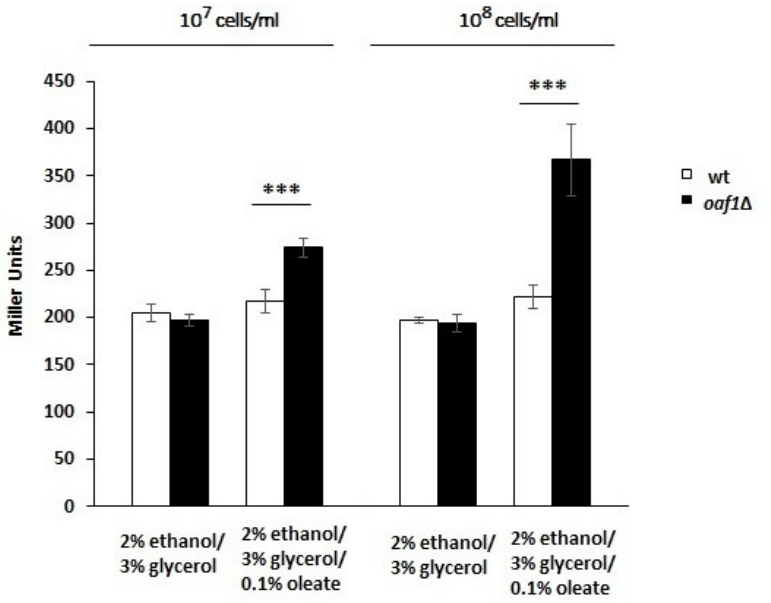

Figure 4.7 – The UPR in *oaf1*Δ cells is induced by oleate. β-galactosidase activity (reported in Miller Units) was monitored in wt and *oaf1*Δ cells bearing the p*URA3*-UPRE-*CYC1-lacZ* plasmid and grown in presence of 2% ethanol/ 3% glycerol ± 0.1% oleate. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

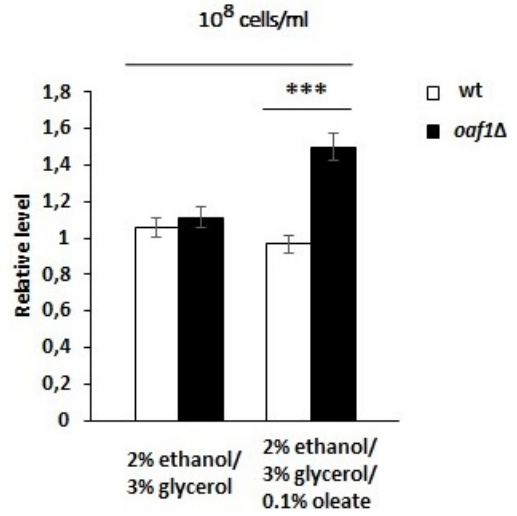

Figure 4.8 – The UPR induced by oleate promotes autophagy in *oaf1*Δ cells. Wt and *oaf1*Δ cells bearing the EGFP-ATG8 construct were grown in presence of 2% ethanol/ 3% glycerol ± 0.1% oleate. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

Mitochondria are sensitive to fatty acids and behave as sensor organelles for lipotoxicity. FFA have been associated to mitochondrial dysfunction (Garbarino *et al.*, 2009; Rockenfeller *et al.*, 2010). As first evidence, an impairment in mitochondrial respiration was reported by the index of respiratory competence (IRC) (Figure 4.9). Wt and *oaf1*Δ cells were cultured in presence of the mixes ethanol/glycerol and ethanol/glycerol/oleate, then plated on YEPD (1% yeast extract, 2% peptone, 2% D-glucose and 2% agar) and YEPG (1% yeast extract, 2% peptone, 3% glycerol and 2% agar) plates at selected time-points, and ultimately IRC was estimated by the ratio of colony-forming units (CFU) (see *Materials & Methods*). Since glucose can be metabolised through fermentation but glycerol necessarily requires respiration, mitochondrial functionality becomes essential for growth on YEPG media. As observed, wt and *oaf1*Δ cells grown in presence and absence of oleate retained initially a comparable respiratory competence (Figure 4.9). In contrast, after a long exposure to oleate, compared to wt cells, *oaf1*Δ cells strongly reduced their clonogenic potential on glycerol, which indicates that mitochondrial respiration is impaired. Moreover, 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) stainings showed that, compared to wt cells, *oaf1*Δ cells present a decrease of the mitochondrial membrane potential in presence of oleate (Figure 4.10). This supported the finding of a decrease in respiration capacities reported by the IRC assay (Figure 4.9). Also, the fluorescence emitted by mitochondria in *oaf1*Δ cells over time turned punctiform (Figure 4.10), indicating that mitochondrial fission is enhanced, another characteristic related to mitochondrial dysfunction (Westermann, 2010; Giedt *et al.*, 2016).

Since reactive oxygen species (ROS) are a hallmark of mitochondrial dysfunction (Ott *et al.*, 2009; López-Otín *et al.*, 2013; Breitenbach *et al.*, 2014, Beach *et al.* 2015), we performed a dihydrorhodamine 123 (DHR123) staining to identifiy if oleate-dependent mitochondrial dysfunction in *oaf1*Δ cells could be characterized by the presence of H_2O_2. Indeed, the sole absence of oleate could prevent in *oaf1*Δ cells a burst in the generation of H_2O_2, while a rapidly increasing amount of *oaf1*Δ $DHR123^+$ cells was visible in presence of oleate (Figure 4.11). Such a high increase in ROS content can be ascribed to a mitochondrial dysfunction but also to an impaired detoxification system, with a consequent increase in sensitivity towards ROS. Therefore, we tested cell resistance to increasing concen-

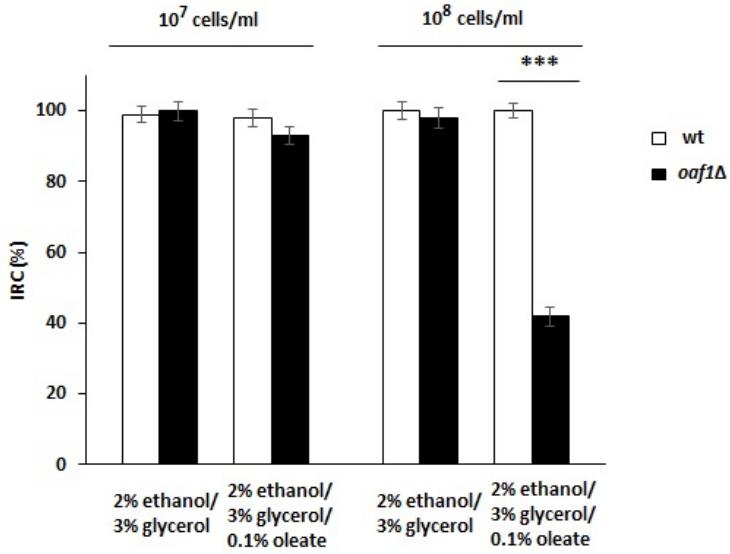

Figure 4.9 – Respiratory competence is decreased in *oaf1*Δ cells exposed to oleate. Wt and *oaf1*Δ cultures at the indicated density points were diluted and samples plated onto YEPD and YEPG plates. IRC was determined as described (see *Materials & Methods*). Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

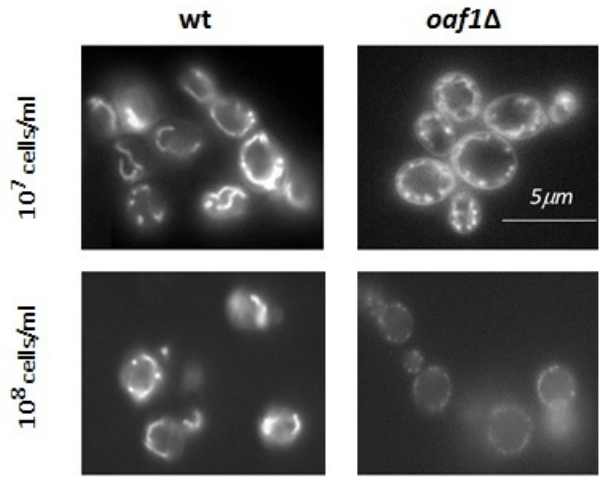

Figure 4.10 – Oleate impairs mitochondrial functionality in *oaf1*Δ. Representative image of wt and *oaf1*Δ cells stained with $DiOC_6$ to visualize mitochondrial membrane potential.

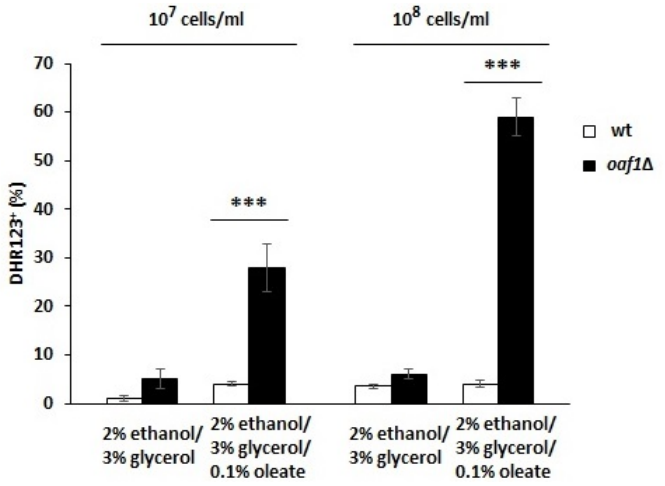

Figure 4.11 – Mitochondrial dysfunction in *oaf1*Δ cells is associated to the presence of ROS. Summary graph of DHR123$^+$ cells determined in wt and *oaf1*Δ cells grown in presence of 2% ethanol/ 3% glycerol ± 0.1% oleate. For the determination of DHR123$^+$ cells, evaluation of about 1000 cells for each sample in three independent experiments was performed. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

trations of H_2O_2. As shown in Figure 4.12, compared to wt cells, *oaf1*Δ mutants were very sensitive and after exposure to 5 mM H_2O_2, vitality could be almost abrogated. Indeed, peroxisomes are regarded as a source of oxidative stress, since H_2O_2 is a major product of β-oxidation (Hiltunen *et al.*, 2003; Schrader & Fahimi, 2006; Antonenkov *et al* 2010). This is not the case of *oaf1*Δ cells, as peroxisome biogenesis and proliferation is impaired (Karpichev *et al.*, 1997), ascribing mitochondria as H_2O_2 producers. As a defence system, yeast possesses a specific peroxisomal catalase encoded in *CTA1* whose expression is controlled by Oaf1:Pip2 in presence of oleate, but also by Adr1 in presence of ethanol (Simon *et al.*, 1991; Kunau & Hartig, 1992; Gurvitz & Rottensteiner, 2006). Moreover, Cta1 was found to be targeted also to mitochondria (Petrova *et al.*, 2004). Worthy of notice, it has to be underlined that in contrast to mammals, in *S. cerevisiae* fatty acids are degraded only in peroxisomes (Kunau *et al.*, 1995; Trotter, 2001; van Roermund *et al.*, 2003). Still, mitochondria are regarded as the major ROS producers, and this increases the importance of Cta1 in oxidative stess protection. In a condition such as that generated by the deletion of

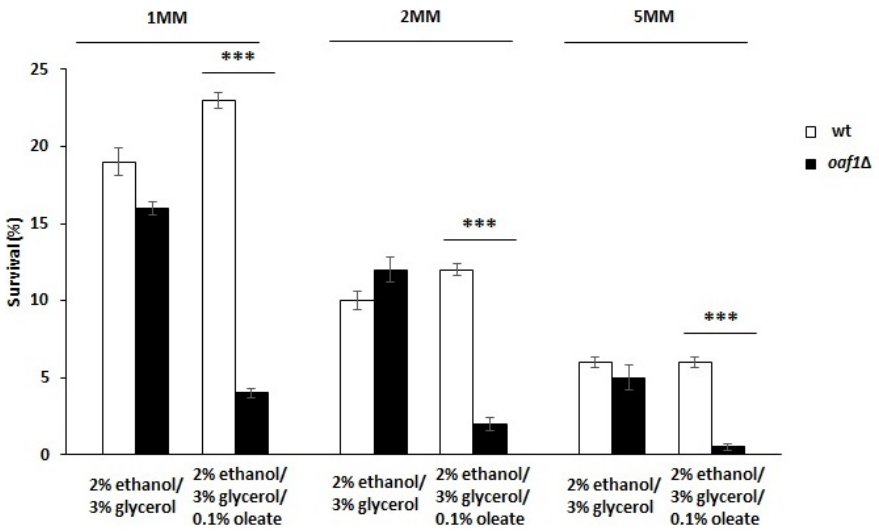

Figure 4.12 – A higher sensitivity towards H_2O_2 characterizes *oaf1*Δ cells. Wt and *oaf1*Δ cells growing in presence of 2% ethanol/ 3% glycerol ± 0.1% oleate were treated with 1 mM, 2 mM or 5 mM H_2O_2 for 120 minutes and plated on YEPD plates. Survival of untreated cells was considered as 100%. Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

OAF1, expression of *CTA1* results impaired, and the control exerted by Adr1 alone is not sufficient to induce levels of expression as the ones guaranteed by Oaf1:Pip2 (Gurvitz *et al.*, 2001). In addition, compared to wt cells, the catalase activity in *oaf1*Δ cells is strongly reduced (Figure 4.13).

4.4 Conclusions

In this article, we show that the lack of *OAF1* can be a decisive factor in presence of oleate as carbon source, on one side because it is essential for growth on fatty acids, on the other side because the negative effects go further the hindrance in using fatty acids as carbon/energy source. In fact, recapitulating, oleate was shown to induce a high content of FFA in *oaf1*Δ cells, associated with an impairment in mitochondrial functions, linked to a decrease in catalase defence and an increase in H_2O_2 content. Further investigations will provide new tesserae regarding the mechanism of lipotoxicity. Moreover, in the context of this thesis, strong evidence

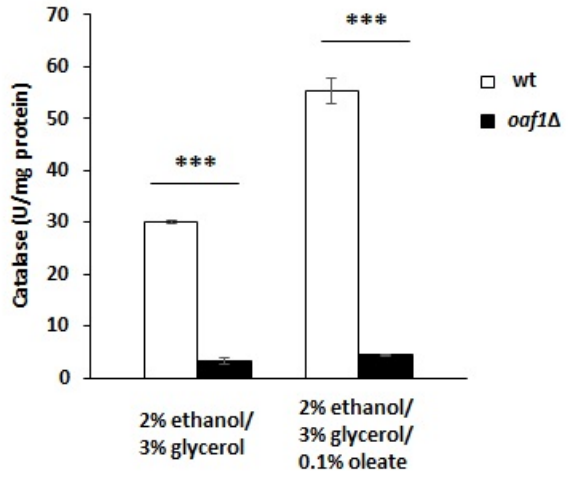

Figure 4.13 – The higher sensitivity towards H_2O_2 of *oaf1*Δ cells is correlated to a decreased catalase activity in presence of oleate. Catalase activity was determined as described (see *Materials & Methods*). Data refer to mean values of three independent experiments. SD is indicated. Statistical significance as assessed by *t*-test is indicated (*** $p \leq 0.001$).

was provided that not just acetyl-CoA as a molecule, but the management of the whole acetyl-CoA metabolism, including events located in its production, such as β-oxidation, are fundamental to regulate cellular longevity, therefore contributing to the view of acetyl-CoA metabolism as a major determinant in the mechanism of aging. On the other hand, data presented here establish a relation between lipotoxicity in yeast and adipocytes, reinforcing the notion of this unicellular eukaryote as a powerful model organism. In the last decades, the budding yeast has proven to be a successful tool to study evolutionarily conserved mechanisms. In fact, this single-celled eukaryote offers a very broad and flexible cellular context, which in turn already resembles or can be manipulated in order to mimic specific traits of mammalian cells, including those acquired through cellular differentiation, thus further increasing its value as a model organism. In agreement, the mammalian PPARs, that are themselves fatty acid sensors that act as transcriptional factors to regulate lipid metabolism, were found to be functional homologues of Oaf1 (Phelps *et al.*, 2010). In this particular study, it is shown that the *oaf1*Δ mutant has many paral-

lelisms with mammalian white adipocytes exposed to an excess of fatty acids. Taken all together, with this evidence, the *oaf1*Δ mutant can be proposed as an efficient model to study white obese adipocytes. Further, a mutant carrying a chimeric Oaf1 protein bearing the ligand-binding domain of mammalian PPARs could be designed and, in the context of and adequate reporter system, used as a tool to evaluate, at an early preclinical stage, the activating power of promising agonists to be used in therapeutic interventions for lipid-related diseases.

4.5 Materials & Methods

Media, strains and growth conditions. All yeast strains used in this study are listed in Table 4.1. All deletions were generated by PCR-based methods (Baudin *et al.*, 1993). 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. 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% glucose (w/v), 2% ethanol (v/v), 3% glycerol (v/v) or 0.1% oleate (v/v) as reported in the text. Auxotrophies were compensated with supplements (*Chapter 3*). 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 in (Vanoni *et al.*, 1983). Doubling time was obtained by linear regression of the cell number increase over time on a semilogarithmic plot.

Strain	Genotype	Source
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	P.P. Slominski
*oaf1*Δ	W303-1A *oaf1*Δ*::HIS3*	This study
GAL-wt	W303-1A [p*URA3*-UPRE-*CYC1-lacZ*]	This study
GAL-*oaf1*Δ	W303-1A *oaf1*Δ*::HIS3* [p*URA3*-UPRE-*CYC1-lacZ*]	This study
W303-1A pATG8-EGFP-ATG8	W303-1A *pATG8::natNT2-pATG8-EGFP*	This study
*oaf1*Δ pATG8-EGFP-ATG8	W303-1A *oaf1*Δ*::HIS3 pATG8::natNT2-pATG8-EGFP*	This study

Table 4.1. Strains used in this study.

Spot assay. Exponential-growth phase cells were dropped (5 μl from six 10-fold dilutions of a starting concentration of 10^8 cells/ml) onto agar plates supplemented with the aforementioned nutrient sources. Plates were incubated at 30 °C for 3-5 days, depending on the carbon source.

Index of respiratory competence (IRC). IRC was measured according to

(Parrella & Longo, 2008) by plating identical samples on YEPD plates (1% yeast extract (w/v), 2% peptone (w/v), 2% D-glucose (w/v) and 2% agar (w/v)) and on YEPG plates (1% yeast extract, 2% peptone, 3% glycerol (v/v) and 2% agar). IRC was calculated as colonies on YEPG divided by colonies on YEPD taken as 100%.

Fluorescence microscopy. Lipid droplets were detected with Nile Red (Sigma) as described in (Greenspan *et al.*, 1985) and analysed with Scion Image (Scion Corporation). Reactive oxygen species (ROS) were detected with dihydrorhodamine 123 (DHR123, Sigma) (Madeo *et al.*, 1999). The Annexin V (ApoAlert Annexin V Apoptosis Kit, Clontech) assay for phosphatidylserine as well as propidium iodide (PI, Sigma) staining for necrotic cells were performed as described previously in (*Chapter 2*). The mitochondrial membrane potential was assessed by staining with 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Invitrogen), according to (Koning *et al.*, 1993). A Nikon Eclipse E600 fluorescence microscope equipped with a Leica DC 350 F ccd camera was used. Digital images were acquired using FW4000 software (Leica).

Lipid composition. Total lipids were extracted according to the Folch method (Folch *et al.*, 1957) and analysed by thin-layer chromatography on silica gel plates (Fluka), using chloroform/acetone 9.6:0.4 (v/v) as solvent as described in (Thomas III *et al.*, 1965). Lipids spots were visualized by exposing the plates to UV light and quantification was performed by densitometric analysis with Scion Image (Scion Corporation).

β-galactosidase activity assay. Cells transformed with the p*URA3*-UPRE-*CYC1-lacZ* (kind gift of Prof. Frank Madeo and Prof. Christoph Ruckenstuhl) were used. For assessing the UPR activation, 10^8 total cells were harvested from cultures over time and pellets were resuspended in 500 μl of Z-Buffer (60 mM Na_2HPO_4*7 H_20, 40 mM NaH_2PO_4*2 H_20, 10 mM KCl, 0.1 mM $MgSO_4$, pH 7.0) supplemented with fresh β-mercaptoethanol (final concentration 40 mM). Afterwards, 50 μl of 0.1% SDS and 50 μl of 100% chloroform were added, and OD_{600} was measured with a plate reader (Genios-Pro, Tecan). The reaction was started due to addition of orthonitrophenyl-β-galactoside (4mg/ml, Z-Buffer as solvent) and stopped after color changing to yellow with 125 μl of 1 M Na_2CO_3. OD_{405} was measured

with a plate reader (Genios-Pro, Tecan) and results were converted to Miller Units (Kippert, 1995) using the following formula:

$$\text{Activity (Miller Units)} = \frac{\text{OD}_{405} * 1000 * \text{dilution factor}}{\text{incubation time[min]} * \text{OD}_{600}}$$

Autophagy measurements. Autophagy was measured using the *GFP-ATG8 protection assay* (Nair *et al.*, 2011). Western blot analysis of strains carrying the GFP-Atg8 fusion protein expressed from its endogenous promoter and in the natural chromosomal locus was performed. These strains were generated by PCR-based methods using a modified pYM-N37 plasmid (kind gift from Prof. Frank Madeo and Prof. Christoph Ruckenstuhl). Cell extracts were obtained by chemical lysis: 10^8 total cells were harvested, washed, respuspended in 150 μl of Lysis Buffer (1.85 M NaOH, 7.5% fresh β-mercaptoethanol) and incubated on ice for 10 minutes. After the addition of 150 μl of 55% TCA, cells were again incubated on ice for 10 minutes. Pellet was recovered by centrifugating at 10 000 g for 10 minutes at 4 °C and resuspendend in 100 μl of FSB (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 100 mM β-mercaptoethanol). After the addition of 1-5 μl of untitrated 1 M Tris added until blue colour was obtained, samples were heated at 95 °C for 5 minutes. Subsequently, samples were spinned down for 10 seconds and used or stored at -20 °C. Western analysis was performed according to standard methods and immunoblotting was performed using a monoclonal mouse anti-GFP antibody, rabbit polyclonal anti-glyceraldehyde-3- phosphate dehydrogenase (GAPDH) antibodies and the respective peroxidase-conjugated affinity-purified secondary antibody (all antibodies were kind gifts from Prof. Frank Madeo and Prof. Christoph Ruckenstuhl). For detection, the ECL system was used (Amersham). Quantification was performed using a ChemiDoc Touch Imaging System (Bio-Rad) and Image Lab (Bio-Rad).

H_2O_2 resistance assay. Exponential growth-phase cells from minimal medium containing either 2% ethanol/ 3% glycerol or 2% ethanol/ 3% glycerol/ 0.1% oleate as carbon sources were harvested and resuspended in an identical media supplemented with 1 mM, 2 mM or 5 mM H_2O_2. The treatments were carried out for 120 minutes at 30 °C and afterwards cells

were plated on YEPD solid media and incubated at 30 °C for 2 days. Colony-forming-units were counted and compared to the untreated control to determine the survival percentage.

Catalase assay. Catalse activity was determined as in (Giannattasio *et al.*, 2005). Briefly, exponential growth-phase cells were washed with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in 300 μl of the same buffer. Cells were broken with glass beads through several cycles of vortex shacking (1 minute) and ice cooling (1 minute). Afterwards, a clear supernatant was obtained by centrifugation. 20-60 μl of this supernatant were used for the catalytic activity assay. Total catalase activity was measured spectrophotometrically by monitoring the disappearance of H_2O_2 (Sigma) at 240 nm (Luck, 1954). One unit of enzyme activity corresponded to the decomposition of 1 μmol of substrate in 1 minute. Protein concentration was estimated using the BCA^{TM} Protein Assay Kit (Pierce).

Statistical analysis. All values are presented as the mean of three independent experiments ± standard deviation (SD). Three technical replicates were analysed in each independent experiment. Statistical significance was assessed by *t*-test. The level of statistical significance was set at a p-value of ≤ 0.001.

4.6 References

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5 Conclusions & Perspectives

Acetyl-CoA has been shown as a key intermediate in the relation between carbon metabolism and aging. The importance of this metabolite is strictly dependent on its cellular localization and on an efficient metabolic flux between cytosol and mitochondria. This notion was pointed out in *Chapter 2* with the analyses effectuated employing the yeast mutant *mpc1*Δ that lacks an essential subunit of the mitochondrial pyruvate carrier (MPC) and is therefore unable to introduce pyruvate in the mitochondria to foster biosynthesis and energy metabolism also through the production of mitochondrial acetyl-CoA. Such an impairment in the metabolic flux rewires metabolism, causing a depletion in the mitochondrial pool of acetyl-CoA precursors, reducing on one hand the mitochondrial metabolic efficiency and increasing on the other hand the level of cytosolic acetyl-CoA precursors such as acetate, overall decreasing CLS. This situation was rescued by the administration of carnitine, a transporter of acetyl groups among cellular compartments. Restoring the flux between cytosol and mitochondria showed that: i) the uptake of pyruvate in the mitochondria is the main road to obtain and metabolise mitochondrial acetyl-CoA, ii) the importance of pyruvate and acetyl-CoA has not to be ascribed to a specific growth phase or metabolism, and further that iii) pyruvate and acetyl-CoA are essential for mitochondrial metabolism in the long-time period to ensure survival. This points out that the distribution of acetyl-CoA and its precursors has a leading role in regulating cellular aging.

In *Chapter 3*, analyses were conducted using the yeast mutant *sir2*Δ, which

lacks the deacetylase Sir2, the founding member of Sirtuins, a family of evolutionarily conserved aging regulators. The absence of such a deacetylase favours acetyl-CoA anabolism by rapidly consuming the cytosolic acetyl-CoA precursor acetate, and further establishes a higher mitochondrial efficiency that promotes longevity. From a metabolic perspective, the decrease in the level of acetate is related to a higher gluconeogenic flux mediated by the acetylated form of Pck1, the rate-limiting enzyme of gluconeogenesis and also target of Sir2. A comparable situation was proven to be obtained also in wild type cells by the administration of NAM, a well-know Sirtuin inhibitor. These results show further the importance of mitochondrial metabolism in the long-time period to ensure survival, and additionally suggest that the clearance of acetyl-CoA and its precursors from the cytosol, eventually metabolised or efficiently passed to other cell compartments, has a key role in aging. In fact, while their accumulation at a cytosolic level decreases CLS, their constant partitioning, to fulfil cellular biosynthetic and energetic needs, ensures an adequate level of all metabolites and a prolonged efficiency of different cellular mechanisms. This points out that the available pool of acetyl-CoA and its precursors, in addition to their distribution among compartments, has a key role in regulating cellular aging. Since acetyl-CoA and its precursors are obtained through carbon metabolism, nutritional sources acquire an essential role in the regulation of survival.

In *Chapter 4*, particular attention was given to fatty acids, a carbon source that directly provides acetyl-CoA units when processed through β-oxidation. For this purpose, we employed the *oaf1*Δ mutant, which lacks Oaf1, the sole transcriptional factor that, in concert with its obligatory partner Pip2, regulates fatty acid β-oxidation and peroxisome proliferation. The inability to use fatty acids as carbon source, united to the capacity to still store fatty acids in triacylglycerols inside lipid droplets, was found to push toward a saturation of these deposits, generating "obese" cells. Also, the saturation of these deposits caused an increase in the level of free fatty acids, in turn promoting cell death. The incapability to obtain acetyl-CoA from fatty acids during time, together with the increase in the free fatty acid level, generated a condition for the *oaf1*Δ mutant characterized by a heavy impairment of mitochondrial functionality. Further, a high resemblance between *oaf1*Δ cells and white obese adipocytes was underlined.

In particular, *oaf1*Δ cells represent a model of non-reversible obesity, and can potentially be used as a basic system for further investigations. In addition, a chimeric Oaf1 protein bearing the ligand-binding domain of mammalian PPARs, functional homologues of Oaf1, could be made. This strategy could provide, in the context of an adequate reporter system, a new tool to evaluate the activating power of promising agonists to be used in therapeutic interventions for lipid-related dysfunctions.

In conclusion, acetyl-CoA has emerged as a fundamental metabolite during aging. The importance of acetyl-CoA is not limited to the molecule itself, but can be extended, pointing out that the whole acetyl-CoA metabolism, comprehensive of production and distribution, is a protagonist in cellular aging. In fact, an impairment in the production of acetyl-CoA from β-oxidation of fatty acids has such a strong effect that it already impacts cells survival during growth. As a second fact, the distribution of acetyl-CoA among compartments, in particular mitochondria, objects of the study reported in *Chapter 2*, testifies how the available pool of acetyl-CoA and its precursors, above a certain level, is harmful instead of helpful as a carbon/energy source. We can speculate that the cell naturally reaches a high level for at least some of these molecules through its own metabolism. On the contrary, in absence of the deacetylase Sir2, acetyl-CoA metabolism is potentiated, favouring on one side the consumption of its intermediates, and on the other side the acetylated state of the proteins that are target of Sir2.
Further, in this scenario, mitochondria surprisingly emerged as valuable sensor organelles able to identify the functional state, and predict the fate, of a cell during aging. These new insights shed further light on the complex process of aging and provide evidence of how carbon metabolism, acetyl-CoA and mitochondria can be used to potentially probe longevity.