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Intermediary metabolism regulates autophagic response of aging cells

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Wahre Wissenschaft lehrt vor allem anderen, zu zweifeln und unwissend zu sein.

Miguel de Unamuno

EIDESSTATTLICHE ERKLÄRUNG

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Graz, am …….. ……..

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Für meine Eltern

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Abstract

Maintaining cellular homeostasis by degradation and recycling of damaged cellular material is crucial for healthy aging. This process is accomplished by autophagy, a highly regulated form of self-digestion that is conserved from yeast to humans. Autophagy is mainly regulated by sensing of nutrients and energy, however a crucial molecular sensor connecting cellular metabolism and age-induced autophagy is not known. In this work, we propose that the nucleo-cytosolic pool of the central energy metabolite acetyl-Coenzyme A (acetyl-CoA) regulates autophagy during aging. Using yeast as a model organism, we show that inhibition of the mitochondrial route of acetate consumption by deletion of the putative CoA-transferase *ACH1* results in the upregulation of the nucleo-cytosolic path of acetyl-CoA generation mediated by the acetyl-CoA synthetase Acs2p. This causes the accumulation of the acetyl-CoA precursor acetate, hyperacetylation of histones, an age-dependent autophagy defect and accelerated chronological aging. While inhibition of known nutrient sensing kinases TOR or Sch9p fails to recover the autophagy deficiency in *ach1* mutant cells, knockdown of *ACS2* potently restores autophagy, histone-acetylation and accelerated aging. Thus, we propose that nucleo-cytosolic acetyl-CoA consistently links carbon metabolism to the regulation of histone acetylation, autophagy and aging.

Kurzzusammenfassung

Die Wahrung der zellulären Homöostase durch Abbau und Wiederverwertung von beschädigten Zellbestandteilen ist essentiell für einen gesunden Alterungsprozess. Autophagie, ein von der Hefe bis zum Menschen hoch konserviertes „Selbstverdauungsprogramm“ ist für diesen Prozess hauptverantwortlich. Dieses wird hauptsächlich durch die An- bzw. Abwesenheit von Nährstoffen und Energieträgern reguliert, wenngleich noch kein Molekül bekannt ist welches als spezifischer Vermittler zwischen Metabolismus und alters-induzierter Autophagie dient. Im Rahmen dieser Arbeit zeigen wir, dass der nucleo-cytosolische Anteil des zentralen Energiemetabolits Acetyl-Coenzym A (Acetyl-CoA) als Regulator für Autophagie während des Alterns von Zellen wirkt. Anhand des Modellorganismus Bäckerhefe zeigen wir, dass ein Erliegen der mitochondrialen Acetat-Verwertung durch Deletion der mutmaßlichen CoA-Transferase *ACH1* zur Hochregulierung der nucleo-cytosolischen Acetyl-CoA Produktion durch die Acetyl-CoA Synthetase Acs2p führt. Dies wiederum geht mit einer Akkumulation von Acetat einher, welches als Vorläufer für Acetyl-CoA dient, und zu gesteigerter Acetylierung von Histonen, einem alters-bedingten Autophagiedefekt sowie einer Reduktion der chronologischen Lebensspanne führt. Während die Inhibition der bekannten auf Nahrungssignale ansprechenden Kinasen TOR und Sch9p den Autophagiedefekt in *ACH1* Knockout-Stämmen nicht beheben können, vermag ein Knockdown von *ACS2* sowohl die Autophagie als auch das Ausmaß der Histonacetylierung auf Wildtypniveau wiederherzustellen, und die verkürzte Lebensspanne wieder zu verlängern. Angesichts dieser Daten schlagen wir ein Modell vor, in dem nucleo-cytosolisches Acetyl-CoA als Bindeglied zwischen zellulärem Kohlenstoffmetabolismus sowie Histonacetylierung, Autophagie und Alterungsprozessen dient.

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1 Introduction

1.1 Yeast in aging and cell death research

Since the discovery of programmed cell death in the budding yeast *Saccharomyces cerevisiae* and the conservation of markers and many mechanisms involved up to the mammalian system, research in this field stepped up enormously (Madeo, Frohlich, and Frohlich 1997; Madeo et al. 1999). Numerous crucial molecular regulators and executors of both apoptotic, as well as necrotic cell death have been described since (Madeo et al. 2004; Eisenberg et al. 2010).

The use of yeast as a model organism for aging and cell death research offers several advantages to other established models such as *D. melanogaster* and *C. elegans*. Other than being non-pathogenic, fast growing and cheap in cultivation, yeast harbors comparatively clear-cut, fast and easy genetics that allow uncomplicated modification of its DNA, which is furthermore very well studied, for yeast being the first eukaryote whose genome was completely sequenced (Zagulski, Herbert, and Rytka 1998; Botstein, Chervitz, and Cherry 1997; Goffeau et al. 1996). Due to its efficient homologous recombination system and the simple mutant isolation, generation of recombinant or knockout strains is quite uncomplicated, and allows fast unbiased screenings also in a high-throughput manner (Wu et al. 2011; Nakaya et al. 2012). All these benefits make yeast a valuable tool for identifying functional hierarchies pathways. A big advantage of yeast particularly for aging and cell death research is that mitochondria, which not only are crucial for cellular energy balance, but also are central executing organelles in cell death, are easily manipulable, either by generating rho^0 strains lacking mitochondrial DNA, or by modulating mitochondrial respiration by simply shifting cultures to different media.

The importance of yeast as a model system for research on aging, cell death and related processes (e.g. autophagy) is even more consolidated, as heterologously expressed mammalian genes involved in these processes in many cases retained their functionality without them having any known orthologs. Using yeast as a "clean room" system to study interactions between heterologously expressed factors involved in cell death pathways, the function of several important players like the tumor suppressor p53 or members of the Bcl-2 protein family such as Bax or Bcl-xL, has been elucidated (Greenwood and Ludovico 2010; Gourlay, Du, and Ayscough 2006).

Recently, yeast has also been increasingly used as a system for studying neurodegenerative disorders, including α-synucleoinopathies, polyglutamine disorders, β-amyloid diseases,

tauopathies and TDP-43 proteinopathies. Heterologous expression of human proteins involved in these diseases has given insights into the mechanistics of cell death pathways relevant to these pathologies (Braun et al. 2010; Braun et al. 2011; Buttner et al. 2013).

Despite the fact that the process of aging and associated cell death is way more complex in humans, yeast has provided crucial insights into pathways involved in aging in mammals.

1.2 Aging in yeast

Using the yeast *Saccharomyces cerevisiae*, two distinct kinds of aging models have to be discriminated (**Figure 1**):

Replicative aging is defined as the number of daughter cells generated by a single mother cell via asymmetric budding. After a limited number of divisions and a short post-replicative state, cells irrevocably succumb to cell death. Aging in this model is based on the asymmetric inheritance of cellular damage by the mother cell, as daughter cells derived from their mother cells usually retain full replicative capacity. Replicative aging of yeast serves as a model for the mortality of mitotic cells higher organisms (Longo et al. 2012; Kaeberlein 2010). In yeast, replicative lifespan is determined by manually separating freshly budded daughter cells from their mother cell and count the number of cell divisions (Steinkraus, Kaeberlein, and Kennedy 2008).

In contrast, the chronological aging is a model for post-mitotic, non-dividing cells in tissues. The chronological lifespan marks the length of time post-diauxic yeast cells stay viable (Fabrizio and Longo 2007). In this aging model, cellular damage is accumulated during stationary phase within a non-dividing cell up to a point at which re-entry into cell cycle is no longer possible, ending in cellular demise and, ultimately, programmed cell death (Kaeberlein 2010; Herker et al. 2004; Fabrizio et al. 2004).

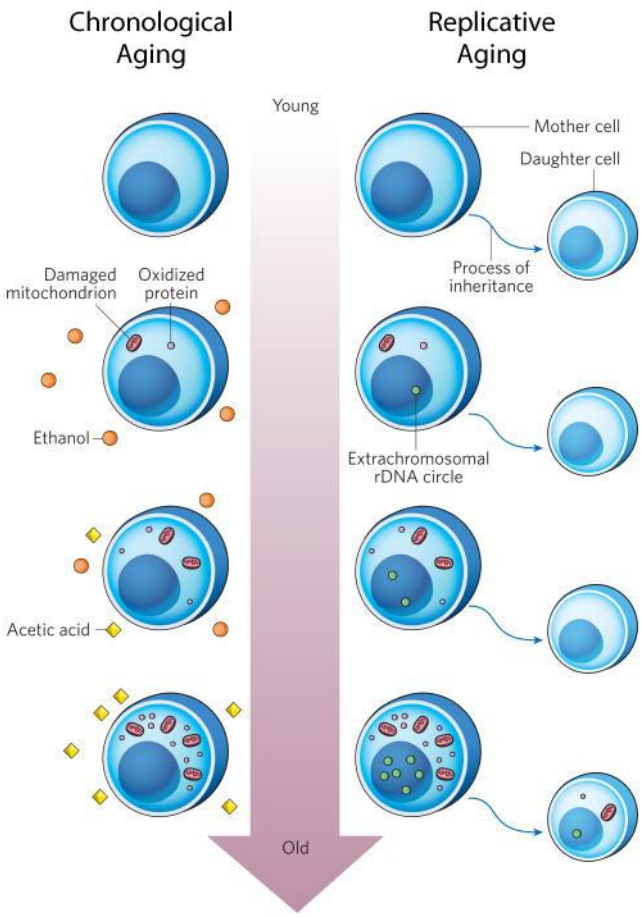

Figure 1: Chronological vs. replicative aging of yeast: Chronological aging refers to the aging of non-dividing cells, and is characterized by the accumulation of damage caused by oxidative stress and pro-aging factors such as acetate over time, until the cell loses its capability to re-enter the cell cycle. Replicative Aging is based on the unequal inheritance of cellular damage in proliferating cells. While mother cells retain most of the age-associated damage during its replicative lifespan, daughter cells generated in the very late replicative lifespan do inherit sufficient damage to succumb to accelerated aging due to breakdown of the asymmetry. Figure adapted from (Kaeberlein 2010).

1.3 The process of aging

In times of ever increasing lifespan, the senescence of the population poses a growing problem for society. Demographics develop towards a population consisting of a growing number of elders, who increasingly suffer from chronical age-associated disorders, often negatively impacting their quality of life as well as their functional output. In fact, aging itself is one of the main driving forces for disease – the chance of contracting one of the "major killers" in western civilization such as heart disease, diabetes and most types of cancer as well as most neurodegenerative diseases, increases drastically upon aging (Kaeberlein 2013; Fontana, Partridge, and Longo 2010).

The process of aging affects all eukaryotic, and possibly also prokaryotic organisms (Nyström 2003; Lindner et al. 2008; Stewart et al. 2005). It is characterized by the accumulation of molecular, cellular and organ damage, resulting in loss of function, susceptibility for disease, and finally death. Lots of research was invested into deciphering the mechanisms of aging since

Denham Harman proposed his “free radical theory of aging” more than 50 years ago, suggesting that aging was caused by free radicals formed as by-products of metabolism. Although reactive oxygen species (ROS) were shown to be involved in various cell death scenarios in many studies, their role as a primal cause for aging and are-related cell death is still controversial (HARMAN 1956; Gems and Partridge 2013). Today, it seems safe to assume that an extensive signalling network that is involved in nutrition sensing, cell growth, stress resistance and autophagy also regulates aging. Several pathways involved in this network, such as the insulin/IGF-I or TOR pathways have already been identified and successfully linked to aging. In this context, numerous both genetic as well as dietary alterations have been shown to significantly increase not only total lifespan, but also so called “healthspan” (defined as the timespan an organism is able to maintain or return to homeostasis in response to challenges; not synonymous with lifespan) in various model organisms ranging from yeast to flies, rodents and even monkeys (Fontana, Partridge, and Longo 2010; Tatar 2009).

The most efficient non-genetic mechanisms to prolong lifespan is dietary restriction, which refers to a reduction in food intake without malnutrition (also refered to as caloric restriction (CR)). While in yeast, worms, flies and mice caloric restriction both extended longevity as well as decelerated the accompanying age-related phenotypes such as cellular demise and decline in function, it is still unclear if CR increases life-span in primates, as studies with rhesus monkeys displayed contrasting results so far (Anderson, Shanmuganayagam, and Weindruch 2009; Bodkin et al. 2003; Colman et al. 2009; Mattison et al. 2012). Consistently, many conserved mechanisms of lifespan extension involve the reduced activity of nutrient-signaling pathways such as the Igf (insulin-like growth factor), TOR (target of rapamycin) and Ras-cAMP-PKA pathways. Most of these pathways regulate longevity directly or indirectly via a multitude of cellular processes involved, for example, in growth and proliferation, translation, stress-response, metabolism, cellular protection and autophagy (Fontana, Partridge, and Longo 2010; Kaeberlein 2010).

1.4 Autophagy

Autophagy, deriving from the Greek words *auto* “self” and *phagein* “to eat”, refers to a well regulated process that involves degradation of cellular compounds by delivering substrates to lysosomes, which is conserved in all eukaryotes. Apart from the ubiquitin-proteasomal system (UPS), which serves for the rapid elimination of proteins when fast adaption is needed, autophagy is the second way of degrading cellular material. However in contrast to the UPS, autophagy is also able to direct excess or defective organelles towards disposal by the lysosome.

For this purpose, cytoplasmatic bulk or whole organelles are engulfed into double-layered membranes, and delivered to the lysosome in vesicular form, so called autophagosomes (Kirkin et al. 2009; Rubinsztein, Mariño, and Kroemer 2011).

Many of the molecular components executing autophagic processes are conserved throughout the eukaryotic domain. These factors are encoded by ATG genes (autophagy related genes), 35 of which are known in yeast to date (Nazarko et al. 2011).

There are several primal functions of autophagy, all of which depict pro-survival functions in eukaryotic cells: A key role of autophagy is as a regulator of organellar homeostasis. As a main source for toxic reactive oxygen species as well as pro-apoptotic mediators, dysfunctional mitochondria which have lost their membrane potential are targeted by autophagy and degraded, probably even in a selective manner (Priault et al. 2005; Twig et al. 2008; Narendra et al. 2008). The degradative function of autophagy also plays a crucial role in the disposal of protein aggregates, which are often associated with neurodegenerative diseases (Ravikumar et al. 2010).

It has been shown that autophagy also plays an important part in the innate immune response and contributes to the elimination of invading pathogens such as bacteria and viruses. Although the machanisms remain mostly unknown, autophagy seems to play a protective role in numerous infectious diseases (Yano et al. 2008; Gutierrez et al. 2004; He and Klionsky 2009).

Finally, in unicellular eukaryotes like yeast autophagy is critical for providing nutrients and energy in times of scarce resources, as it enables recycling of macromolecules (Yang et al. 2006).

In principle, three different types of autophagy can be distinguished: 1) Macroautophagy is the quantitatively most important type of autophagic process, and is often refered to just as “autophagy”. It is characterized by the engulfment of cytosolic bulk or whole organelles by de-novo formed double-membrane vesicles, which fuse with the lysosome for degradation of the sequestered material. Macroautophagy also comprises more distinct forms of autophagy that, depending on the cargo that is prone to degradation, include mitophagy (mitochondria), pexophagy (peroxisomes), ribophagy (ribosomes) or reticulophagy (endoplasmatic reticulum, ER) (Kiel 2010; He and Klionsky 2009; Inoue and Klionsky 2010). Another form of selective autophagy is the cytoplasm-to-vacuole targeting pathway (Cvt), which uses the autophagic machinery to transport the precursor vacuolar hydrolases prApe1 (Aminopeptidase I) and prAms1 (α-mannosidase) from the cytosol to the vacuole. 2) Microautophagy refers to a rather poorly understood mechanism, by which cytosolic material or even larger components are engulfed by the lysosomal membrane itself. Whether micro- or macroautophagy is induced

depends on the organism, environmental conditions and the stimuli that triggers the autophagic response (Kissová et al. 2007; Marzella, Ahlberg, and Glaumann 1981). 3) Chaperone-mediated autophagy (CMA) is a form of selective autophagy that is only described in mammals as yet. Transport of most substrate proteins to the lysosome is dependent on KFERQ-like motifs, the lysosomal membrane associated protein LAMP-2A for translocation, and both cytosolic as well as lumenal members of the hsc70 (constitutively expressed form of heat shock protein of 70 kDa) family. In contrast to other forms of autophagy, vacuolar transport is not involved (Dice 2007; Kaushik and Cuervo 2012). The aforementioned distinct kinds of autophagy are summarized in **Figure 2**.

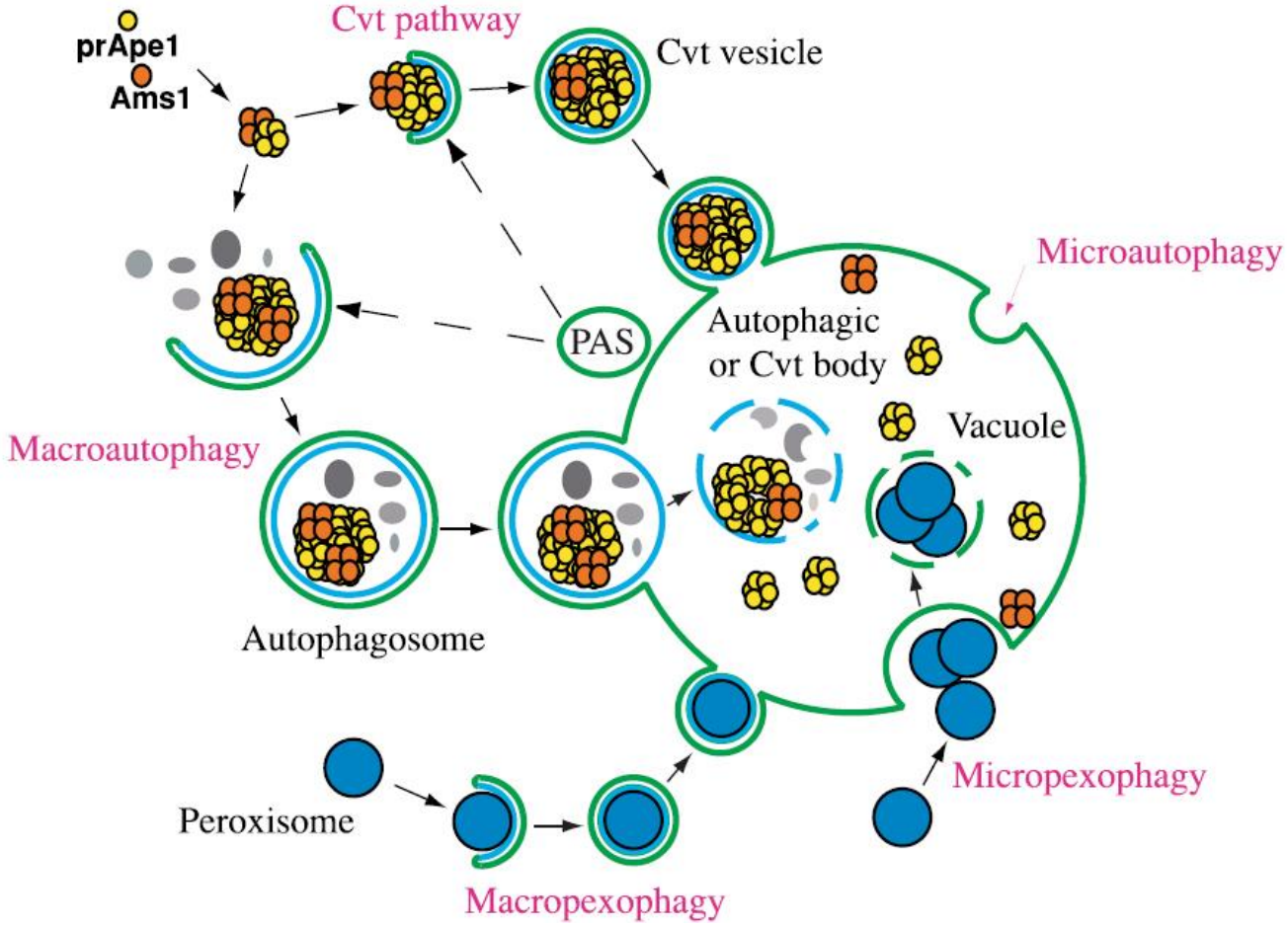

Figure 2: Autophagic processes in yeast. The presumed origin of both autophagosomes and Cvt vesicles is the pre-autophagosomal structure (PAS). While Cvt vesicles predominantly enclose prApe1 and prAms1 for delivery to the vacuole, autophagosomes form by the bulk or selective engulfment of cytosolic components. Similar to autophagosomes, Cvt vesicles also dock and fuse with the vacuolar membrane to release their content into the lumen. During microautophagy, cytoplasmatic material (or selectively, peroxisomes) is engulfed directly by the vacuolar membrane. Under normal nutrient-rich growth conditions the Cvt pathway is active, whereas autophagosomes form upon various stressors or when nutrients are depleted. Figure adapted from (Huang and Klionsky 2002).

As described above, autophagy plays an important role in cellular homeostasis, and its cytoprotective functions are often linked with healthy lifespan and longevity. It is always active at a basal level under normal growth conditions and can further be induced by starvation, growth factor deprivation, hypoxia, ER stress and other stress stimuli such as ROS or high

temperature (T Yorimitsu and Klionsky 2005; Ravikumar et al. 2010; Ferraro and Cecconi 2007). Autophagy is regulated by a wide network of signaling pathways that are particularly influenced by nutrient signaling kinases. Intriguingly, autophagy is not associated with pro-life functions exclusively, but also plays a role in cell death, as its regulating signaling network ties up to cell death pathways at some points (Rubinsztein, Mariño, and Kroemer 2011; Yang and Klionsky 2009a), and the autophagic machinery might even be involved in the execution of specific types of cell death (autophagic cell death). (Denton, Nicolson, and Kumar 2012; Galluzzi et al. 2012)

1.4.1 Molecular mechanism of autophagosome formation

The molecular process of macroautophagy (which will further be just referred to as "autophagy") can be split into mechanistically distinct steps, such as induction, cargo recognition, packaging, vesicle nucleation, expansion and completion, Atg protein recycling, fusion of the autophagosome with the lysosome, breakdown of the cargo and recycling of the resulting macromolecules (Yang and Klionsky 2009b). One of the most critical and intensively studied steps in autophagy is the formation of the autphagosome, which refers to the cargo loaded double layered membrane vesicle, that derives from a de novo formed membrane sac (termed the phagophore or isolation membrane) sequestering parts of the cytosol. Of the more than thirty known autophagy related genes in yeast – many of which orthologs were subsequently found and characterized in mammals-, 18 (ATG1-10, 12-14, 16-18, 29 and 31) are essential for autophagosome formation and thus non-selective autophagy. The rest has functions in selective autophagy and the degradation of autophagic bodies (Mizushima, Yoshimori, and Ohsumi 2011). Rather than being generated by budding from the preexisting membranes of organells, the double-membrane autophagosomes seem to be assembled at specific structures, called the preautophagosomal structure (PAS, also phagophore assembly site) (He and Klionsky 2009).

Atg proteins involved in autphagosome formation can be grouped into several functional units: 1) the Atg1 kinase complex (ULK1/2 in mammals) including its regulators Atg13 and Atg17, which is a direct downstream target of the main autophagy regulatory kinase (m)TORC1 and is responsible for the recruitment of many other Atg proteins to the PAS; 2) the Class III PI3K complex (Phosphatidylinositol 3-kinase), which produces phopsphatidylinositol 3-phosphate (PI3P) at the PAS, and can be inhibited by Bcl-2 in mammals; 3) the Atg2-Atg18 complex, which binds to the PI3K complex and take part in the nucleation process of the phagophore; 4) Atg9, which is involved in nucleation of the phagophore and might also cycle between the PAS and peripheral membrane pools; 5) the Atg8 conjugation system (LC3 in mammals), which links the ubiquitin-like Atg8 to phosphatidyl-ethanolamine (PE) in a lipidation reaction and is critical for

the expansion and closure of the isolation membrane; and 6) the Atg12 conjugation system, which results in the formation of a Atg12-Atg5-Atg16 complex that contributes to the conjugation of Atg8-PE and the elongation of the phagophore (Suzuki et al. 2007; Mizushima, Yoshimori, and Ohsumi 2011; Suzuki et al. 2013).

Apart from these six functional complexes, certain non-Atg components like the secretory and endocytic pathways or the cytoskeletal network might as well carry out crucial functions during autophagy (He and Klionsky 2009).

The core machinery of autophagy is highly conserved from yeast to mammals, however this chapter will focus on the autophagosome formation in *Saccharomyces cerevisiae*. When autophagy is induced by nutrient deprivation, the serine/threonine kinase TORC1, which acts as a central negative regulator of autophagy, is inactivated and its substrate Atg13 gets dephosphorylated. In turn, this enables the binding of Atg13 to Atg1 and the Atg17-Atg29-Atg31 scaffold, resulting in the Atg1-Atg13-Atg17 ternary complex translocating to the PAS and the activation of Atg1 kinase activity, both of which are essential for autophagy induction (Yang and Klionsky 2009b).

The nucleation and assembly of the initial isolation membrane is dependent on the Class III PI3K complex, which contains the proteins Vps34, Vps15, Atg14 and Atg6. In mammals, the function of the Atg6 homologue Beclin-1 is regulated by the anti-apoptotic protein Bcl-2, which also inhibits autophagy (Kang et al. 2011). The PI3K complex produces PI3P and recruits several other PI3P-binding proteins and Atg proteins to the PAS, including Atg21, the Atg2-Atg18 complex, Atg20 and Atg24. The latter two can interact with the Atg1-Atg13-Atg17 complex, which is formed upon autophagy induction. Together with other Atg proteins, the PI3K complex furthermore recruits two ubiquitin-like conjugation systems to the PAS, namely Atg12-Atg5-Atg16 and Atg8-PE, both of which regulate the enlargement of the phagophore. Atg12 is transferred to Atg10 by Atg7 (E1 activating enzyme), and then covalently linked to Atg5, whereupon the conjugate associates with Atg16 and is attached to the isolation membrane. In the second ubiquitin-like conjugation system, Atg8 is first cleaved by the cysteine protease Atg4, transferred to Atg3 by Atg7, and finally linked to the lipid PE, facilitated by the Atg12-Atg5 conjugate (He and Klionsky 2009; Itakura and Mizushima 2010; Mizushima, Yoshimori, and Ohsumi 2011). Atg8 is suggested to be involved in various stages of autophagosome formation and extension and is widely used as a marker for autophagic activity. The extent of Atg8-lipidation (or its mammalian homologue LC3 respectively) for example serves as a reference to quantify autophagy induction (Cheong and Klionsky 2008).

The role of Atg9 in autophagosome formation is still rather poorly understood. Given that it is the only multispan transmembrane Atg protein, and is found at the PAS as well as on other cellular structures such as the ER or the Golgi, it was suggested that Atg9 may cycle between the PAS and peripheral membrane compartments and act as a carrier in supplying membrane during phagophore expansion (Kovács et al. 2007; Chen and Klionsky 2011).

When autophagosome formation is completed, Atg8 is cleaved from PE by Atg4, and is recycled into the cytosol together with various other Atg proteins. After fusion of the autophagosome with the vacuole, the inner vesicle membrane and cargo is degraded by various hydrolases, lipases and proteases, and resulting small molecules are transported into the cytosol and recycled.

1.4.2 Regulation of autophagy

Both in the yeast *Saccharomyces cerevisiae* and in higher eukaryotes autophagy is regulated by signaling pathways sensing nutrients and cellular energy, as well as a response to stress like ROS or ER stress. Pathways involved in sensing of insulin or growth factors, as well as autophagy induction upon pathogen infection or hypoxia however are exclusive for higher eukaryotes. Most of the signaling cascades at some point converge at nutrient responsive kinases, including TOR, protein kinase A (PKA), Sch9 (homologue to the mammalian protein kinase B (PKB)/Akt as well as S6 kinase (S6K)) and the kinase Snf1 (an AMP-activated kinase, homologue to mammalian AMPK), all of which are also involved in regulating aging and lifespan (Fontana, Partridge, and Longo 2010; He and Klionsky 2009). **Figure 3** depicts the most important signaling pathways involved in autophagy regulation in yeast and mammals.

The highly conserved serine/threonine protein kinase TOR acts as a central sensor of nutrient availability, energy status and – in higher eukaryotes – growth factors, and is a crucial negative regulator of autophagy. In *Saccharomyces cerevisiae* TOR exists as two distinct complexes, namely TORC1 and TORC2. While TORC2 mainly regulates spatial cell growth by mediating actin cytoskeleton organization, TORC1 (yeast homologue to mammalian mTORC1) is responsible for temporal cell growth by activating anabolic processes like ribosome biogenesis, translation, transcription and nutrient uptake and by inhibiting catabolic processes like autophagy or the UPS. Sensitivity to the antibiotic immunosuppressive drug and autophagy inducer rapamycin is also mediated by TORC1, whose kinase activity is abrogated by binding of rapamycin-bound FKBP12 (FK506-binding protein of 12 kDa) (Wullschleger, Loewith, and Hall 2006a; Heitman, Movva, and Hall 1991).

Upstream regulation of TOR is best studied for the regulation of mammalian mTORC1 by growth factors via the insulin-PI3K-TSC-Rheb pathway. Upon binding of insulin or insulin-like growth factors (IGF), PI3K and the serine/threonine kinase Akt (also known as protein kinase B (PKB), a mammalian homologue to yeast Sch9), are recruited. Phosphorylation by Akt inhibits the TSC complex (tuberous sclerosis complex), which harbors GTPase activating activity, allowing the small GTPase Rheb to activate mTORC1 (Soulard, Cohen, and Hall 2009; Wullschleger, Loewith, and Hall 2006b; Hay and Sonenberg 2004). The molecular mechanism of TORC1 regulation by nutrients however is yet rather poorly understood. Recent studies suggest that in mammals amino acid signals also converge to either the TSC complex or Rheb as an upstream factor of TOR, however, since TOR in *Saccharomyces cerevisiae* responds to nutrients despite the lack of functional orthologs of Rheb or the TSC complex, (m)TORC1 may be able to sense amino acids directly (Martin and Hall 2005; Hay and Sonenberg 2004).

Another signaling pathway that negatively regulates autophagy in parallel to TOR is the Ras/c-AMP dependent protein kinase A (PKA) system, which is involved in glucose sensing from yeast to mammals. In the presence of nutrients the small G proteins Ras1 and Ras2 are active and stimulate the adenylyl cyclase to produce cAMP, which in turn activates PKA. Similar to the TOR mediated mechanism of autophagy inhibition, Atg13 was shown to be a downstream target of phosphorylation by PKA, preventing the formation of the Atg1-kinase complex, which is crucial for autophagy induction (Budovskaya et al. 2005; He and Klionsky 2009; McEwan and Dikic 2011).

The yeast protein kinase Sch9, the closest homologue of mammalian Akt/PKB and S6K (ribosomal protein S6 kinase, which is a key mediator of mTOR signaling in mammalian cells involved in cell growth), is the third signaling pathway negatively regulating autophagy (as well as replicative and chronological lifespan) in response to nutrients. Here, Sch9 has been suggested to act both in parallel to TOR and PKA and as a direct downstream target of the TOR kinase. In this context, Sch9 has been reported to be required for TORC1 mediated regulation of entry into stationary phase, rRNA transcription and translation initiation, representing functions of Sch9 downstream of TOR (Urban et al. 2007; Wei and Zheng 2009). On the other hand, inactivation of Sch9 and PKA induced autophagy despite TORC1 being active, suggesting that Sch9, PKA and TOR act at least partly independent as well.

Snf1, the yeast homologue to mammalian 5'-AMP-actiavated protein kinase (AMPK), serves as a positive regulator of autophagy, as it links the cellular energy levels by sensing ATP to autophagy regulation. Hence, a decrease in ATP/AMP ratio activates Snf1/AMPK kinase activity resulting in an adaption to metabolic stress and autophagy. While in mammals, AMPK mediates its

autophagy inducing functions via phosphorylation of the TSC complex, and subsequent inactivation of mTORC1, as well as by activation of the cell cycle regulator $p27^{kip1}$, yeast Snf1 is suggested to act via phosphorylation of Atg1 and Atg13 (Inoki, Zhu, and Guan 2003; Hedbacker and Carlson 2008; Liang et al. 2007; Wang et al. 2001).

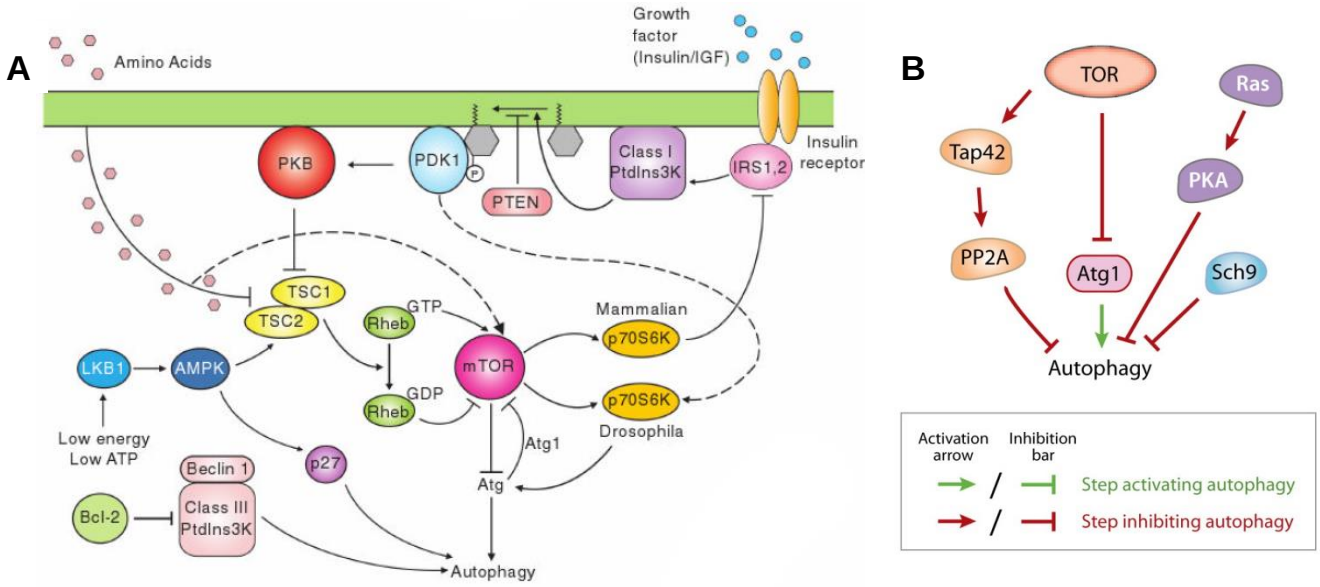

Figure 3: Signaling pathways regulating autophagy in mammals and yeast. (**A**) Activation of mTOR depends on several imputs such as nutrients (amino acids), growth factors (insulin/IGF) or energy (ATP). Stimulation of the insulin receptor leads to activation of a class I phosphatidylinositol 3-kinase (class I PtdIns3K) and subsequent activation of PDK1 and PKB/Akt. Akt inhibits the TSC1/2 complex via phosphorylation, leading to the stabilization of the small GTPase Rheb, which in turn stimulates mTOR, leading to inhibition of autophagy. Inactivation of mTOR by both amino acids as well as ATP functions independently from the insulin signaling pathway. Amino acids are might inhibit autophagy either by inhibition of the TSC1/2 complex, or by directly activating mTOR. Low levels of ATP activate the AMPK pathway, which inhibits mTOR activity by activating TSC1/2. In mammals, the regulation network of autophagy is coupled to the cell death machinery at some points. Most prominently, the antiapoptotic protein Bcl-2 associates with Beclin 1, the mammalian hoolog of Atg6, and inhibits the class III PtdIns3K and thus autophagy. Figure adapted from (Yang and Klionsky 2009a). (**B**) In yeast, several nutrient-sensing kinase-pathways are involved in autophagy regulation. As in mammals, TOR serves as the main negative regulator of autophagy, by inhibiting Atg1 kinase activity. In parallel to TOR, Ras is active under nutrient-rich conditions and leads to the activation of PKA, which in turn inhibits autophagy. The kinase Sch9 (the yeast homologue to mammalian Akt and S6K) serves as a third negative regulator of autophagy in response to nutrients. Figure adapted from (He and Klionsky 2009).

1.4.3 Acetylation in autophagy regulation

Albeit the main pathways involved in autophagy signaling mainly rely on regulators exhibiting kinase activity, also other post-translational modifications play a major role in induction, regulation and fine-tuning of autophagy. Apart from phosphorylation, which was long established to be involved in autophagy regulation, it is ubiquitylation and especially acetylation that are more and more becoming the focus of intense research (McEwan and Dikic 2011; Deribe, Pawson, and Dikic 2010).

The sirtuin family of NAD-dependent histone/protein deacetylases represents some of the most well characterized factors involved in the regulation of both autophagy and aging by modulating the acetyl-proteome. Named after the yeast longevity regulator Sir2, seven mammalian homologues have been identified yet, all of which fulfill important functions in the regulation of

metabolism, growth and differentiation, inflammation, survival, as well as in aging and lifespan extension (Longo and Kennedy 2006; Imai et al. 2000). Overexpression of SIRT1, the best characterized mammalian sirtuin, was shown to induce autophagy in cell culture even in the presence of nutrients, and SIRT1 activity was shown to be necessary for starvation-induced autophagy, as expression of a deacetylase-inactive mutant of SIRT1 inhibited the induction of autophagy under caloric restriction. Resveratrol, a natural polyphenol found in grapes, potently induces autophagy accompanied by longevity in various model organisms by activating SIRT1 and altering the acetyl-proteome (Morselli et al. 2011; Mariño et al. 2011). Furthermore, several components of the core autophagy machinery including Atg5, Atg7 and Atg8 were shown to be direct substrates of SIRT1 deacetylation (I. H. Lee et al. 2008; Salminen and Kaarniranta 2009). These observations correlate with the finding that SIRT1 is also a major player in metabolic processes and that its expression is upregulated upon nutrient deprivation. In this context, mammalian p300 acetyltransferase acts as an opponent to SIRT1 and is responsible for the acetylation of Atg5, Atg7, Atg8 and Atg12. Consistently, down-regulation of p300 leads to elevated autophagy levels, whereas overexpression of p300 inhibits the self-digestion program (I. H. Lee and Finkel 2009).

Generally, the acetylation status of proteins constituting the autophagic machinery seems to be of great importance to the regulation of autophagy (**Figure 4**). Recent studies in yeast revealed that under nutrient-rich conditions, Atg3, Atg5, and Atg8 are acetylated. Dependent on the activity of the histone acetyl-transferase (HAT) Esa1, increased acetylation of Atg3 boosted autophagy, while its deacetylation showed inhibitory effects (Yi et al. 2012). Atg7 on the other hand needs to be deacetylated by SIRT1 in order to induce autophagy under starvation conditions (I. H. Lee et al. 2008, 1).

Although dispensable for induction of autophagy, the histon/protein deacetylase HDAC6 controls the acetylation status of the microtubule network. Both for the selective degradation of aggregate-containing inclusion bodies by speficic forms of autophagy, as well as for autophagosome maturation and traffick, the microbubule component tubulin needs to be acetylated (Iwata et al. 2005; J.-Y. Lee et al. 2010; Geeraert et al. 2010).

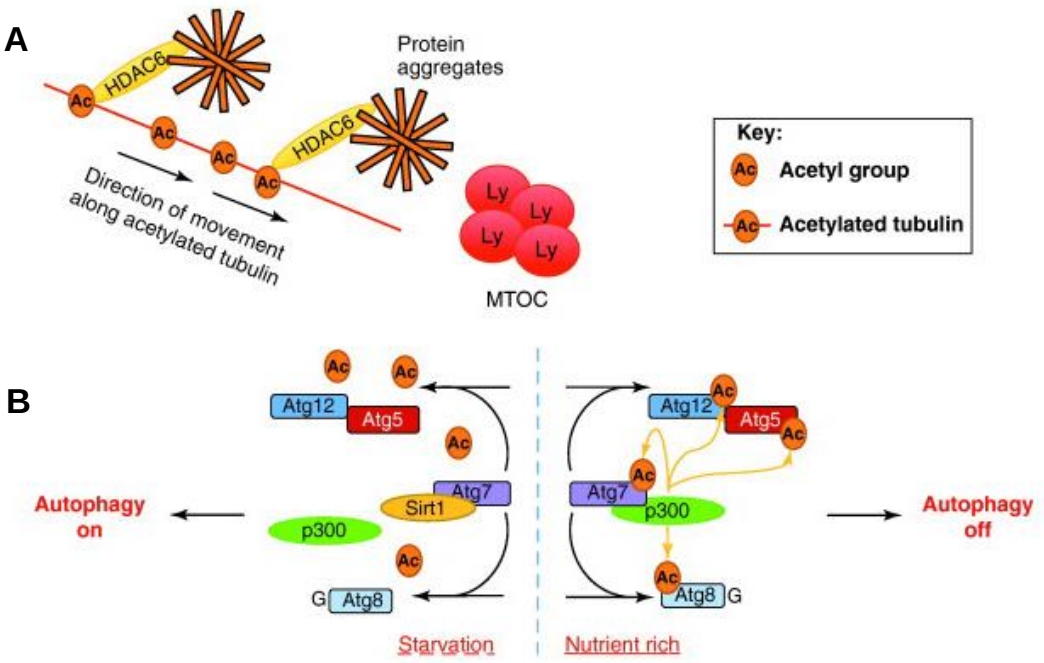

Figure 4: Regulation of autophagy by acetylation. (**A**) The transport of protein aggregates along the cytoskeleton network towards the lysosomes is mediated by adaptor proteins such as HDAC6. This movement along the microtubule depends on the acetylation of tubulin. (**B**) In mammals, regulation of autophagy by acetylation of Atg proteins is mediated by the acetyltransferase p300 and the deacetylase Sirt1. Under nutrient-rich conditions p300 directly interacts with Atg7 and acetylates several key players involved in autophagosome formation such as Atg7, Atg8, Atg12 and Atg5, and thus inhibits autophagy. When nutrients are depleted, p300 dissociates from Atg7 and Sirt1 deacetylates the aforementioned Atg proteins leading to autophagy induction. Figure adapted from (McEwan and Dikic 2011).

The execution of short-term autophagy is a cytoplasmic process that is independent of nuclear factors, which is demonstrated by the fact that cytoplasts (enucleated cells) show markers of functional autophagy when starved (Morselli et al. 2011). Still, there is growing evidence that regulation of long-term autophagy might still depend on epigenetic modulations such as histone acetylation and transcriptional control of autophagy related genes.

One well studied target of SIRT1 deacetylation activity for example is the forkhead box O (FoxO) family of transcription factors. FoxO3 is known to regulate a multitude of stress responses and pro-survival programs in the cell. Deacetylation of FoxO3 by SIRT1 leads to elevated stress resistance, amongst others by induction of autophagy through the transactivation of autophagy-relevant transcripts (Brunet et al. 2004; Warr et al. 2013; Morselli et al. 2010; Sandri 2012). Transcriptional control might also play a role in spermidine-mediated autophagy induction. Spermidine, a natural occurring polyamine and promising anti-aging drug, induces autophagy in a Sir2/SIRT1 independent manner by inhibiting HATs, altering the acetyl-proteome and reducing global H3 histone acetylation while selectively keeping the promotor region of the essential autophagy gene *ATG7* acetylated. This allows for the spermidine-driven transcription of autophagy related genes while maintaining a state of general gene silencing (Eisenberg et al. 2009). In another very recent study, acetylation of lysine 16 of histone H4 regulated by the

balance of hMOF (an acetyltransferase) and SIRT1 activity, was revealed to determine the outcome of autophagy induction either towards cytoprotective autophagy or towards cell death (Füllgrabe et al. 2013; Dang et al. 2009). Several studies furthermore demonstrated that genetic or chemical inhibition of HDAC activity lead to induction of autophagy, further consolidating that protein acetylation in general, a histone acetylation in particular may have important roles in autophagy regulation (Francisco et al. 2012; He and Klionsky 2009).

1.5 Acetyl-CoA links metabolism to the regulation of cellular processes

What unites all known regulatory systems relying on protein acetylation, regardless of the domain of life and the process targeted by this posttranslational modification, is the requirement of a donor substrate for the regulatory transfer of the acetyl group. So far, the only known donor for acetylation reactions is acetyl-coenzyme A (Acetyl-CoA), a universal metabolite found in all organisms.

Acetyl-CoA consists of an acetyl group, which is linked to CoA via an energy-rich thioester bond that facilitates the transfer of the acetyl moiety to correspondent substrates. As a central metabolite itself, acetyl-CoA contributes to the synthesis of important metabolites such as fatty acids, sterols and amino acids, and also serves the production of reduced NADH and $FADH_2$ in the TCA cycle, which in turn feed the electron transport chain to generate ATP.

Given the central role at the intersection of numerous both anabolic and catabolic pathways, acetyl-CoA is an attractive candidate for a regulatory molecule linking the cellular metabolic state to the regulation of metabolic pathways and gene expression by modulating general protein and histone acetylation in eukaryotes (L Cai and Tu 2011; Shimazu et al. 2010).

In yeast, the biosynthesis of acetyl-CoA runs via two metabolic routes feeding two distinct pools of acetyl-CoA within the cell: 1) the nucleo-cytosolic pool of acetyl-CoA is supplied by the acetyl-CoA synthetase Acs2p, which catalyzes the ATP-dependent activation of acetate to acetyl-CoA (Takahashi et al. 2006; Van den Berg and Steensma 1995). 2) the mitochondrial pool of acetyl-CoA is determined by the activity of the mitochondrial acetyl-CoA synthetase Acs1p, and the putative acetyl-CoA hydrolase Ach1p. The latter was shown to actually catalyze the transfer of CoA from succinyl- and propionyl-CoA to acetate to form acetyl-CoA in vivo, rather than hydrolyzing it (Buu, Chen, and Lee 2003; Fleck and Brock 2009). Furthermore, the mitochondrial pyruvate dehydrogenase complex (PDH) converts pyruvate derived from glycolysis to acetyl-CoA via decarboxylation, whereat it depends on the import of pyruvate mediated by the pyruvate transporter Mpc1p (Bricker et al. 2012).

Some of the first links between the regulation of protein acetylation and the availability of acetyl-CoA as an acetyl donor were established in the yeast model organism. Takahashi et al. could show that under standard glucose conditions the nucleo-cytosolic pool of acetyl-CoA is controlled by Acs2p and is rate-limiting for histone acetylation and global transcription. Thus, inactivation of Acs2p lead to histone H3 hypoacetylation accompanied by global down-regulation of transcription (Takahashi et al. 2006). Consistently, the activity of the cytosolic acetyl-CoA carboxylase Acc1p, which uses acetyl-CoA for de novo synthesis of fatty acids, regulates the availability of acetyl-CoA for HATs and thus global histone acetylation (Galdieri and Vancura 2012). Furthermore, the rapid expression of growth genes upon entry into growth was recently shown to depend on the acetylation of histones specifically at these genes, resulting from a rapid increase in acetyl-CoA levels during growth. Acetyl-CoA was therefore suggested to function as a gauge of the cell's metabolic state and to regulate protein and histone acetylation (Ling Cai et al. 2011; L Cai and Tu 2011).

Contrary to yeast, mammalian cells rely on glucose rather than acetate as a major bioenergetic substrate, and consequetly the Acs2p homologue AcsCS1 was shown to only have a minor impact on histone acetylation. In contrast, knockdown of the nucleo-cytosolic ATP-citrate lyase (ACL), which produces acetyl-CoA from citrate derived from glucose via the mitochondrial TCA cycle, lead to a decrease in histone acetylation (Wellen et al. 2009). A very simplified comparison of yeast and mammalian acetyl-CoA metabolism and pools are shown in **Figure 5**.

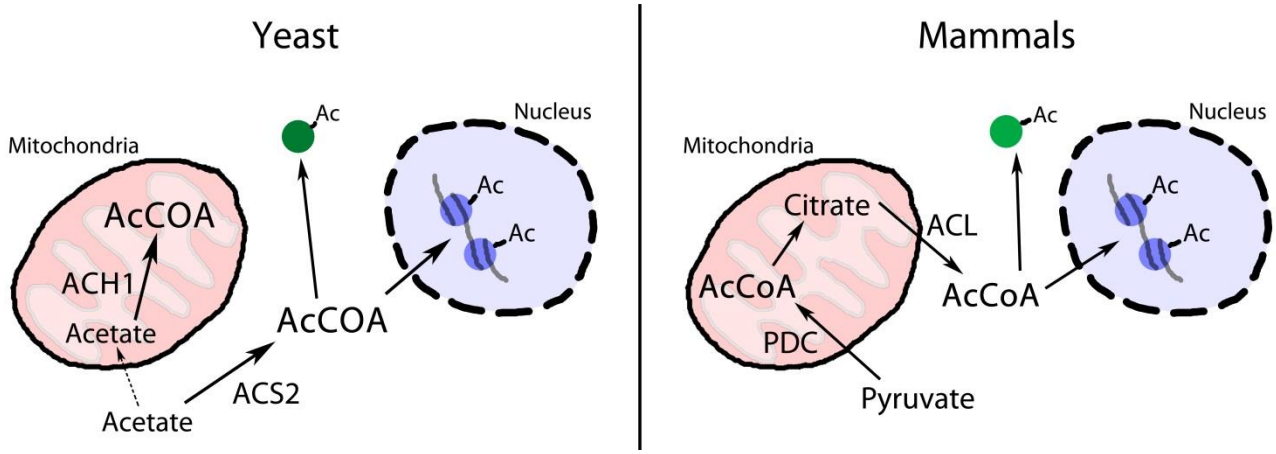

Figure 5: Nucleo-cytosolic Acetyl-CoA is linked to protein acetylation in yeast an mammals. In yeast, acetate is an abundant metabolite and major source for the generation of acetyl-CoA. In mitochondria, acetate is converted to acetyl-CoA by Acs1 and Ach1, while the nucleo-cytosolic pool is supplied by Acs2p. Mammalian cells mostly rely on glucose as a source of energy. In mitochondria, glycolysis derived pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC), which enters the TCA cycle, where citrate is formed. In the cytoplasm, the ATP-citrate-lyase (ACL) converts citrate to acetyl-CoA. In both yeast and mammals nucleo-cytosolic acetyl-CoA was shown to determine histone and protein acetylation (Wellen et al. 2009; Galdieri and Vancura 2012; Takahashi et al. 2006).

1.6 Aim of this study

Given the recent insights into the crucial role of protein and histone acetylation in many cellular processes, including autophagy and aging, this study aimed at examining the possible role of acetyl-CoA biosynthesis pathways in regulating long-term autophagy in an aging yeast model. We therefore hypothesized that the levels of acetyl-CoA may act as an internal indicator for the cellular metabolic state, and serves as substrate for HATs and other protein acetyltransferases leading to alterations in the acetyl-proteome, which regulates autophagy in aging cells. In short, we tried to establish a novel link between acetyl-CoA metabolism, histone acetylation, and the regulation of autophagy. To address this hypothesis, various disruptions of de novo acetyl-CoA synthesis pathways were investigated upon effects on autophagy as well as upon aging phenotypes.

2 Materials

2.1 Strains

Table 1: *S. cerevisiae* strains used in this work.

Name	Genotype	Origin
BY4741	Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Euroscarf
BY4742	Mat α his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Euroscarf
Δ*ach1*	BY4741 ach1::kanMX4	Euroscarf
BY4742 Δ*ach1*	BY4742 ach1::kanMX4	Euroscarf
acs2-WT	BY4741 acs2::HygMX [pHT112, ACS2-CEN-URA3]	Takahashi et al. (2006)
Acs2-Ts1	BY4741 acs2::HygMX [pHT215, Acs2-Ts11-CEN-URA3]	Takahashi et al. (2006)
acs2-WT Δ*atg7*	BY4741 atg7::kanMX4 acs2::HygMX [pHT112, ACS2-CEN-URA3]	Eisenberg et al. (in preparation)
Acs2-Ts1 Δ*atg7*	BY4741 atg7::kanMX4 acs2::HygMX [pHT215, Acs2-Ts11-CEN-URA3]	Eisenberg et al. (in preparation)
BY4741 pATG8-EGFP-ATG8	BY4741 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
Δ*ach1* pATG8-EGFP-ATG8	BY4741 ach1::kanMX4 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
BY4742 pATG8-EGFP-ATG8	BY4741 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
BY4742 Δ*ach1* pATG8-EGFP-ATG8	BY4742 ach1::kanMX4 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
tet-WT	BY4741 URA3::CMV-tTA	Yeast Tet-Promoters Hughes Collection

		(yTHC), ThermoScientific
Name (continued)	**Genotype (continued)**	**Origin (continued)**
tet-ACS2	BY4741 URA3::CMV-tTA pACS2::kanR-tet07-TATA	Eisenberg et al. (in preparation)
tet-WT Δ*ach1*	tet-WT ach1::hphNT1	Eisenberg et al. (in preparation)
tet-ACS2 Δ*ach1*	tet-ACS2 ach1::hphNT1	Eisenberg et al. (in preparation)
tet-WT pATG8-EGFP-ATG8	tet-WT pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
tet-ACS2 pATG8-EGFP-ATG8	tet-ACS2 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
tet-WT Δ*ach1* pATG8-EGFP-ATG8	tet-WT ach1::hphNT1 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
tet-ACS2 Δ*ach1* pATG8-EGFP-ATG8	tet-ACS2 ach1::hphNT1 pATG8::natNT2-pATG8-EGFP	Eisenberg et al. (in preparation)
tet-WT Δ*atg7*	tet-WT atg7::HIS3	Eisenberg et al. (in preparation)
tet-ACS2 Δ*atg7*	tet-ACS2 atg7::HIS3	Eisenberg et al. (in preparation)
tet-WT Δ*ach1* Δ*atg7*	tet-WT ach1::hphNT1 atg7::HIS3	Eisenberg et al. (in preparation)
tet-ACS2 Δ*ach1* Δ*atg7*	tet-ACS2 ach1::hphNT1 atg7::HIS3	Eisenberg et al. (in preparation)
Δ*sch9*	BY4741 sch9::HIS3	Longo et al.
Δ*sch9* Δ*ach1*	BY4741 ach1::kanMX sch9::HIS3	This Work
Δ*sch9* pATG8-EGFP-ATG8	BY4741 sch9::HIS3 pATG8::natNT2-pATG8-EGFP	This Work
Δ*sch9* Δ*ach1* pATG8-EGFP-ATG8	BY4741 ach1::kanMX sch9::HIS3 pATG8::natNT2-pATG8-EGFP	This Work

2.2 Growth media for S. cerevisiae

Medium	Contents
YPD (full media)	1 % yeast extract 2 % bacto peptone 4 % D-glucose (2 % agar)
SMD (minimal media)	0,17 % yeast nitrogen base 0,5 % ammonium sulphate 2 % D-glucose 80 mg/l histidine 200 mg/l leucine 30 mg/l all amino acids except the above mentioned 320 mg/l uracile 30 mg/l adenine (2 % agar) For BY4742 strains additional lysine was added up to a final concentration of 120 mg/l
GNA (Presporulation plates)	1 % yeast extract 3 % Difco nutrient browth 5 % D-glucose 2 % agar
Sporulation media	1 % KAc 0,005 % ZnAc

2.3 Buffers and Solutions

2.3.1 Chemical lysis

Solution	Contents
Lysis buffer	1,85 M NaOH 7,5 % β-mercaptoethanol

Solution (continued)	Contents (continued)
Final sample buffer (FSB), 1x	50 mM Tris/HCl, pH 6,8 2 % SDS 10 % glycerol 0,1 % bromophenol blue 100 mM β-mercaptoethanol
Tris solution	2 M Tris

2.3.2 SDS-Page and Western Blot analysis

Solution	Contents
Electrophoresis buffer	25 mM Tris-HCl, pH 8,3 192 mM Glycine 0,2 % SDS
Separating gel	250 mM Tris-HCl, pH 8,8 0,2 % SDS 12,5 % acrylamide 0,4 % N.N'-methylen-bisacrylamide 0,1 % ammonium peroxodisulfate (APS) 0,01 % N.N.N'.N'-tetramethylethylenediamide (TEMED)
Stacking gel	250 mM Tris-HCl pH 6,8 0,2 % SDS 5 % acrylamide 0,13 % N.N'-methylene-bisacrylamide 0,1 % ammonium peroxodisulfate (APS) 0,01 % N.N.N'.N'-tetramethylethylenediamide (TEMED)
Transfer buffer	10 mM CAPS/NaOH pH 11 10% methanol
TBS (-T)	10 mM Tris-HCl, pH 7,4 150 mM NaCl (0,02 % Triton-X 100)
TST	10 mM Tris-HCl, pH 7,4 150 mM NaCl 0,1 % Tween20

2.3.3 Antibodies

Antibody	Dilution	Company
α-GFP (mouse)	1:5000 in 1x TBS + 1 % milk powder	Roche (1814460)
α-Acs2p (rabbit)	1:5000 in 1x TBS + 1% milk powder	gift by Jeff Boeke (JH4885)
α-GAPDH (rabbit)	1:40000 in 1x TST + 1% milk powder	gift by Günther Daum
α-mouse POD (for GFP)	1:10000 in 1x TBS + 1% milk powder	Sigma (F-9137)
α-rabbit	1:10000 in 1x TBS + 1% milk powder	Sigma (A0545)
α-H3	1:5000 in 1x TBS + 1% BSA	abcam (ab1791)
α-H3K9-Ac	1:5000 in 1x TBS + 1% BSA	Millipore (07-352)
α-H3K14-Ac	1:5000 in 1x TBS + 1% BSA	Millipore (07-353)
α-H3K18-Ac	1:10000 in 1x TBS + 1% BSA	upstate (07-354)
α-rabbit (for H3, H3K9-Ac, H3K14-Ac, H3K18-Ac)	1:3000 in 1x TST + 5% BSA	Cell Signaling (7074)

2.3.4 PI staining

Solution	Contents
PBS	25 mM potassium phosphate buffer, pH 7,0 0,9 % NaCl
1000 x PI	100 µg/ml

2.4 Equipment

Equipment	Company
Analytical balance	Sartorius
Autoclave	Systec
Cell counter CASY™	Schärfe Systems
Centrifuge	Hermle Z400K
Centrifuge (table top)	Heraeus Biofuge fresco

Equipment (continued)	Company (continued)
Centrifuge for 96 well plates	Sigma 3-18K
Colony counter	Scananalyzer Colonycounter Digital m
Electroblotting power supply	Biorad PowerPac300
Flow Cytometer	BD FACSAria™ Flow Cytometer
Flow Cytometer with HTS loader	BD LSRFortessa™ Cell Analyzer
Fluorescence icroscope	Zeiss
Fluorescence plate reader	Tecan GeniosPro
Incubator	INFORS HAT Microtron
pH-meter	Metrohm
Photometer	Genesys
Thermomixer	Eppendorf Thermostat plus
Vortex	Scientific Industries
Water distillation	GFL Dest 2208

3 Methods

3.1 Growth media

Media were prepared with double destilled water and autoclaved at 121°C for 23 minutes. For synthetic media, 10x stocks of amino acid mixtures were prepared, autoclaved and stored at -20°C upon addition to the media just before use. Special mixtures lacking the corresponding amino acids were prepared for selection by auxotrophy markers. Heat sensitive ingredients were added after sterilization when the media had cooled down to approximately 50°C.

For preparation of culture plates, 2% agar was added to the media before autoclaving.

3.2 Cultivation and storage

3.2.1 Liquid cultures

S. cerevisiae was grown in full media (YPD) or synthetic media containing all amino acids at 28° C and constant shaking at 145 rpm, until reaching the desired density.

3.2.2 Plate cultures

Yeast strains were streaked out on YPD agar, and incubated at 28°C for 48 hours. Inoculated plates were stored at 4° C for at least 4 hours up to a maximum of two weeks before use.

3.2.3 Storage

Yeast strains were cultivated on (restrictive) plates for two days at or 28° C. Fresh liquid full medium was then inoculated with the amount equivalent to a single colony. After incubation at the correspondent temperature for about 16 hours, 750 µl of the cell suspension were mixed with an equal volume of sterile 50 % glycerine in a cryotube, and immediately frozen at -80° C for long-term storage.

3.3 General molecular biological and biochemical methods

3.3.1 Chemical lysis

4 OD_{600} units of cells were harvested in Eppendorf tubes, washed once with 1 ml ddH_2O and frozen at -20° C. For lysis, pellets were thawed on ice, resuspended in 200 µl lysis buffer. After incubation on ice for 10 minutes, 200 µl 55 % TCA were added and samples were mixed by inverting the reaction tube. Following another 10-minute incubation on ice, samples were

centrifuged at 10.000 g for 10 minutes at 4° C, and the supernatant was removed completely by aspiration. Pellets were resuspended in 200 µl 1x FSB and 1-7 µl untitrated 2 M Tris solution was added until blue colour was obtained. The probes were heated for 5 minutes at 95° C, spinned down for 10 seconds and used immediately, or stored at -20° C for further use.

3.3.2 SDS-Polyacrylamide gel electrophoresis

Separation of proteins for further western blotting analysis was performed using discontinuous SDS-polyacrylamide gel electrophoresis. Resolving gels (12,5 % acrylamide) were poured, covered with butanol and polymerized overnight. If not used immediately, gels were wrapped in kitchen roll drenched in 1x Tris-Glycin-SDS running buffer and stored at 4° C for up to three weeks.

Upper stacking gel (5 % acrylamide) was poured right before sample preparation.

Electrophoresis was performed in Tris-Glycin-SDS running buffer at a current of 10 mA for focussing of proteins in the stacking gel, and at 20 mA after entering the resolving gel.

3 µl of Fermentas PageRuler™ Prestained Protein Ladder served as a standard for determination of molecular weight of protein bands in subsequent immunolotting analyses.

3.3.3 Immunoblotting analysis

SDS-polyacrylamide gel electrophoresis was followed by transfer of proteins to a PVDF (Millipore) membrane using the tank-blot-system of Amersham Biosciences (Mighty Small Transphor) and CAPS transfer buffer. Prior activation of the PVDF membrane was done in methanol for 1 minute, followed by a washing step in ddH_2O for 1 minute. Transfer was performed at 240 mA for at least one hour at constant stirring. In order to block unspecific binding, blotted PVDF membranes were incubated in blocking solution for one hour (3 % milk powder in 1x TBS at room temperature) or overnight (1 % milk powder in 1x TBS at 4° C) under shaking conditions. Incubation with primary and secondary antibodies was performed at room temperature and under shaking conditions for at least one hour each, with three intermediate and concluding washing steps in 1x TBS-T for 5 minutes.

Detection was performed using the ECL-System (chemoluminescence) from Amersham Biosciences. Membranes were covered in reaction solution for 1 minute and exposed to X-ray films for 10 seconds up to one hour, depending on signal intensity.

3.3.4 Autophagy tests and cell death assays

3.3.4.1 Chronological agings

All aging experiments were performed at 28° C and constant shaking. Overnight cultures were prepared in SMD media with all amino acids (for all experiments using BY4742 strains additional lysine was added to a final concentration of 120 mg/l). For the main culture 10 ml of SMD+AA media were inoculated to an OD_{600} of 0,05 in 100 ml baffled flasks. Inoculation of the main cultures marks time point zero for the chronological agings.

3.3.4.2 Clonogenicity assay

Clonogenic survival of yeast cultures were determined using survival platings. Cell density of chronolonically aging cultures were measured with a CASY cell counter system in a 1:10.000 dilution in 1x Casyton. The detection gate for yeast cells was set between 1,5 and 15 µm; 2 separate measurement cycles with 200 µl were performed for each sample, using a 60 µm capillary. Subsequently, a volume corresponding to 500 cells was taken from a 1:10.000 dilution of the main culture in ddH_2O and plated on YPD agar. Plates were incubated at 28° C for 48 hours and colonies were counted.

3.3.4.3 PI staining

To determine the fraction of dead cells during yeast aging, cells were stained with PI (propidium iodide), a dye that is specific for cells which lost their membrane integrity, a typical marker of necrosis.

About $5x10^6$ cells were transferred in 96 well plates, and stained with 250 µl PI/1x PBS (1:1.000). After incubation for 5 minutes in the dark, plates were centrifuged at 4000 rpm for 5 minutes, and the supernatant was removed by tipping. Pellets were resuspended in 250 µl 1x PBS and the samples were analyzed by through flow cytometry (FACS analysis). 30.000 events were recorded for each probe.

3.3.4.4 Induction of autophagy by rapamycin treatment

In order to boost autophagy, rapamycin (1,1 mM in DMSO stock solution) was diluted with water and added to the main cultures at a concentration of 20 nM. A correspondent dilution of DMSO in water was added to control cultures.

3.3.4.5 Fluorescence microscopy

Fluorescence microscopy was used to detect and monitor cellular autophagy rates in strains bearing GFP-tagged Atg8p from its endogenous promotor. To rule out dead cells from the analysis, samples were co-stained with PI.

Depending on cell density, 100 - 300 µl of cell culture were centrifuged at 10.000 rpm for 1 minute and resuspended in 250 µl of a 1:1000 dilution of PI in 1x PBS (pH 7,0). After 1 minute of incubation in the dark, cells were spinned down (14.000 rpm, 30 sec.), and 1,5 µl of the cell pellet were transferred onto a microscope slide and covered with a cover slip. For detection of GFP- and PI-fluorescence, eGFP- and dsRED-filter were used at exposure times of 5.000 ms for GFP, and 150 ms for PI, respectively.

3.3.5 Determination of histone-acetylation

3.3.5.1 Sample collection and lysis

The acetylation of Histone H3 was detected using a immunoblot approach with antibodies specific for acetylated lysyl residues at the N-terminus of H3, as previously described (Eisenberg et al. 2009; Kao and Osley 2003). Cells corresponding to 12,5 OD_{600} were harvested at given timepoints (10.000 rpm, 1 min), wasched with 500 µl 20% TCA and immediately frozen in liquid nitrogen. Samples were stored at -70°C until further usage. For mechanical lysis, samples were thawed on ice, resuspended in 200 µl 20% TCA and transferred to Eppendorf tubes containing 0,4 g acid washed glass beads. Samples were vortexed at maximum speed for 2 minutes at 4°C, washed twice with 500 µl 5 % TCA and the corresponding supernatants were collected. Following an incubation on ice for 10 minutes, samples were centrifuged (15.000 rpm, 15 min), the supernatant discharged and the pellets resuspended in 250 µl 1x Lämmli buffer. 2 M Tris was added for neutralization of the samples, before they were heated at 95° C for 5 min, zentrifuged (14.000 rpm, 10 min), aliquoted and stored at -80° C for further use.

3.3.5.2 Immunoblotting of histones

For histone blots, 15 % SDS gels were used. After transfer of the proteins to the PVDF membrane (220 mA, 75 minutes), membranes were blocked in 1x TBS + 1 % milk powder overnight at 4° C. Membranes were incubated with primary and secondary antibodies for 1 hour each, with three intermediate washing steps with deionized water for 2-3 minutes. Before detection, the blots were again washed with deionized water twice, and finally once with 1x TBS + 0,05 % Tween + 0,002% Triton.

3.3.6 Acetic acid measurements

The concentration of extracellular acetate in the media was determined using the Acetic Acid (Acetate Kinase Manual Format) kit (Megazyme). The manufacturer's protocol was adapted for 96 well plates in a total volume of 200 µl and subsequent readout in the TECAN plate reader.

3.3.7 Generation of strains via yeast mating and sporulation

Mating of haploid strains of opposite mating type harbouring the desired markers was performed by combining cell material of both strains on a YPD agar plate. After 24 hours of incubation at 28° C, cells were streaked out on UHL plates in order to select for diploids. Diploids were cultivated at 28° C for 24 hours, before streaking them out on GNA presporulation plates, which were incubated at the same conditions. For sporulation, 3 ml of sterile filtered liquid sporulation medium was inoculated with cell material equivalent to one colony in eprouvettes and incubated at 25° C for 3-5 days under shaking conditions.

3.3.7.1 Tetrade dissection

1,5 ml of sporulation culture were harvested (13.000 rpm, 1 minute), washed twice in ddH_2O and resuspended in 500 µl ddH_2O. 2 µl β-glucuronidase / arylsulfatase (4,5 U/ml) were added and after incubation at room temperature for 30 minutes, cells were washed twice with 500 µl ddH_2O and resuspended in 500 µl ddH_2O. 10 µl of the suspension were dropped to the edge of a thick YPD agar plate, and tetrads were dissected using a micromanipulator.

3.3.7.2 Confirmation of clones

Strains derived from mating and subsequent sporulation were tested for presence of met15Δ0 and lys2Δ0 markers by stamping on UHL+Met and UHL+Lys plates (SMD agar plates containing uracile, histidine, leucine and either methionine or lysine). Additional markers like NatNT2 (for endogenous GFP-tagging of Atg8) were determined on corresponding selection media.

4 Results

4.1 Disruption of mitochondrial de novo Acetyl-CoA biosynthesis pathways shortens chronological lifespan

In mitochondria, three distinct routes of Acetyl-CoA formation are known. On the one hand, Acetyl-CoA-Synthetase Acs1p and the putative Acetyl-CoA-Hydrolase and CoA-Transferase Ach1p are responsible for mitochondrial acetate utilization and formation of Acetyl-CoA, while the mitochondrial pyruvate carrier 1 (Mpc1p) on the other hand is essential for pyruvate uptake and thus for mitochondrial pyruvate decaboxylation pathways. To test if limitation of de novo Acetyl-CoA production in the mitochondria affected cellular aging, knockout mutants of genes encoding the aforementioned proteins involved in mitochondrial Acetyl-CoA biosynthesis were tested in regard to their chronological lifespan. However, since Acs1p is poorly expressed when cells are grown on glucose (van den Berg et al. 1996), and deletion of *ACS1* (Δ*acs1*) shows no effects on aging and lifespan (data not shown, unpublished data (Eisenberg et al. in preparation)), its role in cellular aging seems to be rather minor. For this reason, the experimental focus of this work lay on deletion strains of *ACH1* (Δ*ach1*) which were tested in chronological aging experiments.

4.1.1 Deletion of ACH1 leads to shortened chronological lifespan

Ach1p is a 64 kDa protein with mitochondrial localization and was initially thought to execute Acetyl-CoA hydrolysis in order to prevent toxic effects of Acetyl-CoA accumulation in mitochondria (F. J. Lee, Lin, and Smith 1990; Buu, Chen, and Lee 2003). However, since hydrolysis of the high energetic thioester-bond would entail the loss of two ATP necessary for the bond formation and thus result in a waste of energy, the enzyme was subject to further research and finally recharacterized to exhibit CoA-transferase rather than hydrolysis activity. Showing high specific activity for CoASH transfer from succinyl-CoA and propionyl-CoA to acetate, a role in acidic acid detoxification was suggested for Ach1p, turning it into an important source of mitochondrial Acetyl-CoA formation (Fleck and Brock 2009).

Effects on aging upon deletion of *ACH1* (Δ*ach1*) were monitored in the long living BY4741, as well as in the rather short lived BY4742 strain background, using established methods of determination of survival like clonogenicity assays and flow cytometric analysis of propidium iodide (PI) stained cultures (**Figure 6**).

In both strain backgrounds, deletion of *ACH1* results in a shortened chronological lifespan, indicated on the one hand by a loss of clonogenicity, and by an increasing amount of PI stained cells during aging. Due to the nature of the clonogenicity assay, the decreasing survival of Δ*ach1* gets apparent already during early days of aging, while PI staining reveals an augmentation of the necrotic population after day 5 of aging, compared to wild type controls.

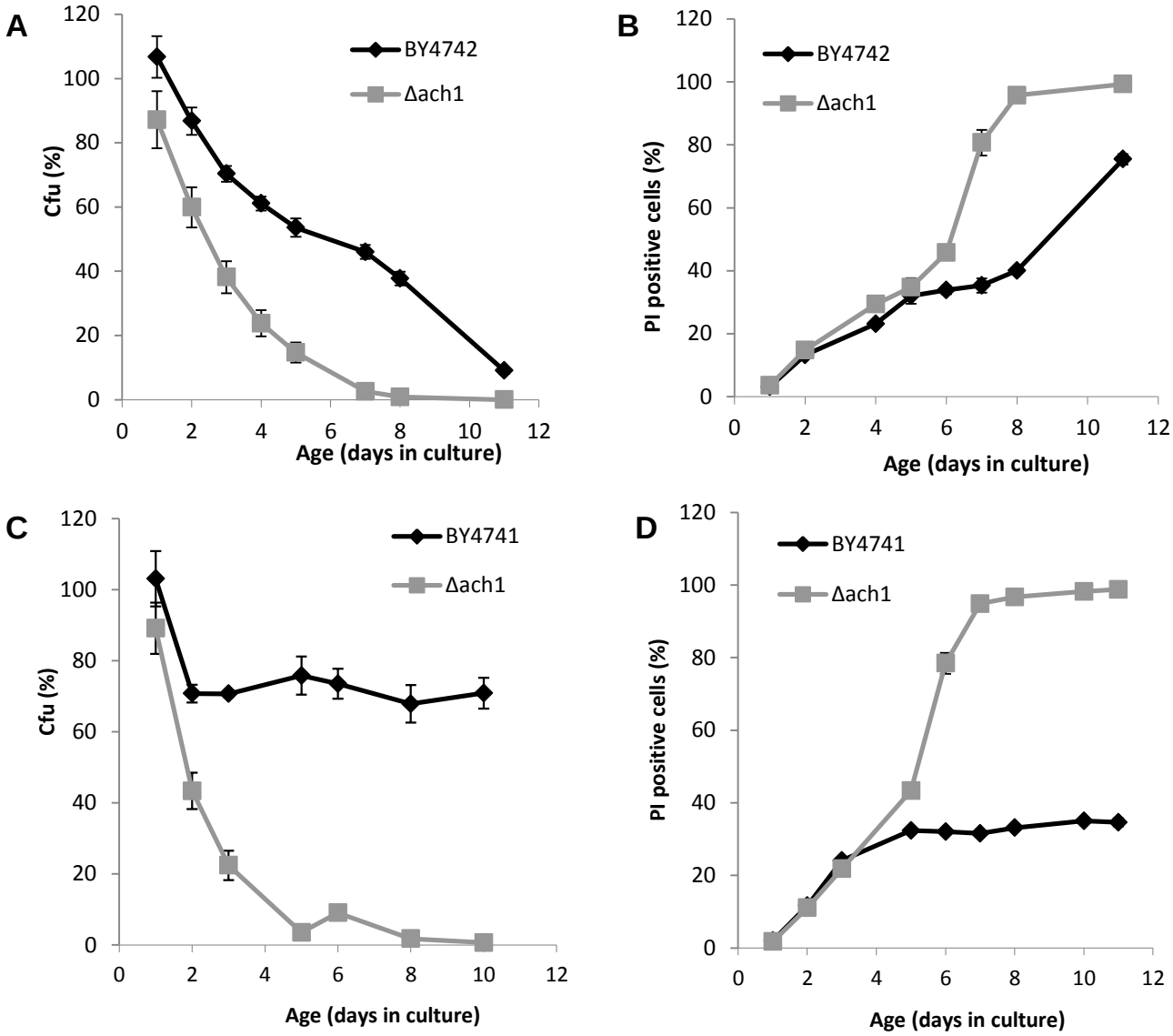

Figure 6: Deletion of ACH1 decreases chronological lifespan. (A-D) Chronological lifespan (CLS) analysis were performed in SMD+AA (120 mg/l lysine) (A, B) and SMD+AA (C, D) respectively, comparing wild type cells to Δ*ach1* cells. Survival was measured using clonogenicity assays (A, C) and glow cytometry quantification of propidium iodide (PI) stained cells (B, D). Lifespan was determined in two different strain backgrounds, the short living BY4742 (A, B) and the long living BY4741 (C, D). Data represent means ±SEM (n = 4) of representative aging experiments.

Consistent with this data, our group could show that deletion of *MPC1* leads to accelerated aging and a decreased chronological lifespan in a similar manner as *ach1* deleted cells, suggesting that mitochondrial acetyl-CoA generation in general is required for healthy aging.

4.2 Repression of Acs2p-mediated nucleo-cytosolic Acetyl-CoA biosynthesis pathway does not affect yeast lifespan

Given that lifespan of yeast is severely limited upon disruption of mitochondrial acetate utilization pathways, we next asked if the nucleo-cytosolic pool of acetyl-CoA might affect yeast aging and lifespan. In yeast, the main source of acetyl-CoA that can be used for histone-acetylation is the acetyl-CoA synthetase 2 (Acs2p), which is located both nuclear and cytosolic, and catalyzes the ATP-dependent ligation of coenzyme A and acetate (Starai and Escalante-Semerena 2004; Takahashi et al. 2006). In order to achieve a repression of the nucleo-cytosolic acetyl-CoA synthesis, we therefore followed two distinct approaches: (i) by knockdown of *ACS2* using a repressable tet-promotor system, and (ii) by using a temperature-sensitive mutant of Acs2p.

4.2.1 Knockdown of Acs2p does not prolong yeast lifespan

Interfering with *ACS2* or its gene product in order to repress the nucleo-cytosolic acetyl-CoA biosynthesis is not a trivial matter, since Acs2 is essential for growth on glucose (Van den Berg and Steensma 1995; van den Berg et al. 1996). Hence, aging phenotypes of *ACS2* knockout strains cannot be monitored under standard aging conditions. To circumvent this problem, we used BY4741 strains carrying the *ACS2* gene under the control of a doxycycline repressible promotor, allowing knockdown of *ACS2*. Since the extent of *ACS2* knockdown directly correlates with the degree of growth arrest, very moderate knockdown conditions were chosen by titrating doxycycline to optimal conditions (1 ng/ml doxycycline), resulting only in a slight, but significant reduction of cell densities while still maintaining close to normal growth (**Figure 7 A**). Furthermore, knockdown of *ACS2* was controlled via immunoblot analysis, detecting a slight reduction in Acs2p protein level after 24 hours of cultivation (**Figure 7 B**).

On the basis of these knockdown conditions, cells were chronologically aged under standard glucose conditions and survival rates were determined.

However, while disruption of the mitochondrial routes of acetate consumption lead to accelerated aging, down-regulation of the nucleo-cytosolic acetyl-CoA synthetase barely affected yeast survival under the tested conditions as determined by clonogenicity (**Figure 7 C**).

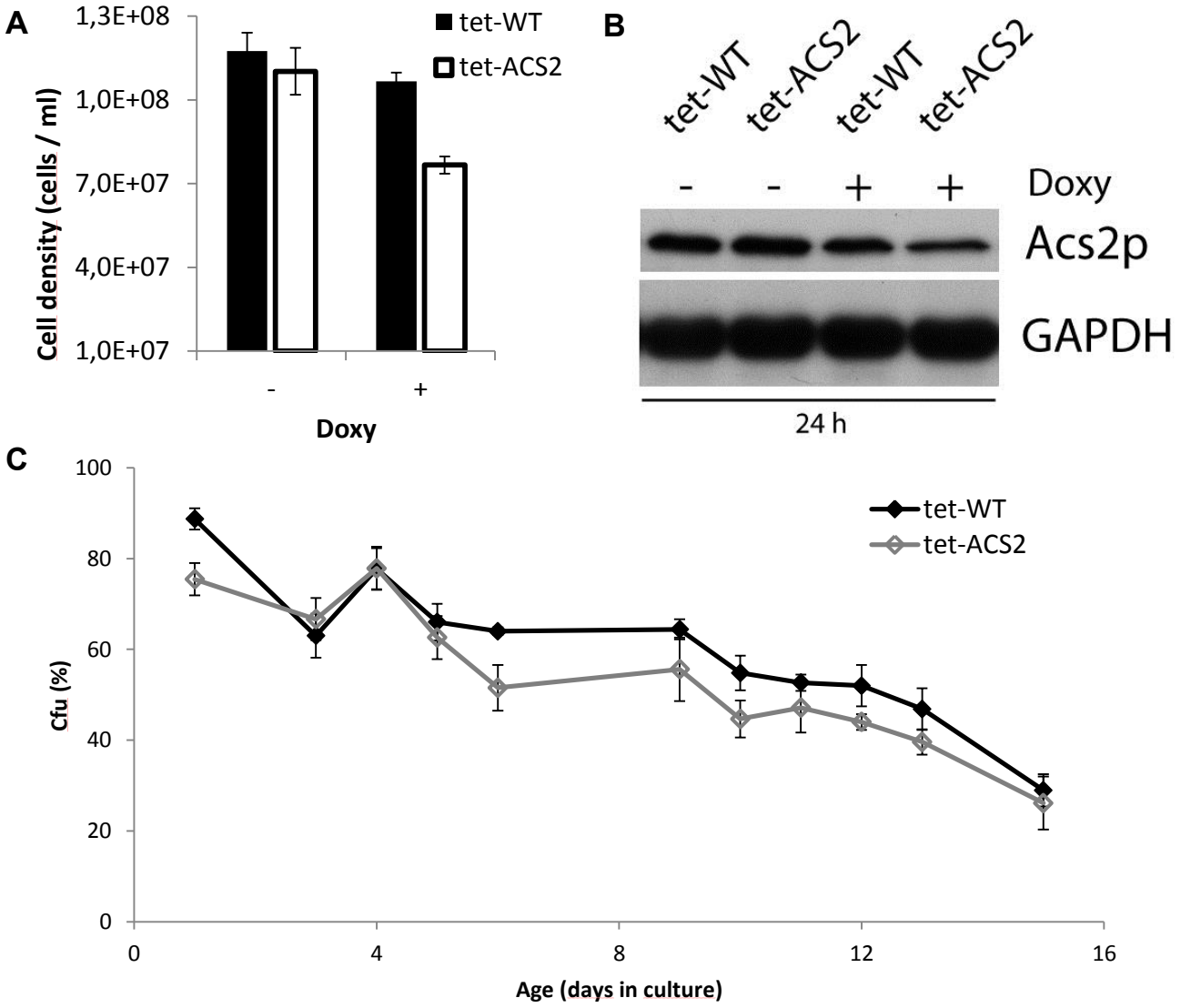

Figure 7: Moderate knockdown of ACS2 does not affect chronological lifespan. (A) Knockdown of *ACS2* was controlled by measuring cell densities using a CASY cell counter. Cells were grown in SMD+AA with (1 ng/ml doxycycline; + Doxy) and without doxycycline (- Doxy) and cell densities were determined after 24 hours of chronological aging. Data represent means ± SEM (n = 4). **(B)** Representative immunoblot of protein extracts from wild type (tet-WT) and *ACS2* knockdown cells (tet-ACS2) depicted in (A) using specific antibodies for Acs2p and GAPDH as a loading control. **(C)** Chronological aging was performed in SMD+AA with 1 ng/ml doxycycline. Survival was determined at given timepoints using clonogenicity assay. Data represent means ± SEM (n = 4) of a representative experiment.

4.2.2 Inactivation of Acs2p counteracts pro-aging effect of enhanced nucleo-cytosolic Acetyl-CoA production

To further characterize the effects of a down-regulation of nucleo-cytosolic Acetyl-CoA production, a temperature-sensitive mutant of Acs2p (Acs2-Ts1) was tested in regards to phenotypes during chronological aging. Acs2-Ts1 harbors four point mutations, resulting in D81N, K205R, C267W and E385K substitutions in the amino acid sequence, that lead to decreased enzymatic activity while maintaining protein stability. The mutant and wild type Acs2p were expressed from a pHT215 plasmid from their original *ACS2*-promotors in a Δacs2 background. Compared to its wild-type control (WT-Acs2), Acs2-Ts1 was reported to show

significant loss of histone acetylation and down-regulation of global transcription, suggesting a central role for Acs2p generated Acetyl-CoA for histone acetylation and gene expression (Takahashi et al. 2006).

During chronological aging, inactivation of Acs2p (Acs2-Ts1) leads to a drastic survival benefit when compared with its wild type control (**Figure 8**). However, it is important to note that in comparison to its BY4741 background origin, the WT-Acs2 control shows heavily increased cell death during aging. Western-blot analysis revealed that compared to the BY4741 wild type, both the WT-Acs2 and the Acs2-Ts1 strains show overexpression of their respective Acs2p protein version. Although Acs2p is expressed from its original promotor in these strains, expression from a centromeric plasmid, which often occurs in more than one single copy per cell, and is uncoupled from the chromatin status of the cell, may explain these elevated Acs2p levels.

Hence, this data suggests, that the temperature-sensitive Acs2-Ts1 mutation rather recovers the reduced lifespan of the WT-Acs2 strain, than leading to a real lifespan prolongation.

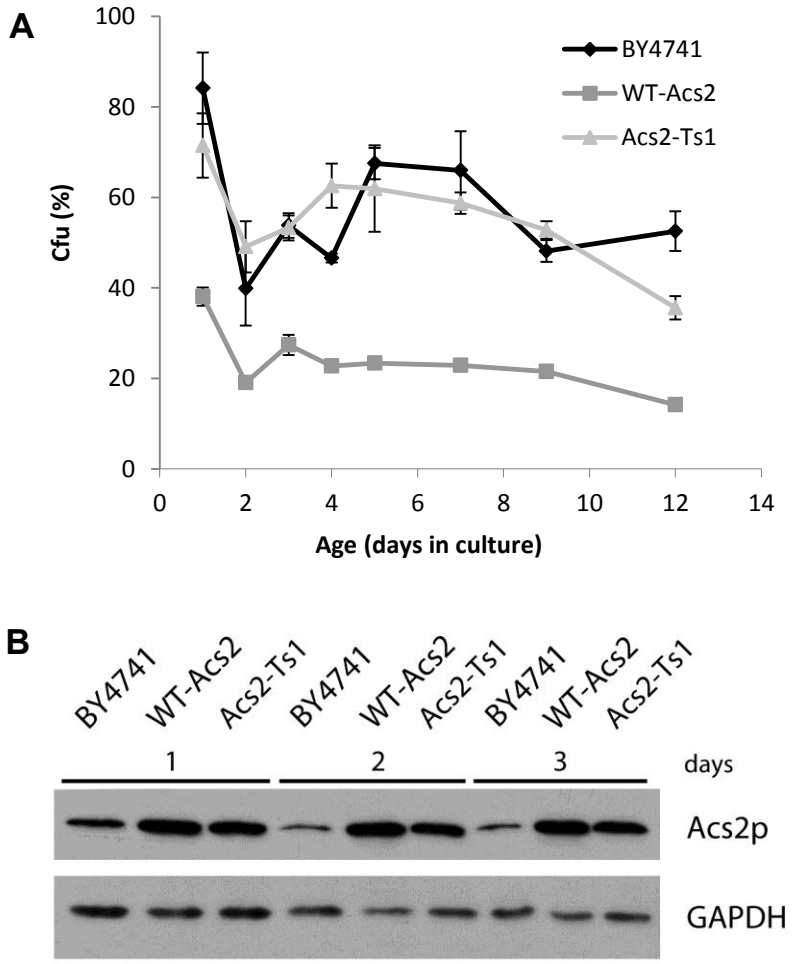

Figure 8: Inactivation of Acs2p rescues loss of viability upon overrepresentation of Acs2p. (A) Chronological aging in SMD+AA of BY4741 wild type cells and Δ*acs2* cells expressing wild type Acs2p (WT-Acs2) and a temperature sensitive mutant (Acs2-Ts1). Survival was determined at indicated timepoints measuring colony forming capacity in clonogenicity assays. Data represent means ± SEM (n = 4). **(B)** Immunoblot analysis using antibodies specific for Acs2p and GAPDH as a loading control of lysates from the strains depicted in (A). A representative blot is shown.

4.2.3 Beneficial effect of Acs2-Ts1 on survival is partly autophagy-dependent

It had previously been shown, that hypoacetylation of histones is associated with longevity and enhanced rates of autophagy during aging. For example, the known autophagy inducer and HAT inhibitor spermidine leads to decreased acetylation at various histone H3 lysine residues and prolongs lifespan of various model organisms in an autophagy dependent manner. Also, the simultaneous inactivation of histone acetyl transferases *IKI3* and *SAS3* leads to similar beneficial effects on yeast lifespan (Eisenberg et al. 2009).

Based on these findings, we checked whether the beneficial effects of Acs2-Ts1 on lifespan were dependent on autophagy. For this purpose, the Acs2-Ts1 strain and its wild type control (WT-Acs2), each bearing an additional knockout of *ATG7*, a gene required for the execution of macroautophagy, were chronologically aged and clonogenicity was determined (**Figure 9**).

Interestingly, deletion of *ATG7* completely abrogates the pro-survival effect of Acs2-Ts1 in the long term, and survival drops down to wild type level. In the rather short lived WT-Acs2 background however, Δ*atg7* has only small further effects. Thus, the advantageous effect of Acs2p inactivation on survival, accompanied by histone deacetylation, is in fact autophagy-dependent.

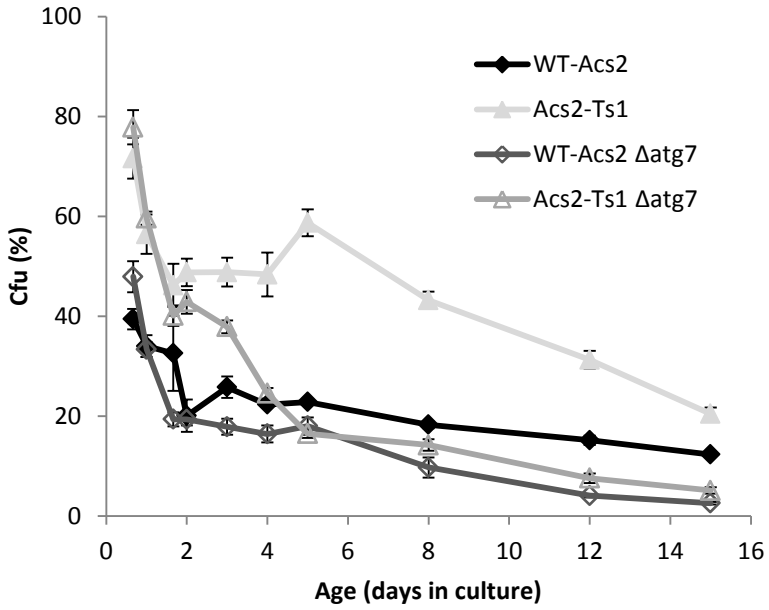

Figure 9: Inactivation of Acs2p rescues loss of viability by Acs2p overexpression in an autophagy-dependent manner. Chronological aging in SMD+AA of cells cells expressing wild type Acs2p (WT-Acs2) and a temperature sensitive mutant (Acs2-Ts1) without and with additional deletion of the essential autophagy gene ATG7 (WT-Acs2 *Δatg7* and Acs2-Ts1 *Δatg7* respectively). Survival was determined by colony forming capacity (clonogenicity). Data represent means ± SEM (n = 4) of a representative aging experiment.

4.3 Inhibition of mitochondrial Acetyl-CoA production by ACH1 deletion causes upregulation of nucleo-cytosolic Acs2p-pathway

Since the abatement of Acetyl-CoA generation in the mitochondria may lead to an accumulation of metabolic precursors such as acetate within the cytosol, corresponding cytosolic pathways might be activated to further metabolize these in turn. We therefore hypothesized that the diminishment of mitochondrial Acetyl-CoA production by deletion of *ACH1* causes an upregulation of nucleo-cytosolic pathways of acetate-utilization.

It has been described earlier, that Δ*ach1* cells show highly increased acetate levels in media after diauxic shift, probably as a result of reduced usage in the mitochondria (Orlandi, Casatta, and Vai 2012; Fleck and Brock 2009). Consistent with the fact that Acs1p is only poorly expressed on glucose and that its deletion lacks any effect of lifespan, the deletion of *ACS1* in the background of Δ*ach1* does not alter the medium acetate concentrations significantly. This suggests that the Acs1p-pathway cannot compensate for the abrogated mitochondrial acetate utilization in *ach1* mutant cells (data not shown, unpublished data (Eisenberg et al. in preparation)).

To see if the nucleo-cytosolic Acs2p-pathway, executing the ATP-dependent conversion of acetate and coenzyme A to acetyl-CoA, was influenced by *ACH1* deletion, Acs2p protein levels were analysed via western blot (**Figure 10**).

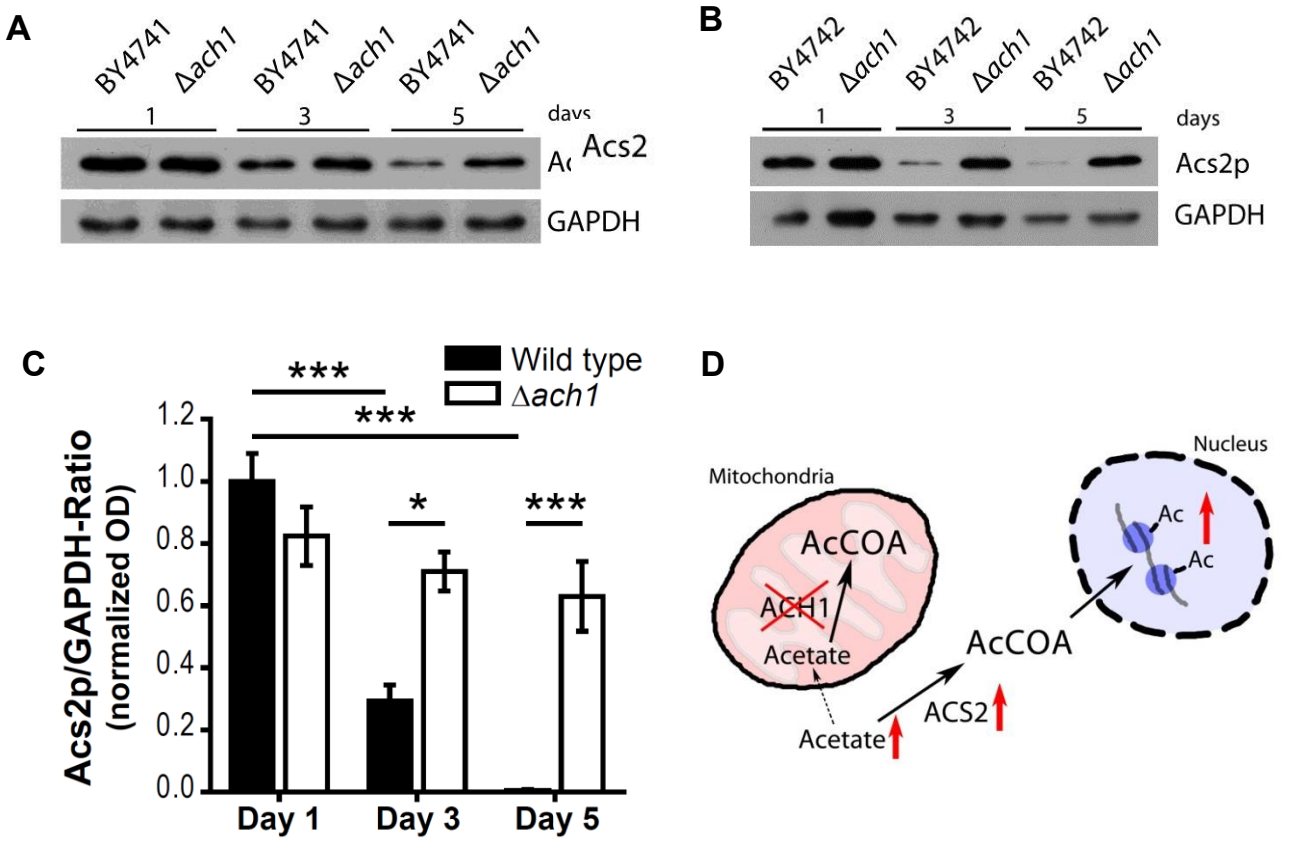

Figure 10: Deletion of *ACH1* causes upregulation of the nucleo-cytosolic Acs2p pathway. (A, B) Representative immunoblot analyses of protein extracts from BY4741 wild type (A) as well as BY4742 wild type cells (B) and their respective *ACH1* deletion strains. Blots were performed using α-Acs2p and α-GAPDH (loading control) antibodies. Cells have been chronologically aged in SMD+AA and samples were taken at indicated timepoints. **(C)** Densitometric quantification of immunoblots depicted in (A). Data was plotted by Tobias Eisenberg and represents mean ± SEM (n = 4). ***p < 0,001, *p < 0,1. **(D)** Graphical abstract on how deletion of *ACH1* leads to upregulation of the nucleo-cytosolic path of acetyl-CoA generation. The associated accumulation of acetate and histone acetylation is covered in chapters 4.6.5 and 4.7.

While in both corresponding wild type strains – the long lived BY4741 as well as in the short lived BY4742 -, protein levels of Acs2p decreased consequently during the first five days of aging, deletion mutants of *ACH1* displayed significantly higher Acs2p levels when compared to the wild type controls. Intriguingly, Δ*mpc1* mutant cells show similarly increased Acs2p-levels, suggesting that upon disruption of mitochondrial Acetyl-CoA synthesis pathways, nucleo-cytosolic Acs2p-pathway is upregulated (Eisenberg et al. in preparation).

To ensure that the accumulated Acs2p, which was detected in the *ACH1* mutant cells by western blotting, was functionally active enzyme, Acetyl-CoA synthetase (ACS) activity was measured using an established enzyme assay (van den Berg et al. 1996). In order to exclude any mitochondrial Acetyl-CoA synthetase activity from the assay, measurements were undertaken in Δ*acs1* strain background. In line with the previous results, deletion of *ACH1* caused an age-dependent increase in ACS activity when compared to the corresponding controls (Eisenberg et al. in preparation).

4.4 Deletion of ACH1 leads to age-dependent autophagy defect

It had been shown before that deacetylation of histones is generally linked with healthy aging and a prolonged lifespan (Imai et al. 2000; Lin, Defossez, and Guarente 2000; Longo and Kennedy 2006), and that hypoacetylation of histone H3 is associated with enhanced autophagy during aging (Eisenberg et al. 2009; Mariño et al. 2011). Since *ACH1* knockout cells show increased protein acetylation in general and hyperacetylation at N-terminal lysyl residues of histone H3 in specific (Eisenberg et al. in preparation), Δ*ach1* cells were tested for possible autophagy defects.

Autophagosome delivery and autophagic body lysis, two markers essential for functional autophagic flux, were monitored using an established GFP-Atg8p processing assay (Cheong and Klionsky 2008). Vacuolar degradation of endogenously expressed N-terminally EGFP-tagged Atg8p was monitored using epifluorescence microscopy as well as immunoblot analysis (**Figure 11**).

While young cells (day 1) of both wild type and Δ*ach1* showed high numbers of autophagic cells (as indicated by vacuolar localization of EGFP), only wild type cells retained the ability to transport Atg8p to the vacuole via autophagy during aging. In contrast, *ach1* mutant cells developed a strong defect in autophagy, which is definitive from day three onwards during chronological aging. It is important to note, however, that this age-dependent defect is not a direct consequence of premature cellular demise of Δ*ach1* knockout cells, since cell death occurs later on in the aging, as can be seen in propidium iodide counterstaining of microscopy samples (see **Figure 11 A**) and survival assays (see chapter 4.1.1).

Apart from showing reduced vacuolar localization of EGFP-Atg8p, *ACH1* knockout cells showed increased accumulation of the fusion construct at small dot-like structures, supposedly pre-autophagosomal structures (PAS). This might hint at a defect in the autophagosomal formation cascade in aging Δ*ach1* cells, occurring downstream of Atg8p recruitment to the PAS (Suzuki et al. 2007; Suzuki et al. 2013).

Immunoblot analyses of cultures harboring the endogenous EGFP-Atg8p fusion construct confirmed the autophagy-deficient phenotype of aged *ach1* mutant cells. While wild type cells show constant to increasing amounts of free, proteolytically liberated EGFP derived from autophagic transport to the vacuole, no free EGFP could be detected in Δ*ach1* cells from day three on (**Figure 11 C**).

Together, these data suggest that mitochondrial acetate utilization by Acetyl-CoA production through Ach1p is crucial for maintaining autophagic flux during aging.

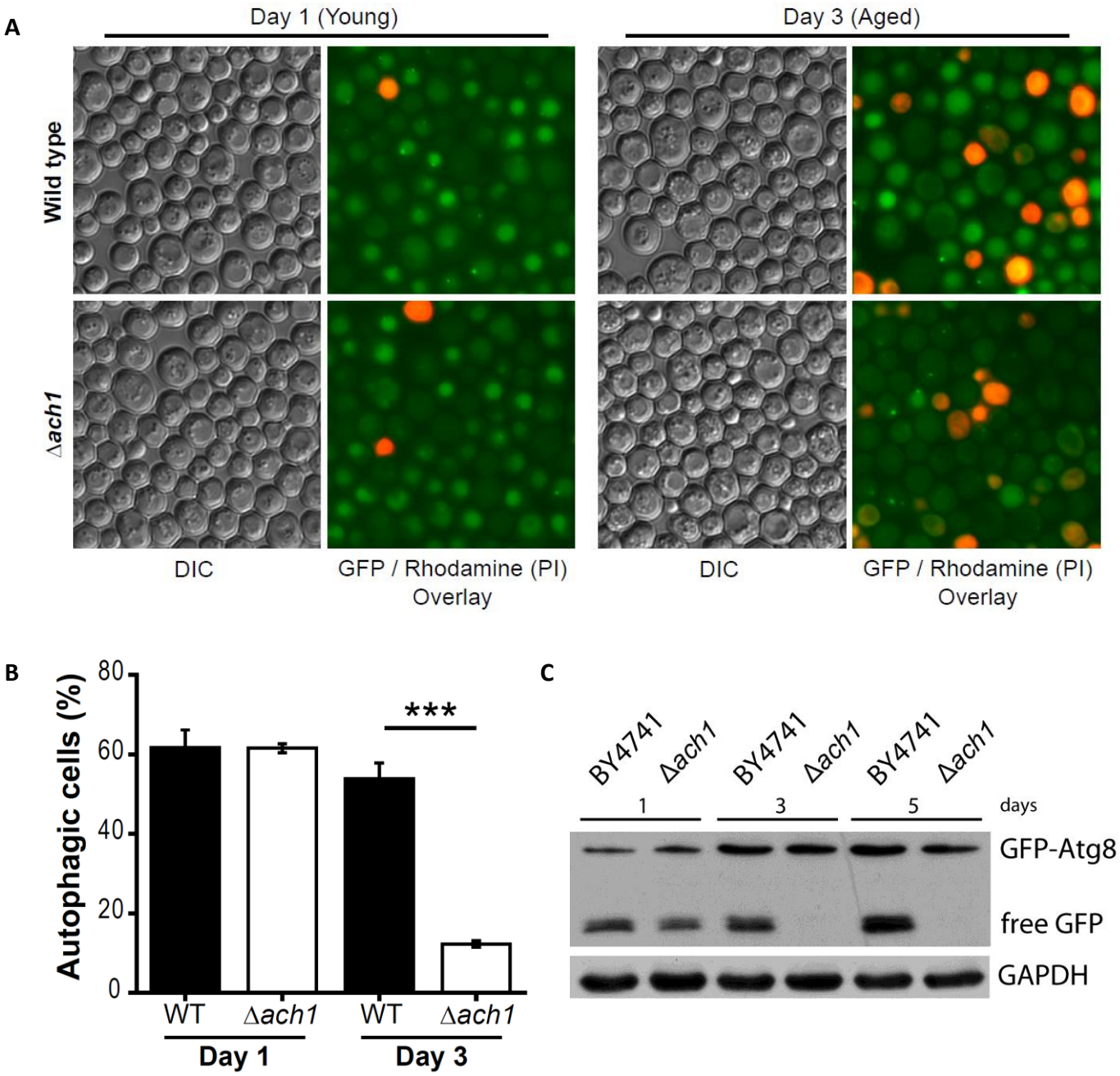

Figure 11: Deletion of *ACH1* causes age-dependent autophagy defect. (A) Representative fluorescent microscopy pictures of wild type (WT) and *ACH1* deleted (Δ*ach1*) cells expressing an EGFP-Atg8p fusion protein from its natural Atg8 promotor (pATG8-EGFP-ATG8). Samples were counterstained with propidium iodide (PI) to visualize dead cells. Cells were aged in SMD+AA with samples being microscoped at indicated timepoints. **(B)** Quantification of autophagic cells depicted in (A). 150-300 cells were counted for each replicate. Autophagic cells were defined as cells exhibiting a clear vacuolar localization of EGFP fluorescence. Data was plotted by Tobias Eisenberg and represents mean ± SEM (n=4). ***p < 0,001. **(C)** Representative western blot derived from lysates of cells depicted in (A), using specific antibodies to detect GFP and GAPDH (as a loading control). Free GFP indicates the functionality and quantity of autophagic flux.

4.5 Inhibition of nucleo-cytosolic Acetyl-CoA generation promotes autophagy

Since *ach1* mutant cells showed a severe defect in autophagy that correlates with an upregulation of the nucleo-cytosolic Acs2p mediated path of Acetyl-CoA production, we next investigated if the inhibition of Acs2p might lead to elevated autophagy levels in wild type cells.

To investigate this question, we again resorted to strains harboring the EGFP-Atg8p construct as well as the *ACS2* gene under a doxycycline-repressable tet-promotor sytem, using the same culture conditions as for the lifespan experiments (see chapter 4.2.1).

Epifluorescence microscopy revealed a massive translocation of cytosolic EGFP-Atg8p to the vacuole upon *ACS2* down-regulation after three days of aging. Both the number of autophagic cells, defined as cells with clear vacuolar localization of EGFP, as well as the intensity of the EGFP signal increased, indicating a strong induction of macroautophagy.

Western blotting analysis with an anti-GFP antibody corroborated these results, as free EGFP signals increased upon *ACS2* knockdown during later days of aging when compared to the wild type control (**Figure 12**).

All in all, these results suggest thatinhibition of nucleo-cytosolic Acetyl-CoA production induces autophagy, and that nucleo-cytosolic Acetyl-CoA is an inhibitor of autophagy during yeast aging.

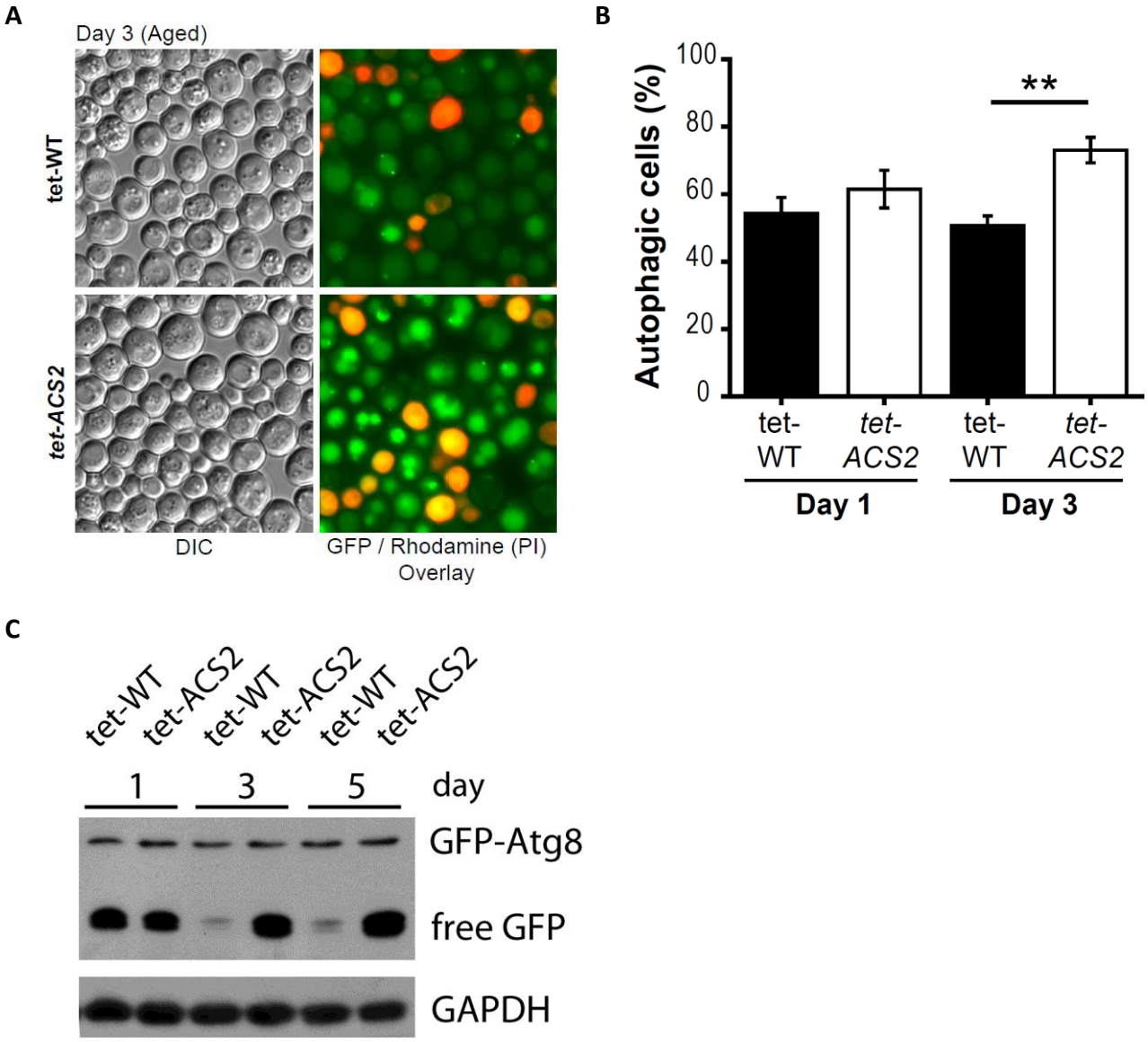

Figure 12: Down-regulation of nucleo-cytosolic acetyl-CoA production boosts age-induced autophagy. (A) Fluorescence microscopy of wild type (tet-WT) and *ACS2* knockdown (tet-ACS2) cells expressing EGFP-Atg8 from its natural promotor (pATG8-EGFP-ATG8). Cultures were chronologically aged for three days in SMD+AA with 1 ng/ml doxycycline to induce *ACS2* knockdown. Cells were counter-stained with propidium iodide (PI) to visualize dead cells. Representative micrographs are shown. **(B)** Quantification of young (day 1) and aged (day 3) cells depicted in (A). Micrographs were blind counted with 150-300 cells being analyzed per replicate. Data was plotted by Tobias Eisenberg and represents means ± SEM (n = 4). **p < 0,01. **(C)** Immunoblot analysis of lysates generated from strains depicted in (A) after 1, 3 and 5 days of aging. Antibodies specific for GFP and GAPDH (loading control) were used. A representative blot is shown.

4.6 Knockdown of ACS2, but not inhibition of Tor- and Sch9-signalling re-establishes autophagy in Δach1

To get further insight into how the Acetyl-CoA mediated autophagy regulation relates to other known autophagic signaling pathways, we tested if the autophagy defect of *ach1* mutant cells was ameliorated by inhibition of kinase signaling pathways involved in regulation of autophagy. The target of rapamycin (TOR)- and Sch9p-kinase pathways represent two well characterized signaling cascades involved in nutrient sensing, cell division and growth, that both negatively

regulate autophagy. It had been shown that genetic or pharmacological inhibition of either of these pathways potently induces autophagy and can promote lifespan extension in yeast and other model organisms (He and Klionsky 2009; Madeo, Tavernarakis, and Kroemer 2010).

4.6.1 Inhibition of TOR by Rapamycin fails to restore autophagy in *ach1* mutant cells

The conserved Ser/Thr kinase TOR regulates a multitude of cellular processes such as cell growth and metabolism in response to environmental conditions. As a central negative regulator of autophagy, inhibition of TOR with rapamycin leads to autophagy dependent lifespan extension in *S. cerevisiae* (Wullschleger, Loewith, and Hall 2006a).

To test if the autophagy defect and the accelerated chronological aging of Δ*ach1* knockout cells were abrogated upon inhibition of TOR, cultures of wild type cells and ach1 mutant cells were treated with rapamycin using established conditions (Alvers et al. 2009). Since rapamycin does not show a life-prolonging phenotype in the long-living BY4741 wild type strain, all experiments involving TOR inhibition were carried out using the short lived BY4742 strain background. Rapamycin was added to the media right before inoculation to a final concentration of 20 nM, a concentration where no growth arrest of treated cells is detectable, but clear phenotypes associated with TOR inhibition were described for wild type cells (Alvers et al. 2009).

Tracing of EGFP-Atg8 fluorescence in epifluorescence microscopy shows a clear age-dependent decrease in vacuolar EGFP localization in *ach1* mutant cells, indicating a similar loss of autophagic function in both the BY4742 and the BY4741 background, when *ACH1* is knocked out (see chapter 4.4). Furthermore, treatment with rapamycin does not restore autophagy in aging Δ*ach1* cells: while in young *ach1* knockout cells (day 1) that still show signs of functional autophagy (vacuolar localization of EGFP), rapamycin treatment only shows a slight induction of autophagy flux, the decline in vacuolar EGFP-positive cells upon aging could not be impeded. In contrast, wild type cells showed a massive induction of autophagy during early aging, which remained at high levels during first three days of aging (**Figure 13 A, B**).

The defect in autophagy in Δ*ach1* knockout cells and the inability to restore this deficiency with rapamycin was furthermore confirmed with immunoblot analysis. While BY4742 wild type cells expressing EGFP-Atg8 from its endogenous promoter showed an increase in free-EGFP/EGFP-Atg8p-ratio upon rapamycin treatment, indicating a boost of autophagic flux. No free EGFP was detectable in aged *ach1* mutant cells, however (**Figure 13 C, D**).

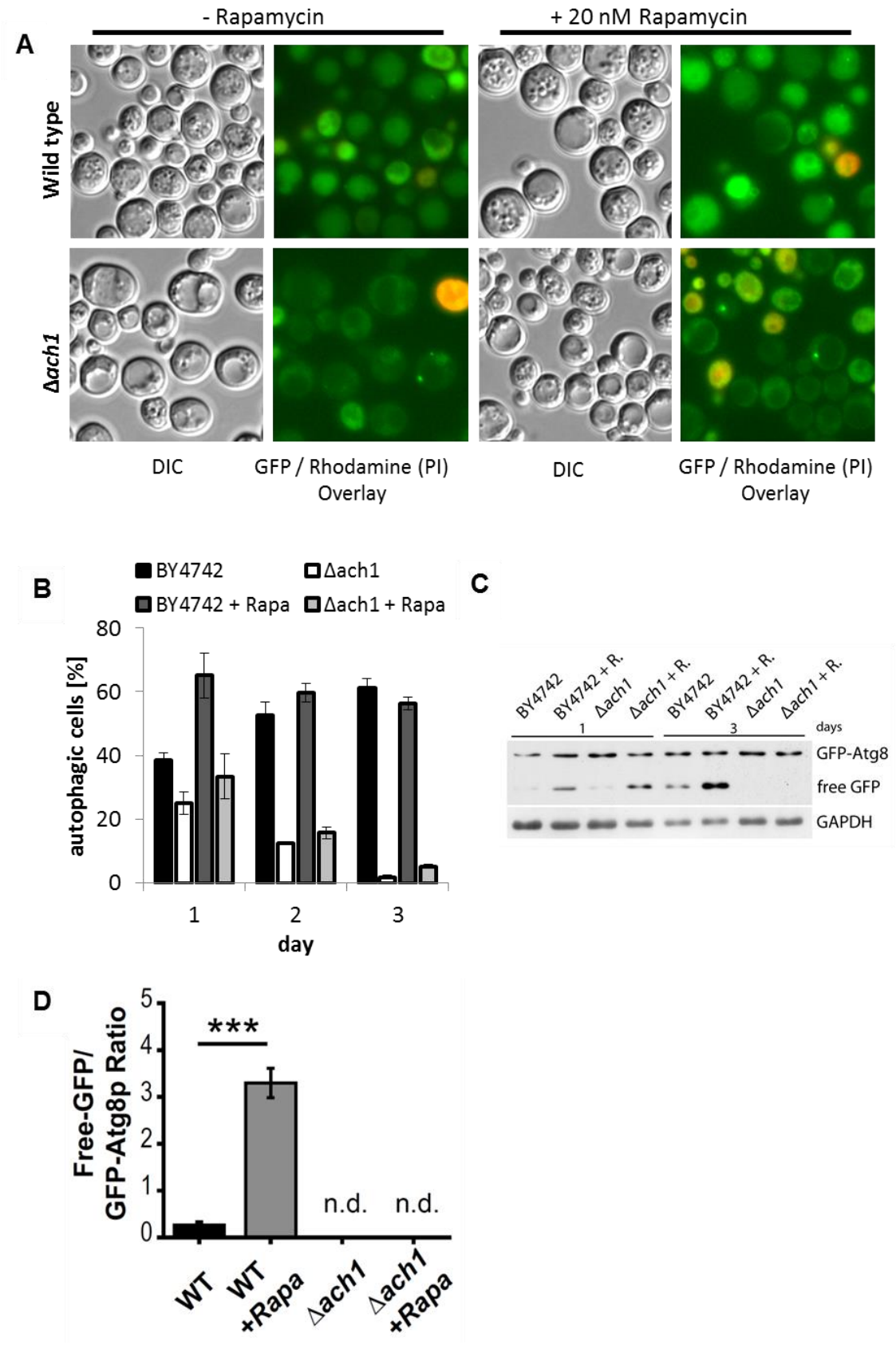

Figure 13: Rapamycin does not restore autophagy in aged Δ*ach1* cells. (A, B) Representative fluorescence microscopy (A) and corresponding quantification (B) of wild type (BY4742) and Δ*ach1* cells supplemented with or without 20 nM rapamycin after 3 days of chronological aging. For microscopic analysis (A) cells were co-stained with propidioum iodide (PI) to visualize dead cells. 150-400 cells per replicate were counted for quantification of micrographs (B), with autophagic cells being defined as cells exhibiting clear vacuolar localization of EGFP fluorescence. Data represent means ± SEM (n = 4). **(C, D)** Representative immunoblot analysis and corresponding densitometric quantification of lysates derived from cells depicted in (A). GFP and GAPDH (loading control) were detected with specific antibodies. Data in (D) was plotted by Tobias Eisenberg and represents means ± SEM (n = 4). ***p < 0,001.

4.6.2 Administration of Rapamycin prolongs lifespan of wild type cells, but not of Δ*ach1* cells

Lifespan extension by rapamycin was shown to be dependent of autophagy (Alvers et al. 2009). As shown above, inhibition of TOR was not able to restore autophagy in *ach1* mutant cells when monitored via EGFP-Atg8 processing. To further consolidate this data, survival of *ACH1* knockout cells and the corresponding wild type was determined using clonogenicity assays and PI staining followed by flow cytometry analysis (**Figure 14**).

Consistent with the missing amelioration of autophagy deficiency in aging Δ*ach1* cells upon rapamycin treatment, cells deficient in the mitochondrial way of acetyl-CoA utilization showed no increase in clonogenicity in survival platings, and no decrease in PI positive, necrotic population, when administered with 20 nM of rapamycin. In contrast, TOR inhibition prolonged lifespan in the wild type, as shown by an increase in cfu, and a decrease of necrotic cell population during aging, when compared to untreated controls.

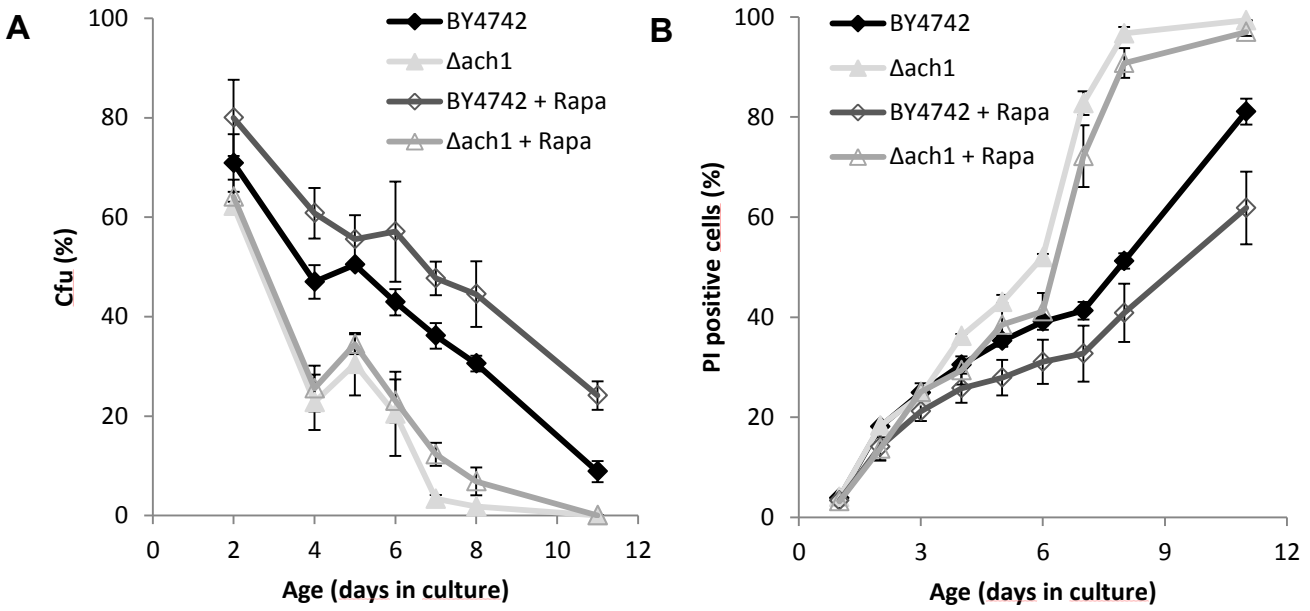

Figure 14: TOR inhibition does not prolong lifespan of *ach1* deleted cells. Chronological lifespan analyses were done in SMD+AA (120 mg/ml lysine) using BY4742 wild type and *ACH1* knockout (Δ*ach1*) cells supplemented with or without 20 nM of rapamycin. Survival was determined at indicated timepoints by **(A)** colony forming capability and by **(B)** flow cytometric analysis of propidium iodide (PI) stained cultures. Data represent means ± SEM (n = 4).

4.6.3 Deletion of *SCH9* does not restore Δ*ach1* autophagy defect

Next to TOR, the protein kinase Sch9p, the yeast ortholog of mammalian S6 kinase, is a central negative regulator of autophagy in yeast. Deletion of *SCH9* was shown to prolong yeast lifespan, and simultaneous inactivation of PKA lead to an increase of autophagy (Fabrizio et al. 2001; Yorimitsu et al. 2007).

To check if inactivation of Sch9p signaling pathway could restore the age dependent autophagy defect of *ACH1* mutant cells, double mutants Δ*ach1* Δ*sch9* were created and autophagy levels were monitored via EGFP-Atg8 liberation assay and microscopic localization of EGFP-Atg8 (**Figure 15**).

In wild type BY4741 cells, deletion of *SCH9* resulted in a massive accumulation of EGFP-Atg8 in vacuoles during the first two days of aging, indicating a substantial increase in autophagic flux. Correspondingly, western blot analysis showed a strong signal for free EGFP when *SCH9* is knocked out in the wild type background. Interestingly, this effect wears off by day three of chronological aging. While disruption of the *SCH9* kinase pathway was able to boost autophagy during early Δ*ach1* chronological aging (day 1), it could not abrogate the age-dependent autophagy defect, as hardly any vacuolar EGFP-positive cells were detectable in epifluorescence microscopy in advanced aging (day 3), and no free EGFP was detectable in immunoblot analysis.

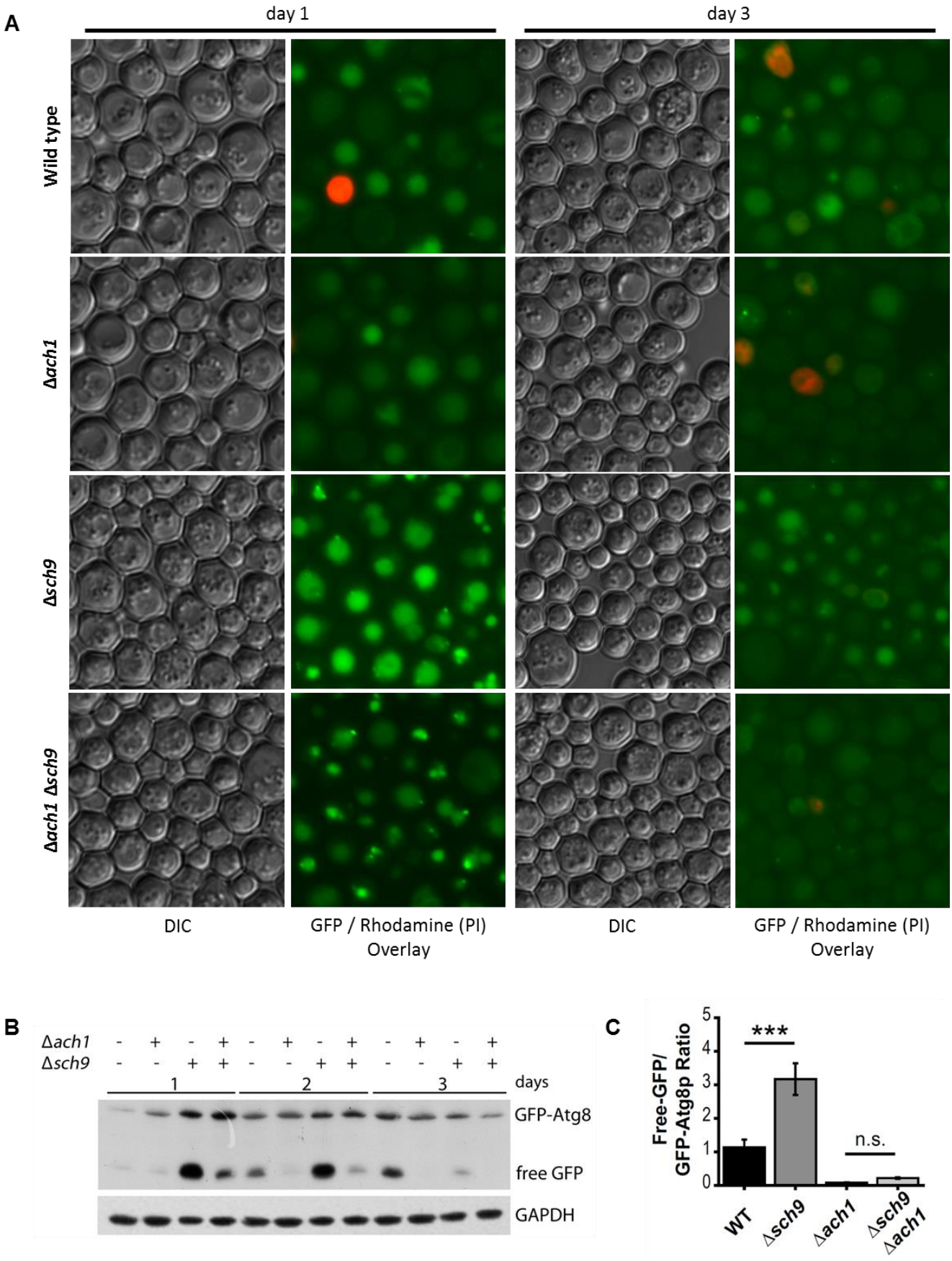

Figure 15: Deletion of *SCH9* does not reinstate autophagy in aged Δ*ach1* cells. (A) Representative fluorescence micrographs of wild type, Δ*ach1*, Δ*sch9* and Δ*ach1* Δ*sch9* double mutant cells expressing an EGFP-Atg8 fusion from its natural promoter (pATG8-EGFP-ATG8). Cells were aged in SMD+AA and microscoped at indicated timepoints after co-staining with propidium iodide (PI) to visualize dead cells. **(B, C)** Representative immunoblot (C) and corresponding densitometric quantification (C) of whole cell lysates of cells depicted in (A). Blots were probed with specific antibodies for GFP and GAPDH (loading control). Data in (C) was plotted by Tobias Eisenberg and represents means ± SEM (n = 4). ***p < 0,001, n.s. = not significant.

4.6.4 Δ*sch9* delays aging in Δ*ach1* and wild type cells

Similar to TOR, the kinase Sch9p is involved in nutrient sensing and was shown to be a central regulator of cell growth and metabolism, autophagy and both replicative and chronological aging in yeast. Deletion of *SCH9* or its homologues Akt and S6 kinase increases the lifespan of yeast, as well as *C. elegans* and *Drosophila* respectively (Hertweck, Göbel, and Baumeister 2004; Kapahi et al. 2004).

To see if additional disruption of *SCH9* signaling leads to decelerated aging in the background of Δ*ach1*, despite the inability to recover the autophagy defect, Δ*ach1* Δ*sch9* double mutants were created and chronologically aged. Clonogenicity, as well as the size of the necrotic population of the culture was determined throughout aging.

In the long living BY4741 wild type, increased survival rates upon *SCH9* impairment were only visible at very late timepoints (day 12 and later) in colony forming capacity; PI staining furthermore revealed beneficial effects during early aging (until 7 days of aging). When compared to Δ*ach1* single mutant cells, Δ*ach1* Δ*sch9* displays ameliorated survival during the whole course of aging, showing increased clonogenicity and reduced PI positive necrotic populations (**Figure 16**). Since death kinetics however show a similar progression as in the Δ*ach1* single mutant, this benefit in survival may be interpreted as a delay of cell death, rather than a full rescuing effect. The role of autophagy concerning these survival benefits in aging scenarios involving the inactivation of Sch9p kinase however remains to be elucidated.

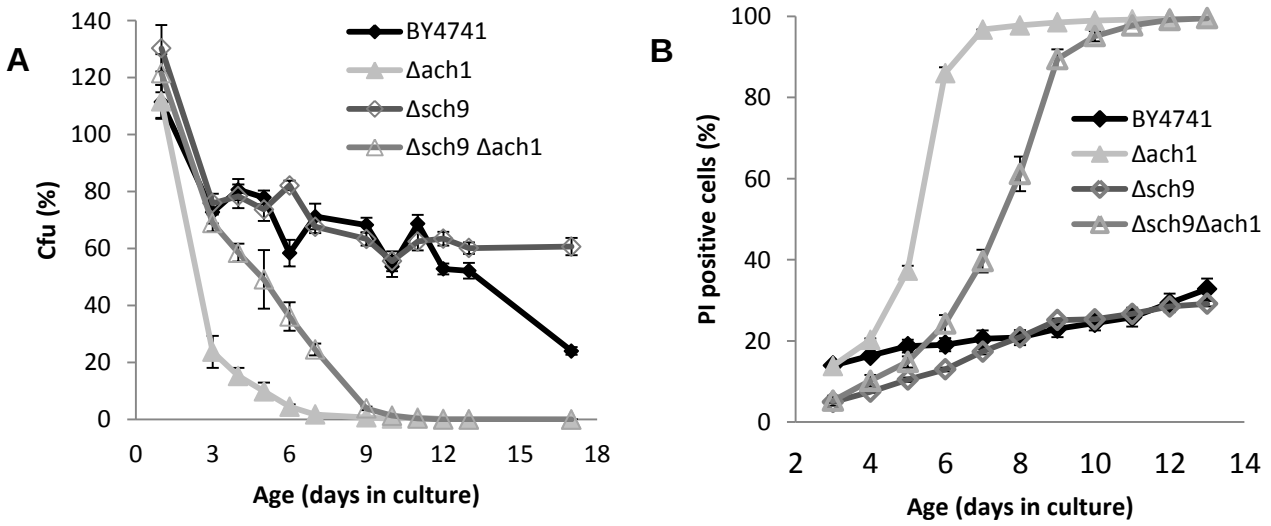

Figure 16: Deletion of *SCH9* delays aging in ach1 knockout cells. Chronological lifespan of BY4741 wild type cells and Δ*ach1*, Δ*sch9* and Δ*ach1* Δ*sch9* mutant strains was determined by clonogenicity assays (A) and flow cytometric quantification of propidium iodide (PI) stained cells (B). Cells were grown in SMD+AA and samples were analyzed at indicated timepoints. Data represents means ± SEM (n = 4) of representative experiments.

4.6.5 Knockdown of *ACS2* abrogates autophagy deficiency in Δ*ach1*

Given the findings that the autophagy defect in cells lacking *ACH1* correlates with elevated Acs2p protein levels, and that down-regulation of Acs2p boosts autophagy levels in wild type cells, we hypothesized that the Δ*ach1* autophagy defect is mediated by Acs2p, the nucleocytosolic source of acetyl-CoA generation. To test this, the doxycycline repressible tet-ACS2 promotor was combined with the *ACH1* deletion, and chronological agings were performed, using the same knockdown conditions already established for Acs2p.

Monitoring EGFP-Atg8 localization using epifluorescence microscopy and EGFP-liberation via immunoblot analysis, knockdown of *ACS2* distinctly reestablished autophagy in the background of aged Δ*ach1* cells. Down-regulation of *ACS2* augmented autophagy in cells lacking *ACH1* during all monitored timepoints, shown by an increased number of autophagic cells, a gain in EGFP-fluorescence as well as immunoblot intensities (suggesting elevated Atg8 expression upon down-regulation of *ACS2*), and an increase in free EGFP/EGFP-Atg8p ratio. At day three, when the defect in autophagy machinery becomes definitive in cells lacking *ACH1*, down-regulation of *ACS2* restored the number of autophagic cells as well as the free EGFP/EGFP-Atg8 levels back to wild type level. As a control, knockdown of *ACS2* in wild type background alone again displays strongly elevated levels of autophagy surpassing those of tet-WT controls (**Figure 17 A-D).**

To exclude that the autophagy defect in Δ*ach1* cells is due to possible toxic effects of the extensive acetate accumulation described earlier (Orlandi, Casatta, and Vai 2012; Eisenberg et al. in preparation), the concentration of extracellular acetate was determined. *ACH1* knockout cells showed massively elevated levels of acetate in the media after 48 hours of aging, and this accumulation of acetate was not affected by simultaneous down-regulation of *ACS2* (**Figure 17 E**). This argues in favor of a causal role of the nucleo-cytosolic Acs2p mediated pathway of acetyl-CoA formation in the autophagy defect of Δ*ach1* cells.

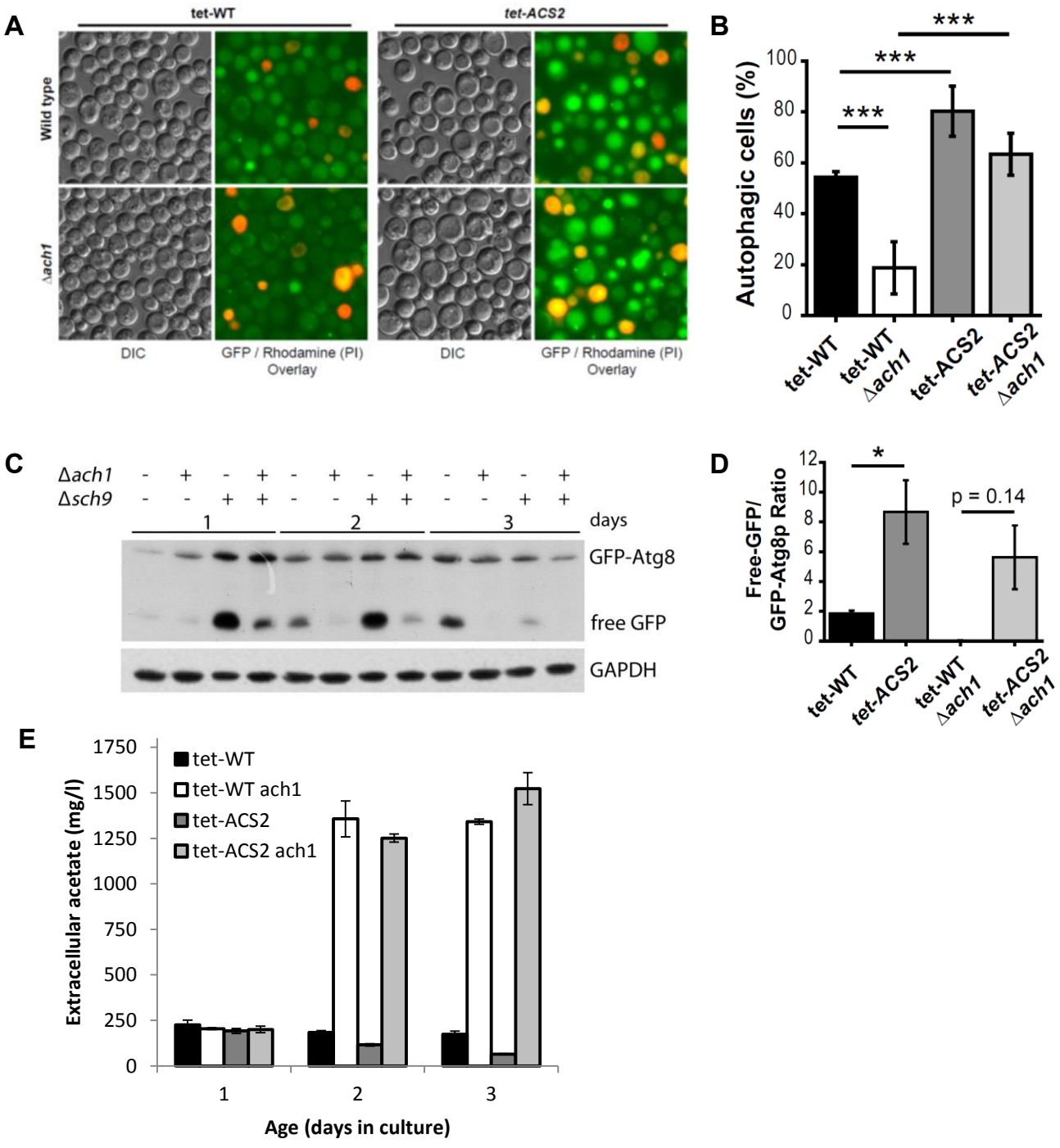

Figure 17: Down-regulation of the Acs2p way of acetyl-CoA generation restores autophagy in *ach1* mutant cells. (A) Fluorescence microscopy pictures of wild type and *ach1* knockout (Δ*ach1*) cells with or without additional knockdown of *ACS2* (tet-ACS2), chronologically aged for 3 days in SMD+AA with 1 ng/ml doxycycline to achieve knockdown. Counterstaining with propidium iodide (PI) was used to visualize dead cells. (B) Quantification of cells depicted in (A). Micrographs were blind counted with autophagic cells being defined as cells exhibiting clear vacuolar localization of GFP fluorescence. Data was plotted by Tobias Eisenberg and represents means ± SEM (n = 4). (C, D) Representative immunoblot analysis (C) and corresponding densitometric quantification (D) of cell lysates from strains depicted in (A). Blots were probed with antibodies specific for GFP and GAPDH (loading control). Quantification data (D) was plotted by Tobias Eisenberg and represents means ± SEM (n = 4). (E) Determination of extracellular acetic acid accumulation in media of chronologically aged cells depicted in (A). Data represents means ± SEM (n = 4).

4.7 Down-regulation of ACS2 restores histone H3 hyperacetylation caused by ACH1 deletion

Based on the premise that down-regulation of *ACS2* reinstated autophagy in Δ*ach1* cells, we next checked wether the overrepresentation of the Acs2p mediated nucleo-cytosolic way of acetate consumption was also causal for the hyperacetylation in *ACH1* deleted cells. Therefore, the acetylation status of three lysine residues of histone H3 (K9, K14 and K18), which were previously shown to be hyperacetylated upon *ACH1* deletion (Eisenberg et al. in preparation), was determined in the background of *ACS2*-knockdown via immunoblotting. While all investigated lysine residues of histone H3 showed increased acetylation in Δ*ach1* cells after three days of chronological aging when compared to wild type controls (tet-WT), simultaneous down-regulation of *ACS2* markedly reduced acetylation almost back to wild type level (**Figure 18**).

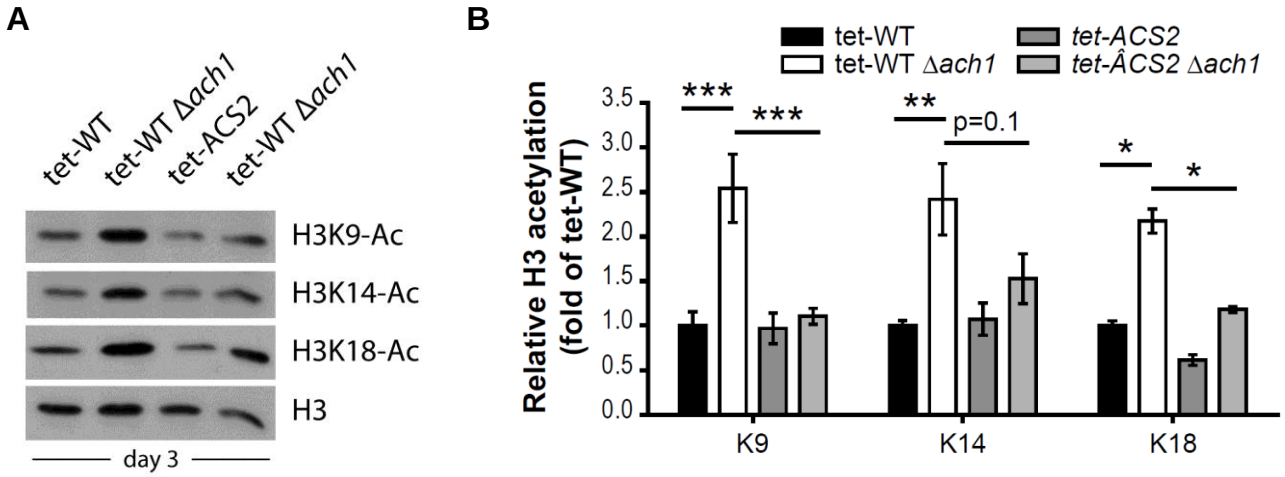

Figure 18: *ACS2* knockdown restores hyperacetylation of histone H3 in *ach1* deleted cells. Representative immunoblot analysis **(A)** and corresponding densitometric quantification **(B)** of whole cell acid extracts of wild type and Δ*ach1* cells combined with or without knockdown of *ACS2* (tet-ACS2). Cells were chronologically aged to day 3 in SMD+AA with 1 ng/ml doxycycline. Blots were probed with antibodies specific for acetylated N-terminal H3 lysyl residues K9, K14 and K18 as well as for total histone H3 (loading control). Densitometric quantification of relative acetylation was calculated as K-Ac/total H3 ratios normalized to ratios of WT. Data was plotted by Tobias Eisenberg and represents means ± SEM (n = 8).

4.8 Aging of ACH1 knockout cells is ameliorated by ACS2 knockdown

Since literature exhibits numerous evidence to the crucial role of autophagy for healthy aging, and our data suggests that down-regulation of the nucleo-cytosolic, Acs2p-mediated path of acetate consumption reinstates autophagy in *ACH1* knockout cells, we tested if knockdown of *ACS2* ameliorated Δ*ach1* aging. To address this question, *ach1* mutant cells harboring the doxycycline repressible tet-ACS2 promotor, were chronologically aged under the same

knockdown conditions that restored autophagy, and survival was determined via clonogenicity assays and flow cytometry analysis of PI stained necrotic cells.

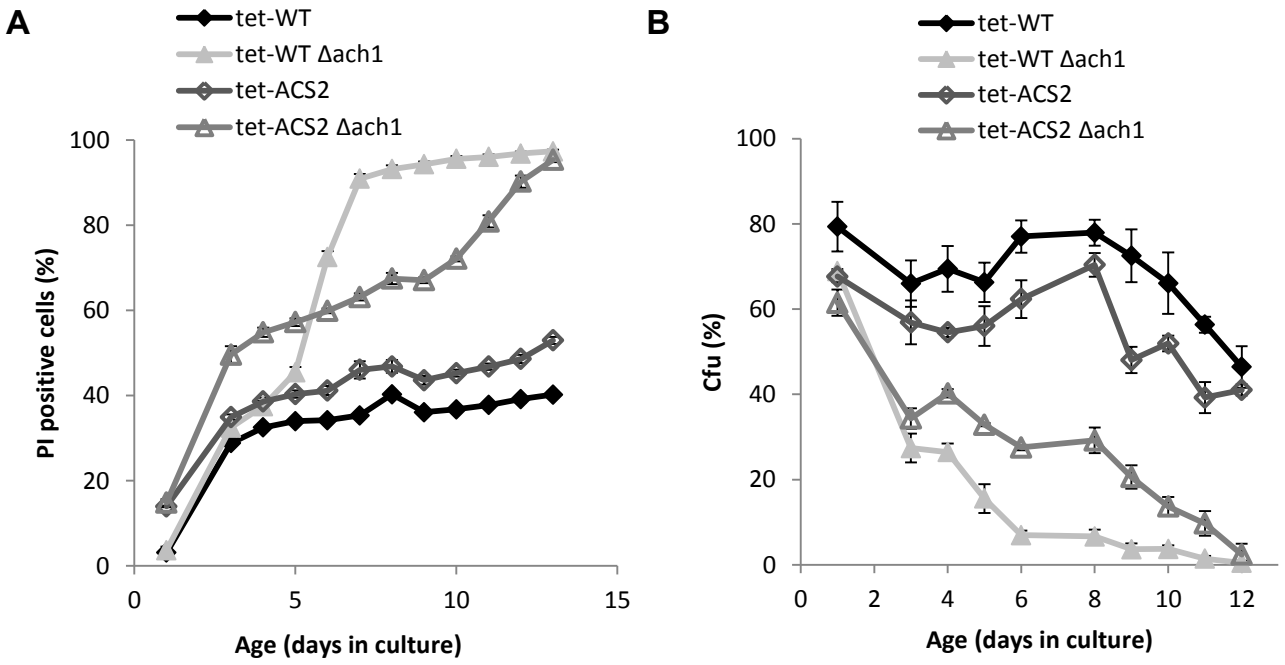

Figure 19: Knockdown of *ACS2* prolongs lifespan of Δ*ach1* cells. Chronological lifespan analyses were done in SMD+AA with 1 ng/ml doxycycline of wild type (tet-WT) and *ACH1* knockout (Δ*ach1*) cells with and without knockdown of *ACS2* (tet-ACS2). Survival was determined by colony forming ability **(A)** and flow cytometric quantification of propidium iodide (PI) stained cells **(B)**. Data represents means ± SEM (n = 4).

While knockdown of *ACS2* could not fully rescue Δ*ach1* death back to wild type control (tet-WT) levels, clonogenicity was considerably ameliorated, and markers of cell death were reduced (**Figure 19**). In contrast to suppression of Sch9p function, which in fact delayed premature cell death in ach1 mutant cells, knockdown of *ACS2* not only provoked a higher maximum lifespan of *ACH1* knockout cells, but also altered Δ*ach1* death kinetics towards what rather resembles that of wild type cells.

4.9 Beneficial effects of ACS2 knockdown on Δach1 chronological aging are autophagy dependent

Considering that down-regulation of *ACS2* ameliorates aging and survival of cells bearing a defect in mitochondrial acetate utilization (Δ*ach1*), we wanted to know to which extend the restoration of the accompanying defect in autophagy was responsible for the survival benefits upon *ACS2* knockdown.

To address this question, a deletion of *ATG7*, a gene essential for autophagosome formation, was introduced into the tet-ACS2 Δ*ach1* strain, and again, cells were chronologically aged, using the

same *ACS2* knockdown conditions established before. As a control, all original knockdown strains were included in the same experiment.

While knockdown of *ACS2* prominently ameliorated survival of *ach1* mutant cells both in clonogenicity assays as well as flow cytometric quantification of PI stained necrotic cells, the disruption of autophagosomal machinery by *ATG7* deletion completely abrogates the beneficial effects of *ACS2* suppression (**Figure 20**). This strongly suggests, that the reestablishment of autophagy by *ACS2* knockdown in cells lacking *ACH1* is indeed crucial for the beneficial effects on lifespan, and that consequently, the loss of autophagy by *ACH1* deletion is – at least to a certain extend – responsible for the adverse effects upon disruption of mitochondrial acetyl-CoA production by Ach1p.

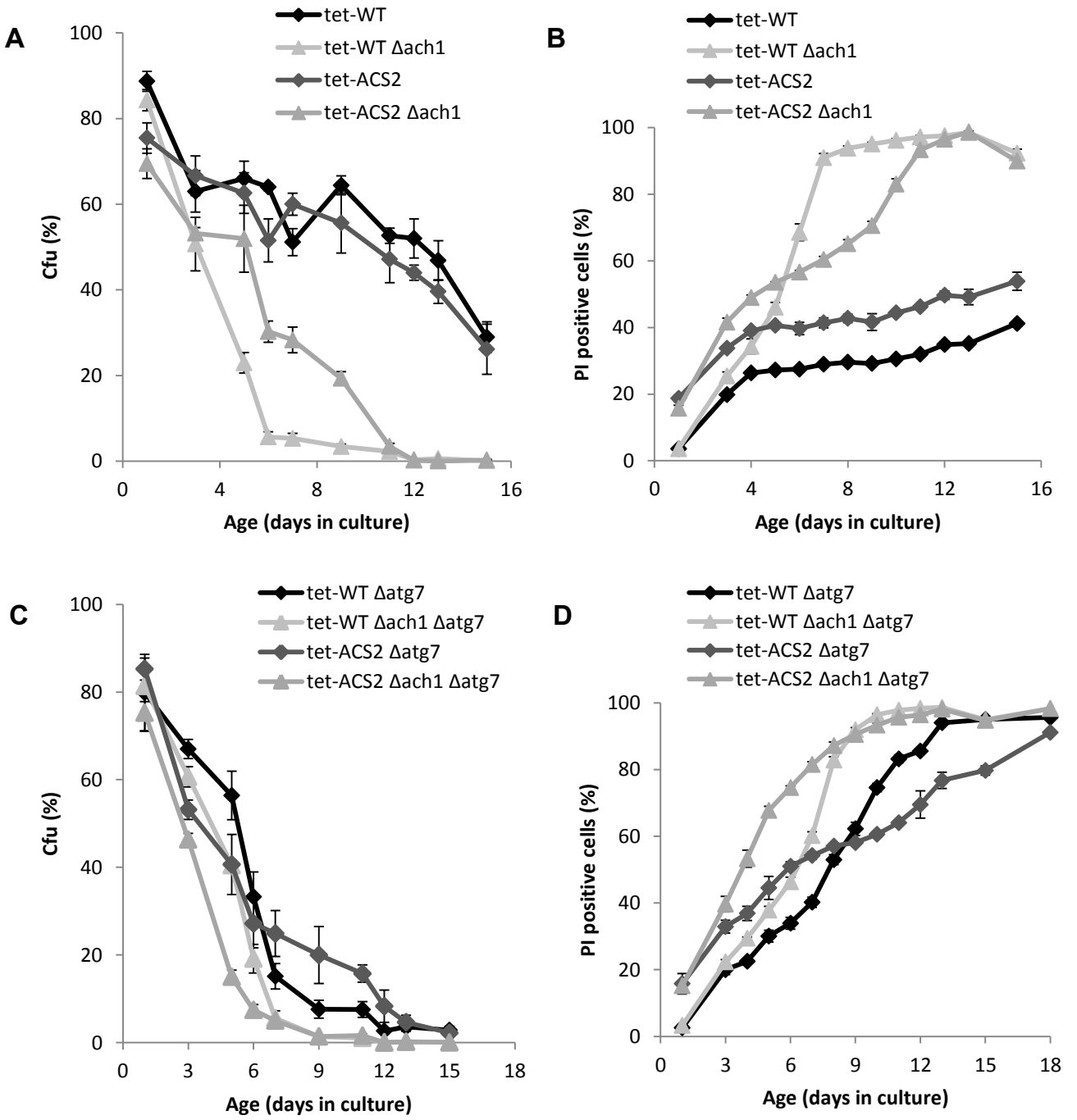

Figure 20: Knockdown of *ACS2* partly restores survival of *ach1* mutant cells in an autophagy-dependent manner. (A, B) Chronological aging was performed in SMD+AA supplemented with 1 ng/ml doxycycline using wild type and Δ*ach1* cells combined with or without additional knockdown of *ACS2* (tet-ACS2). Survival was determined by clonogenicity (A) and flow cytometric analysis of propidium iodide (PI) stained cells (B). Data represents means ± SEM (n = 4), a representative experiment is shown. (C, D) Chronological aging was done similar to (A, B), but in the background of deletion of *ATG7* (Δ*atg7*), which is essential for the execution of autophagy. Data represents means ± SEM (n = 4) of a representative experiment.

5 Discussion

Given the growing recognition of protein and histone acetylation and their impact on a multitude of cellular processes such as metabolism, cell cycle and growth as well as stress response, various recent studies linked the regulation of some of these processes to energy metabolism, in particular the metabolism of acetyl-CoA, which is the only known donor for these acetylation reactions yet (Wellen et al. 2009; Galdieri and Vancura 2012; Takahashi et al. 2006; Zhao et al. 2010; Choudhary et al. 2009). In this present work we outline a novel link between different ways of acetyl-CoA generation, histone acetylation and aging and the associated process of autophagy. For these processes, we demonstrate that a functional and balanced acetyl-CoA metabolism is crucial.

5.1 The nucleo-cytosolic Acs2p mediated acetyl-CoA production suppresses age-induced autophagy

In order to evaluate the influence of the two separate acetyl-CoA pools in the yeast cell on the regulation of autophagy, deletions or knockdowns of genes involved in both the mitochondrial and nucleo-cytosolic ways of acetyl-CoA production were investigated. We found that disruption of the mitochondrial route of acetate consumption by deletion of the putative CoA-transferase *ACH1* lead to a severe, age-dependent autophagy defect which fully manifests after three days of chronological aging in both strain backgrounds tested (the short living BY4742 and the long living BY4741 strain). According to our data, this autophagy defect is due to an upregulation of the nucleo-cytosolic, Acs2p mediated way of acetyl-CoA generation upon disruption of the mitochondrial acetate utilization, which manifests itself in the increase of Acs2p protein level as well as enzyme activity during aging. The fact that down-regulation of the nucleo-cytosolic way of acetyl-CoA generation itself potently boosts age-induced autophagy in wild type cells, and that *ACS2* knockdown reinstates autophagy in Δ*ach1* cells suggests that Acs2p is causal for autophagy inhibition in this scenario.

5.2 Inhibition of autophagy by nucleo-cytosolic acetyl-CoA causes accelerated aging

Just like the pro-survival functions of autophagy during conditions of cellular stress, nutrient depletion or aging have been demonstrated in a multitude of studies, disruption of the autophagy machinery is known to disturb cellular homeostasis leading to premature cell death (Madeo, Tavernarakis, and Kroemer 2010; Eisenberg et al. 2009; Rubinsztein, Mariño, and

Kroemer 2011). Consistent with these findings, inhibition of autophagy by the hyperfunction of the nucleo-cytosolic acetyl-CoA production pathway in *ach1* knockout cells is accompanied with accelerated aging and premature loss of clonogenicity and cell death. Similar, deletion of MPC1, which channels mitochondrial acetyl-CoA generation through the pyruvate dehydrogenase complex, leads to loss of autophagic function (Eisenberg et al. in preparation) and decreased survival. Knockdown of ACS2 in the background of ACH1 deletion not only restored autophagy during aging, but also ameliorated survival, proving that Acs2p activity is at least partly causal for accelerated aging as well. Strikingly however, down-regulation of ACS2 also improved lifespan of *Δatg7* cells positively to some extent, suggesting that alteration of the nucleo-cytosolic acetyl-CoA pool affects aging in yet unknown ways independently of autophagy as well. Since modulation of Acs2p drastically alters both autophagy as well as survival during aging without significantly affecting acetate levels (as measured by determining the extracellular acetate in the medium), suggesting that the pro-aging effect of acetate is at least partly a result of Acs2p mediated conversion to nucleo-cytosolic acetyl-CoA.

Moreover it is intriguing that ACS2 knockdown did not improve overall survival in wild type cells despite of massive induction of autophagy during aging. A temperature-sensitive mutant of Acs2p resulting in lowered enzyme activity (Acs2-Ts1) however protected from cell death induced by Acs2p overexpression in the corresponding wild type control (WT-Acs2) in an autophagy-dependent manner. All in all this data on the one hand suggests that full Acs2p activity is not required for healthy aging under conditions still allowing adequate growth, but its inactivation instead may protect from the detrimental effects of Acs2p overexpression. On the other hand these results indicate that autophagy per se may not suffice to promote longevity but instead requires the additional (de-)activation of other cellular processes which are not affected by *ACS2* down-regulation in this case. Another possible explanation for the lack of life-span extension upon *ACS2* knockdown could be that detrimental effects of *ACS2* down-regulation (overly depletion of Acs2p in lag or early log-phase causes growth arrest and cell death) might interfere with putative beneficial effects of elevated autophagy levels on aging. Optimization of knockdown conditions for *ACS2* under various growth or stress conditions might clarify the role of autophagy upon *ACS2* down-regulation in wild type cells.

5.3 Epigenetic control of Atg transcription and protein acetylation are possible mechanisms of autophagy regulation by nucleo-cytosolic acetyl-CoA

The main regulatory mechanisms of autophagy regulation involving the sensing on nutrients rely on the activity of protein kinases (Kaeberlein et al. 2005; Tomohiro Yorimitsu et al. 2007; Urban et al. 2007). However, while a moderate *ACS2*-knockdown was sufficient to reinduce autophagy in Δ*ach1* cells with upregulated nucleo-cytosolic acetyl-CoA generation, neither the inactivation of the negative regulators TOR kinase (by application of rapamycin), nor deletion of the *SCH9* kinase were able to recover the autophagy defect in Δ*ach1* cells in the long run. Consistently, premature cell death of *ACH1* knockout cells was largely unaffected by TOR inhibition, and only delayed upon *SCH9* deletion. This suggests that autophagy regulation by nucleo-cytosolic acetyl-CoA takes effect downstream of both TORC1 and Sch9p, and that reduced activity of the Acs2p-mediated path of acetate utilization is required for long-term induction of autophagy by inhibition of kinase signaling.

Although the exact mechanism by which acetyl-CoA intervenes in autophagy regulation still remains ambiguous, our data provides indications that epigenetic alterations and protein acetylation might be involved. Over-activation of Acs2p in ach1 knockout cells leads to increased protein acetylation in general, increased acetylation of N-terminal lysyl residues of histone H3 in particular, and reduced transcription of ATG genes, all of which could be reversed by *ACS2* knockdown (Eisenberg et al. in preparation). Thus, the age-dependent autophagy defect in *ach1* mutant cells might be caused by depletion of one or more essential component(s) of the autophagic machinery. Further investigation will be necessary to prove this and in case identify the limiting factor(s).

To sum up, this data suggests that autophagy might be mediated by epigenetic regulation of ATG gene transcription. Although the process of autophagosome formation is a cytoplasmatic process which even cytoplasts are able to perform (Morselli et al. 2011), the long-term maintainance of autophagy during aging might rely on epigenetic and transcriptional alterations as well. Previous studies with the natural polyamine spermidine back up this supposition, since spermidine mediated autophagy induction involves the inhibition of HATs, resulting in global histone deacetylation and ATG gene trancrtiption (Eisenberg et al. 2009). Just recently it was shown that acetylation of lysine 16 of histone H4 regulates the outcome of autophagy as well as the lifespan of yeast cells (Füllgrabe et al. 2013; Dang et al. 2009), which further strengthen the hypothesis that epigenetic histone-acetylation is involved in acetyl-CoA mediated regulation of autophagy.

Apart from histone modifications, also protein acetylation might contribute to regulation of autophagy by Acs2p generated acetyl-CoA. Several components of the autophagic machinery have been shown to be targets of acetylation; for some of them physiological functions could be identified. While Atg3 for example needs to be acetylated to promote autophagy, deacetylation of Atg7p is necessary for autophagy induction (I. H. Lee et al. 2008; Yi et al. 2012). Profound changes in the acetyl-proteome such as they can be seen in Δ*ach1* cells with upregulated nucleo-cytosolic acetyl-CoA generation, might as well affect Atg proteins and thus the cell's capability to induce or maintain autophagy during aging. It is up to future work to determine the acetylation status of the aforementioned Atg proteins and to elucidate if they are affected by Acs2p mediated acetyl-CoA generation. Co-localization studies of Atg proteins may furthermore help to reconstruct at which step in autophagosome formation the over-activation of Acs2p causes the malfunction. **Figure 21** displays a graphical overview on acetyl-CoA metabolism, and its implication in the regulation of autophagy and aging, based on this work.

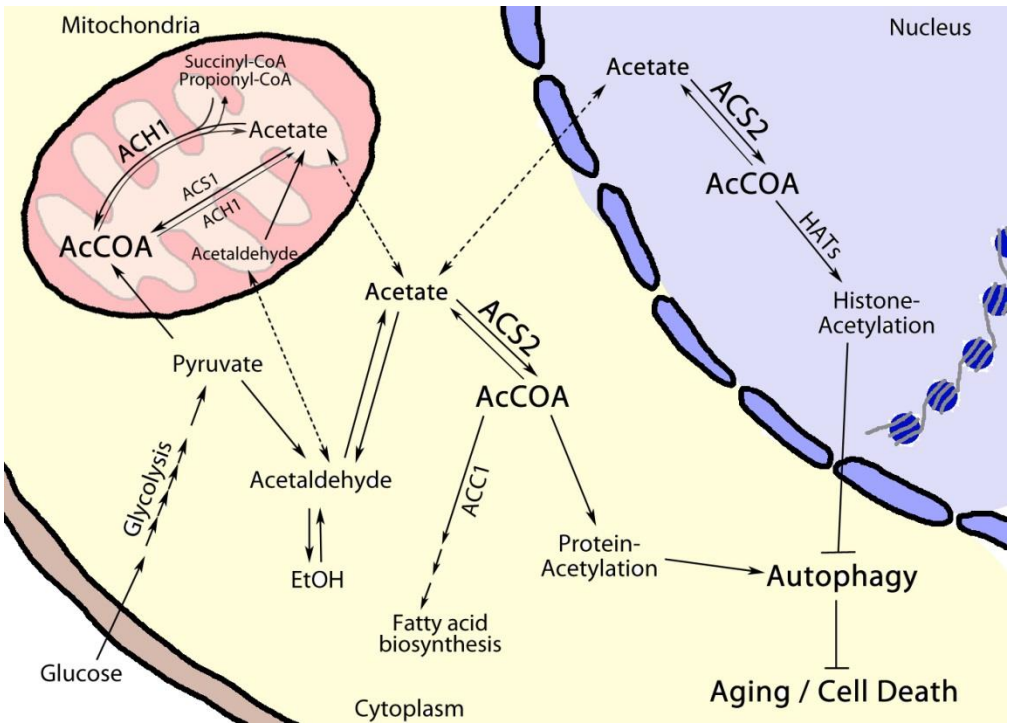

Figure 21: Acetyl-CoA is pivotally integrated in carbon and energy metabolism, protein acetylation and autophagy regulation. As a central intermediate in carbon and energy metabolism, acetyl-CoA (AcCOA) connects numerous metabolic pathways, such as glycolysis, fatty acid biosynthesis and respiration. In yeast, acetyl-CoA is generated separately in mitochondria and the cytosol, with the latter pool being available as donors for acetylation reactions. Disruption of the mitochondrial way of acetyl-CoA generation by *ach1* deletion is associated with the accumulation of acetate and an upregulation of Acs2p, the nucleo-cytosolic source of acetyl-CoA. We show that Acs2p is causal for the loss of autophagy and clonogenicity as well as for the hyperacetylation of histone H3 upon *ACH1* deletion. Given the importance of epigenetic modifications on transcription and acetylation on protein function and the regulation of cellular processes, we speculate that Acs2p mediated acetyl-CoA generation regulates autophagy by controlling the transcription of rate-limiting Atg genes, and/or by determining the acetylation status of the respective gene products. Consequently, we propose that nucleo-cytosolic acetyl-CoA influences aging and lifespan by modulation of autophagy.

6 Conclusion

To sum up, this work identifies the nucleo-cytosolic acetyl-CoA as a potent and thus far unrecognized inhibitor of age-induced autophagy. Furthermore, a decreasing activity of Acs2p, the nucleo-cytosolic source of acetyl-CoA, is required for healthy aging. Since neither inhibition of TOR kinase activity nor *SCH9* deletion were able to induce autophagy when Acs2p was upregulated, regulation of autophagy by nucleo-cytosolic acetyl-CoA appears to act downstream of known kinase signaling pathways and seems to dominate over them. Given that the Acs2p-pathway is involved in histone and protein acetylation, we propose that acetyl-CoA mediated autophagy regulation might be controlled by epigenetic alterations of ATG gene transciption and/or protein acetylation. As the only known donor for acetylation reactions acetyl-CoA hereby represents a promising link between energy metabolism, epigenetic regulation, autophagy and aging. Our data contributes to the growing recognition of acetylation as a crucial post-translational modification in the regulation of cellular processes, and provides new insights in how nutrition pathways might intersect with autophagy and aging.

7 Abbreviations

AA	all Amino Acids
AMPK	AMP-activated kinase
Atg	Autophagy related gene
AMP	Adenosine MonoPhosphate
ATP	Adenosine TriPhosphate
cfu	Colony forming units
CLS	Chronological Lifespan
CMA	Chaperone Mediated Autophagy
CoA	Coenzyme A
Cvt	Cytoplasm to vacuole targeting
ddH_2O	Double destilled water
dH_2O	Deionized water
DIC	Differential Interference Contrast microscopy
DMSO	DiMethyl SulfOxide
DNA	DeoxyriboNucleic Acid
dsRED	RED fluorescing protein drFP583 from *Discosoma*
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmatic Reticulum
EDTA	EthyleneDiamineTetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
GFP	Green Fluorescence Protein
HAT	Histone Acetyl-Transferase
HDAC	Histone DeACetylase
IGF	Insulin-like Growth Factor
mA	Milli Ampere

mM	MilliMolar
µM	MicroMolar
OD	Optical Density
ONC	Overnight Culture
PAS	Pre-Autophagosomal Structure
PDH	Pyruvate DeHydrogenase complex
PE	PhosphatidylEthanolamine
PI	Propidium Iodide
PI3K	PhosphatidylInositol 3-Kinase
PKA	Protein Kinase A
PVDF	PolyVinyliDenFluoride
RLS	Replicative Lifespan
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SEM	Standard Error of the Mean
SMD	Synthetic Minimal medium with Dextrose
TBS	Tris Buffered Saline
TCA cycle	TriCarboxylic Acid cycle
TEMED	N,N,N,N'-TEtraMethylEthyleneDiamine
TOR	Target Of Rapamycin
TSC	Tuberous Sclerosis Complex
TST	Tris buffered Saline with Tween
U	Unit
UPS	Ubiquitin-Proteasome System
V	Volt
YPD	medium with Yeast extract, Peptone and Dextrose

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