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Mitochondria are involved in the anti-aging effects of a natural flavonoid

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Für meine Familie

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Abstract

Aging is the greatest risk factor for developing diseases, and the constantly increasing life expectancy is always accompanied with an increase in age-related diseases, from neurodegeneration to diabetes and cancer. The aim of aging research and the search of potential anti-aging drugs is to extend our healthspan and to slow down the aging process itself to fight age-associated diseases. Natural substances like flavonoids are promising candidates for the use as anti-aging substances. In the baker's yeast *Saccharomyces cerevisiae* we could identify a flavonoid, 2-hydroxychalcone (2-HC), which shows cytoprotective properties and increased viability. This effect could also be transferred to *Drosophila melanogaster*, a complex multicellular organism. This implies that the protective effects of 2-HC are conserved throughout species. Furthermore, we were able to demonstrate a mitochondrial involvement underlying the cytoprotective action of 2-HC. 2-HC treatment leads to an upregulation of the expression of various mitochondrial proteins to significantly increased respiration, preconditioning cells for growth under respiratory stress conditions. Altogether, we have characterized 2-HC as a putative anti-aging drug candidate.

Kurzzusammenfassung

Altern ist der größte Risikofaktor für die Entwicklung von Krankheiten, und unsere stetig steigende Lebenserwartung ist begleitet von der Zunahme altersbedingter Krankheiten wie Neurodegeneration, Diabetes oder Krebs. Ziel der Altersforschung und der Suche nach möglichen Anti-Aging-Substanzen ist es, die Gesundheitsspanne zu erweitern und den Alterungsprozess selbst zu verlangsamen, um altersbedingte Krankheiten zu bekämpfen. Natürlich vorkommende Substanzen wie Flavonoide sind vielversprechende Kandidaten für die Anwendung als Anti-Aging-Mittel. Wir konnten in der Bäckerhefe *Saccharomyces cereviseae* ein Flavonoid identifizieren, 2-Hydroxychalcon (2-HC), welches protektive, lebensverlängernde Effekte zeigt. Diese Ergebnisse sind auf die Fruchtfliege *Drosophila melanogaster* übertragbar, einen multizellulären, komplexen Organismus, in welchem 2-HC die mittlere Lebensspanne verlängern konnte. Dies deutet auf einen evolutionär konservierten Rettungseffekt hin. Darüber hinaus suggerieren die hier präsentierten Ergebnisse eine mitochondriale Beteiligung am positiven Einfluss von 2-HC. Wir konnten bei 2-HC Behandlung eine Hochregulation verschiedenster mitochondrialer Proteine beobachten. Zusätzlich konnte nachgewiesen werden, dass 2-HC zu einer signifikant erhöhten Atmung führt und das zelluläre Überleben unter respiratorischen Stressbedingungen verbessert. Zusammenfassend haben wir 2-HC als eine vielversprechende Anti-Aging-Kandidatsubstanz charakterisiert.

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1 Introduction

1.1 Model organisms

1.1.1 Yeast as a model organism

The budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote, thus containing membrane-bound organelles like a nucleus, mitochondria and an endomembrane system. Cell division under normal laboratory conditions takes place every 90 minutes, which makes it a rapidly growing organism with very simple growth requirements and cheap cultivation (Duina et al. 2014).

S. cerevisiae was the first eukaryote to be fully sequenced in 1996 and many of its molecular pathways have been characterized (Goffeau et al. 1996). Today, of the ~6000 yeast genes the biological role of ~85% is known (Botstein und Fink 2011). Humans and the budding yeast share common ancestors and their evolutionary paths divided about one billion years ago (Douzery et al. 2004). Out of all the genes of *S. cerevisiae*, around 50% were shown to have homologues in human. Recently 414 essential yeast genes were replaced with their human orthologues and almost half of them could be successfully complemented, showing that the genes seem to retain ancestral functions (Kachroo et al. 2015).

A main advantage of yeast is the easy manipulation of the genome to create gene knock-outs or other modifications, which is a very expensive and time consuming process in other mammalian systems (Botstein 1997). Several deletion libraries allow rapid high throughput screenings, which continuously lead to a better understanding of the roles of a gene in a biological pathway or the identification of drug candidates. Based on this, *S. cerevisiase* contributed a lot to the elucidation of mechanisms like the regulation of the cell cycle, autophagy, pathways for cell death and energy metabolism, and also to the understanding of diseases (Beach et al. 1982; Madeo et al. 1997; Dolinski und Botstein 2007).

1.1.2 Fruit fly as a model organism

Drosophila melanogaster, the fruit fly, has been studied for many years and due to its wide range of genetic and molecular tools to analyze gene functions it has evolved to a widely

used model organism. Drosophila is a multicellular organism with many similarities to humans in development as well as in behavior. More than 50% of the genes have human homologues and 75% of the genes that cause diseases in humans can be found in the fruit fly (Chien et al. 2002). Other benefits of working with Drosophila are the relatively easy and inexpensive handling, the short lifespan of about 2-3 months and a reproduction cycle of only days. Additionally there are only few ethical and safety issues and genetic modifications are easy to generate and allowed. (Jennings 2011; Sun et al. 2013)

A female fly can lay more than 100 eggs per day, and in about 10 days (at 25°C) an embryo develops into a fertile adult. The four stages of the life cycle are egg, larva, pupa, and fly. An embryo takes about one day to develop in the egg before eclosing, the larva then pupates after around 5 days of eating and growing and undergoing three molts. Then the metamorphosis to the adult fly takes about four more days (Jennings 2011).

1.2 Programmed cell death

Programmed cell death (PCD) plays an important role in maintaining homeostasis, the balance between growth and death, which is essential for life in all organisms. In a healthy adult it is estimated that around 10 billion cells are produced and killed per day (Renehan 2001). Cells undergo PCD if DNA is damaged and cannot be repaired but it is also an essential process to eliminate redundant cells from embryonic development, autoreactive cells of the immune system and virally infected cells. Dysregulation of PCD in either direction leads to pathologies like cancer, autoimmune diseases or neurodegenerative diseases (Broker et al. 2005).

PCD can be divided into apoptosis, necrosis and autophagic cell death. Apoptosis is the least interfering PCD and is a very controlled process that is energy-dependent. Nuclear fragmentation takes place and chromatin condenses, the cells shrink and fragments are packed into apoptotic bodies (Broker et al. 2005). Necrosis is associated with cell swelling and disruption of the membrane which can lead to loss of cytoplasmic content and inflammatory processes. There also exists a controlled way of necrosis (programmed necrosis), which is dependent on the kinases RIP1 and RIP3 (Han et al. 2011; Zong und Thompson 2006). It is not always possible to distinguish between apoptosis and necrosis

since they have overlapping regulators and mediators, e.g. the failure of apoptosis can lead to necrosis (Zong und Thompson 2006).

1.2.1 Apoptosis

There are two different signaling cascades that can lead to apoptosis: the intrinsic and the extrinsic pathway (Elmore 2007). The extrinsic pathway can be activated by extracellular stimuli, when a ligand binds to a death receptor on the surface. Those receptors are members of the tumor necrosis factor (TNF) receptor gene family and their role is to transmit the signal into the cell, leading to the activation of a cascade signaling process (Locksley et al. 2001; Ashkenazi und Dixit 1998). A death-inducing signaling complex (DISK) is formed and leads to an autocatalytic activation of procaspase-8, which results in the activation of downstream effector caspases (Kischkel et al. 1995; Elmore 2007).

In apoptosis initiation through the intrinsic pathway, the mitochondria play an essential role. Intracellular stimuli like toxins, free radicals or damaged DNA lead to loss of mitochondrial membrane potential and hence to the release of mitochondrial proteins and pro-apoptotic factors into the cytosol. On the other hand there are stimuli that lead to apoptosis when they are absent, like growth factors or cytokines that usually lead to suppression of apoptosis (Elmore 2007). The regulators of those mitochondrial events are mainly members of the Bcl2 protein family, among them are the pro-apoptotic proteins Bax, Bac, and Bid as well as anti-apoptotic factors like Bcl-2 (Cory und Adams 2002).

Pro-apoptotic stimuli lead to the release of cytochrome c and Smac/Diablo into the cytoplasm. Cytochrome c assembles with Apaf-1 (apoptosis activating factor 1) and the procaspase-9 to form the apoptosome. The apoptosome then triggers an autocatalytic activation of procaspase-9 and hence a cascade of caspases (Hill et al. 2004). Factors like Smac/Diablo lead to the inhibition of IAPs (inhibitor of apoptosis proteins) (Du et al. 2000). In a later stage of apoptosis other pro-apoptotic proteins are released from the mitochondria, the apoptosis inducing factor (AIF) and the endonuclease G (EndoG). They work in a caspase-independent fashion and translocate directly into the nucleus where they trigger DNA fragmentation (Ly et al. 2015; Joza et al. 2001). Both cascade pathways, intrinsic and extrinsic, end in the activation of the executor

caspase-3, which activates and recruits endonucleases and proteases which lead to the final demise of the cell (Slee et al. 2001).

1.2.2 Programmed cell death in yeast

If simple unicellular organisms like yeast perform PCD this equates to suicide and seems to be counterintuitive. However, Madeo et al. could describe in 1997 an apoptotic phenotype in a yeast mutant. This cdc48 mutant, that has defects in cell division, showed chromatin condensation, externalization of phophatidylserine and DNA fragmentation, which are characteristics of apoptosis (Madeo et al. 1997). Subsequently many homologues to mammalian mediators of apoptosis were discovered in yeast. A yeast caspase (*YCA1*) was identified, which shows structural homology to caspases in mammals (Madeo et al. 2002). An apoptosis inducing factor (*AIF1*) is present in the mitochondria, like in its mammalian counterpart it translocates into the nucleus (Wissing et al. 2004) and a homologue to EndoG, *NUC1* was identified (Buttner et al. 2007). This demonstrates that apoptosis in yeast exists and furthermore, that the process and the regulation is highly conserved. This makes *S. cerevisiae* an adequate model organism for further research on apoptosis.

Apoptosis in a unicellular organism isn't that paradoxical as it sounds at first glance. A single yeast cell always has to be seen as part of a population. The death of a unique cell that is damaged or old can lead to an evolutionary advantage for the whole population and a survival benefit for younger and fitter cells. This term is called altruistic death, an individual performing suicide for the benefits of other clones, to ensure the survival of their own population (Madeo et al. 1997; Buttner et al. 2006).

1.3 Autophagy

The word autophagy is of Greek origin and can be translated as "self-eating". The autophagic machinery is the recycling system of the cell and leads to self-digestion. It is a pathway that is conserved from yeast to higher organisms and functioning autophagy is essential for health and aging. A decrease in autophagic activity could be related to different pathologies like neurodegeneration, cancer and other age-associated diseases (Del Roso et al. 2003; Nixon 2013; Carroll et al. 2013). Moreover, in older organisms a decrease in autophagy can be observed, and it seems that autophagy is at least partly

involved in the increase of health- and lifespan through caloric restriction (Melendez et al. 2008; Pallauf und Rimbach 2013; Hansen et al. 2008).
Autophagy can remove waste that accumulates inside the cell (including misfolded proteins, cellular aggregates, or damaged organelles) and simultaneously provide the cell with energy and nutrients from the degraded components (Pallauf und Rimbach 2013). A functioning cell continuously undergoes autophagy at a basal level, but it can be tuned depending on energy levels, growth factors and nutrients. Nutrient deprivation and starvation of the cell leads to an induction of autophagy (Hansen et al. 2008).

Autophagy can be divided into macroautophagy, microautophagy and chaperone-mediated autophagy (Pallauf und Rimbach 2013). In macroautophagy, double-membraned autophagosomes are formed around cytoplasmic parts which subsequently fuse with lysosomes, building an autolysosome where the degradation occurs. Besides the unselective macroautophagy, there are also selective forms of macroautophagy known, which degrade specific organelles or cellular components. Among them are mitophagy (degradation of mitochondria), xenophagy (defense against microorganisms), ribophagy (degradation of ribosomes) or pexophagy (degradation of peroxisomes) (Reggiori et al. 2012). In Microautophagy the components from the cytosol are directly sequestered by the lysosome without the formation of an autophagosome through inversions of the lysosomal membrane. Recently also selective forms of microautophagy have been reported (Melendez et al. 2008; Reggiori und Klionsky 2013). In chaperone-mediated autophagy targeted proteins get directly into the lysosome if they are in complex with chaperone proteins (Kaushik und Cuervo 2012). In all forms of autophagy it comes to degradation of the content through lysosomal hydrolases.

1.3.1 Autophagy in yeast

Most of the genes, mechanisms and processes of autophagy as well as their regulation could be first described in yeast and 38 autophagy related genes (*ATGs*) have been discovered in yeast so far. In mammalian cells the formation of autophagosomes was observed already in the 1950s, but the involved genes were mostly identified later on through homologies with *ATGs* in yeast (Lamb et al. 2013; Reggiori und Klionsky 2013). This proves that autophagy is highly conserved through evolution and makes yeast a

suitable organism to study autophagic processes that could be relevant for pathophysiology in humans.

Unselective Macroautophagy

Most of the Atgs act downstream of the central regulator of macroautophagy, the Ser/Thr kinase Tor (target of rapamycin). Tor can regulate autophagy directly through phosphorylation of Atg proteins or through a signaling cascade. The autophagy initiation complex can form when Atg1 and Atg13 get phosphorylated. This leads to a binding of Atg1, Atg13, and Atg17, and the complex then interacts with the PAS (pre-autophagosomal structure) (Kamada et al. 2000; Kawamata et al. 2008). Successive recruitment of further Atg proteins to the PAS leads to the formation of the autophagosome. The transmembrane protein Atg9 is essential for the formation of the autophagosome probably through the regulation of autophagy initiation by recruiting Atg proteins and by organizing the PAS (Noda et al. 2000; Mari et al. 2010). During the generation of the autophagosome, lipid bilayers from different donor sources are added to the phagophore to expand it and form the double membrane around the cargo. Involved in those steps are - among others – the PtdIns 3-kinase complexes with its proteins Vsp15, Vsp34, Atg6 and Atg14, which are needed for the production of PI3P (phosphatidylinositol 3 phosphate). The PI3P can then be bound by Atg18, Atg20, Atg21 and Atg24 (Kihara et al. 2001). Furthermore, the protein complex consisting of Atg12, Atg5 and Atg16 is needed to build the autophagosome and Atg8 is essential its maturation. Atg8 is usually a cytoplasmic protein, but can get membrane-associated upon coupling to phosphatidylethanolamine (PE), a process that is carried out through the proteins Atg4, Atg7, Atg3 and the Atg5-Atg12 complex (Geng und Klionsky 2008; Reggiori und Klionsky 2013).

After the completion of the autophagosome around the cytoplasmic cargo, most Atgs dissociate to let the autophagosome fuse with the vacuole. Involved in this fusion process are different SNARE proteins, proteins of the Rab family, and others (Ishihara et al. 2001; Darsow et al. 1997; Reggiori und Klionsky 2013) . Upon the fusion of the outer membrane of the phagosome with the vacuole membrane, the inner vesicle is released into the lumen. The membrane is lysed by the lipase Atg15 and hydrolyses degrade the cargo.

Resulting products are recycled back into the cytosol with the help of vacuole membrane permeases like Atg22 (Reggiori und Klionsky 2013; Yang et al. 2006).

Mitophagy – a selective form of macroautophagy

In selective macroautophagy specific organelles are degraded. Bulk cytoplasm is not sequestered since the membrane is already in close apposition to the cargo. The core machinery is the same than in unselective macroautophagy, but some Atgs are specific (Okamoto et al. 2009). Selective types of macroautophagy in yeast depend on the scaffold protein Atg11 that can bind to different cargos like mitochondria or peroxisomes (Mao et al. 2013).

Mitophagy seems to occur at a specialized PAS and the cargo recognition in mitophagy works through the mitochondria autophagy receptor Atg32, a mitophagy-specific protein that is not needed for other types of autophagy (Kanki et al. 2009; Okamoto et al. 2009). Atg32 is a transmembrane protein that is located in the outer membrane of the mitochondria. It can bind to the N-terminal propeptide of Ape1 and then interact with the scaffold protein Atg11 and with the central component of the autophagic machinery Atg8 to induce mitophagy. The complex Atg32-Atg11-Atg8 seems to be the initiator complex that is formed to bring the mitochondria to the core autophagy machinery (Okamoto et al. 2009). Other modulators of mitophagy in yeast are the mitochondrial outer membrane protein Uth1 (Kissova et al. 2004), the mitochondrial protein phosphatase Aup1 (Tal et al. 2007), and the mitogen-activated protein kinases Slt2 and Hog1 (Mao et al. 2011).

Microautophagy

Contrary to macroautophagy, in microautophagy parts of the cytoplasm are directly sequestered by the vacuole/lysosome. No autophagosome has to be built and the vesicle membrane is derived from the vacuole. Inversions of the vacuole membrane lead to the formation of an autophagic tube and following uptake of the cargo, delivery into the lumen of the vacuole and to degradation (Reggiori und Klionsky 2013).

Little is known about the mechanisms and the regulation of microautophagy and there is no evidence that the Atg proteins are involved in the unselective process (Mayer 2008). The vacuolar transporter chaperone (VTC) complex was shown to be essential for microautophagy, since a deletion blocks the uptake into vacuoles. Upon nutrient limitation,

the VTC complex starts to accumulate at the vacuoles, distributed inhomogenously in the membrane (Uttenweiler und Mayer 2008; Uttenweiler et al. 2007). The EGO complex, out of the proteins Ego1, Gtr2 and Ego3, seems to be involved in the control of microautophagy (Uttenweiler und Mayer 2008).

Several forms of selective microautophagy have been described, including micropexophagy, micronucleophagy and micromitophagy (Reggiori et al. 2012). In yeast, it is still discussed whether micromitophagy exists, but electron microscopy studies have shown evidence that the vacuole membrane can sequester mitochondria (Kissova und Camougrand 2009).

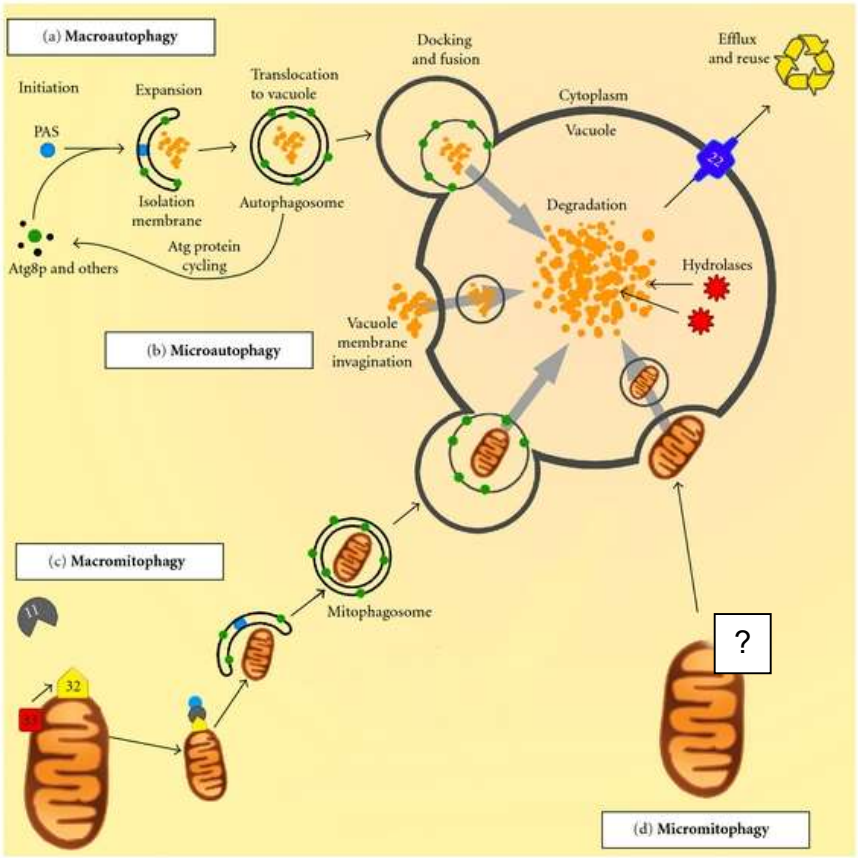

Figure 1 Overview of autophagic mechanisms in yeast. (a) Unselective macroautophagy sequesters components of the cytoplasm into autophagosoms and delivers them into the vacuole where degradation takes place. Atg proteins are involved. (b) Microautophagy takes up cytoplasmic compounds directly into the vacuole through invaginaton of the vacuolar membrane. (c) Selective uptake of mitochondria through a macroautophagic mechanism. Atg32 and Atg11 are involved to get the mitochondria into contact with the core autophagy machinery. (d) Removal of mitochondria through selective microautophagy, the mechanisms remains unclear. Adapted from (May et al. 2012)

1.3.2 Autophagy in flies

The autophagic regulatory pathways are evolutionary conserved. In Drosophila homologues of most of the *ATGs* were found (list of orthologues in Melendez und Neufeld 2008). The autophagic machinery and the process are the same in yeast and flies; macroautophagy, microautophagy and chaperone-mediated autophagy are also described in the fruit fly (Cuervo 2008).

In Drosophila, like in other animal cells, autophagy is highly important for development and differentiation, playing a role in cellular remodeling (Melendez et al. 2008). While some mutations lead to embryonic lethality, others are viable but show defects later on. Null mutations of Atg1, Atg18 and Atg6 lead to lethality prior to eclosion (Scott et al. 2004), whereas flies with mutations in Atg7 are viable but short-lived, sensitive to stress conditions and accumulate aggregates in neurons (Juhasz et al. 2007).

1.4 Aging

Aging is mostly defined as an age-progressive degeneration of physiological functions, resulting in increasing age-dependent mortality and decreasing age-specific fertility (Medawar 1955; Rose 2010). Until today it is not clear why we age, since aging is very complex and many theories of aging are known. Two main hypotheses that explain why an organism ages are widely accepted. The mutation accumulation theory says that somatic mutations accumulate after the reproductive age, when the cost of reparation is higher than the one of reproduction (Medawar 1955). The theory of antagonistic pleiotropy suggests that genes which increase the fitness in early life can have harmful effects later in life (Williams 1957).

Lots of theories suggest that damage of DNA is the most important driver of aging, but also the accumulation of waste in the cells is connected to aging (Gensler und Bernstein 1981; Freitas und Magalhaes 2011).

Caloric restriction can extend lifespan in different organisms, including yeast, flies, worm and rodents, and also leads to a decrease in age-associated diseases (Fontana et al. 2010). Defects in growth signaling pathways lead to the same effect: reduced

insulin/insulin-like growth factor-like signaling (IIS), which is activated in response to nutrients, promotes lifespan in *C. elegans* and *D. melanogaster* but also in nematodes and mice (Clancy et al. 2001; Junnila et al. 2013). The target of rapamycin (TOR) also seems to be involved in this mechanism, since it is activated in response to nutrients. Tor inhibits autophagy and reduced Tor signaling through the inhibitor rapamycin or genetic modifications leads to extended lifespan in yeast, worms, flies and mice (Johnson et al. 2013).

1.4.1 Aging in yeast

Various findings in yeast concerning aging could be transferred to higher model organisms. Lifespan extension through caloric restriction could be demonstrated in yeast and also various genes involved in yeast aging have homologues in other organisms (Johnson et al. 2013; Fontana et al. 2010; Longo und Fabrizio 2012). The conserved pathways and similarities in aging make yeast to a suitable organism for aging studies.

In yeast, aging can be studied by analyzing the replicative lifespan (RLS) or the chronological lifespan (CLS). Replicative aging is the potential of an individual mother cell to divide, which mimics the aging of mitotic human cells. RLS can therefore be quantified by the bud scars on the mother's cell surface, which stand for the number of daughter cells, the mother cell has produced (Steinkraus et al. 2008; Longo und Fabrizio 2012; Fabrizio et al. 2001). Instead, chronological aging represents aging of post-mitotic cells of higher organisms and therefore non-dividing mammalian cells. It can be easily determined by the survival time of a liquid cell population (Longo und Fabrizio 2012). Here, the life span of yeast cells is determined by the period of time where no cell division occurs. During this time, cells accumulate damages, leading to a cellular decline and ending in programmed cell death (Fabrizio und Longo 2003).

CLS and RLS interact with and affect each other, indicating overlapping mechanisms (Fabrizio et al. 2001; Wei et al. 2008; Ashrafi et al. 1999; Laun et al. 2006). CLS can reduce RLS by affecting growth but also by reducing the RLS of mother cells. The major pro-aging pathways are the TOR/Sch9 and the Ras/adenylate cyclase/PKA pathways , which lead to earlier death in CLS as well as in RLS models (Fabrizio et al. 2001; Longo

und Fabrizio 2012). On the other hand, there are several genes that increase CLS and reduce RLS and vice versa (Laun et al. 2006; Ashrafi et al. 1999).

1.4.2 Aging in flies

Drosophila is widely used in aging research, since aging pathways are conserved between flies and human, and many homologous genes could be discovered that modulate lifespan (Fontana et al. 2010). A major advantage is, that it is very easy to manipulate the gene expression: with the Gal4-UAS system genes can be overexpressed and knockdowns can be generated with RNAi (RNA interference) (Sun et al. 2013).

Besides the genetic alterations, many environmental factors have an impact on the lifespan of fruit flies, such as diet, temperature, or physical stress.

As well as in other organisms, caloric restriction leads to an extension of lifespan in Drosophila (Fontana et al. 2010). This shows a high importance of diet composition and food intake. The most commonly used diet is the sugar-yeast extract (SY) diet, and differences in the composition of those two components show a high variation in lifespan. Therefore, measurement of the food intake should be carried out along aging experiments to be sure that the effect is not through a change in food intake (Sun et al. 2013).

In aging studies there are various behavioral and physiological assays that are often conducted together. A measurement of the locomotor activity through climbing assays, which can be an indication of health span, or measurement of stress resistance are commonly used (Sun et al. 2013; Jennings 2011). Stressors can be starvation, heat or cold shock or oxidative stress. Better resistance to stress is often correlated to better survival, however longevity cannot always be associated with stress resistance (Rose et al. 1992; Harshman et al. 1999).

1.5 Mitochondria in Aging

Mitochondria are organelles that are involved in various essential functions: they regulate energy homeostasis and redox balance and are involved in different signaling pathways, including PCD (McBride et al. 2006; Tait und Green 2013). The main metabolic pathways where mitochondria are classically involved in are oxidative phosphorylation and

tricarboxylic acid cycle (TCA), which can regulate each other (Amigo et al. 2016; Yuan et al. 2016). During recent years, increasing evidence shows that major phenotypes of aging are linked to mitochondrial dysfunction (Grunewald et al. 2010). When mutations accumulate during aging due to mitochondrial turnover, this leads to a heteroplasmic population of mtDNA copies which can promote different pathologies. Aging is associated with a decline in mitochondrial mass and function; on the other hand, higher mitochondrial respiratory capacity correlates with a better stress resistance of the cell (Herbener 1976; Stocco und Hutson 1978; Yen et al. 1989). However, it remains unsure if this phenotype of decrease in mitochondrial mass and function is a consequence or a cause of the aging process (Bratic und Larsson 2013; Amigo et al. 2016).

Mitochondria play the central role in energy metabolism, since they produce ATP during oxidative phosphorylation. In the electron transport chain, which consists out of four respiratory chain complexes (I-IV), electrons get transferred to different acceptors to finally reduce oxygen to water. The electron transfer is coupled to proton translocation from the mitochondrial matrix into the intermembrane space through proton pumps. Those pumps are the complexes I, III, IV in mammals and in yeast only complex III and IV. The proton gradient leads to a proton motive force, which is used by the ATP synthase for the phosphorylation of ADP to ATP (Bratic und Trifunovic 2010).

Mitochondria are very dynamic organelles and undergo a constant fusion and fission. This fusing to other mitochondria continuously leads to a change in size and shape and they are able to exchange genetic and protein material between them. Fission helps to separate dysfunctional material of the mitochondria and is tightly correlated to mitophagy, which degrades and removes dysfunctional mitochondria (Mannella 2008). Several studies have shown that highly fragmented material is linked to various pathologies (Mortiboys et al. 2008; Knott et al. 2008). Centenarians, on the other hand, show more mitochondrial mass, which is fused to a higher degree. (Amigo et al. 2016)

There are different theories of why we age, one of them is the Mitochondrial Free Radical Theory of Aging. This theory proposes that reactive oxygen species (ROS), which originate during aerobic metabolism, lead to oxidative damage. This free radical damage accumulates over time in cells and could be causative for aging. During aging mtDNA

mutations accumulate and mitochondrial function declines, which in turn leads to an increase in ROS production (Bratic und Larsson 2013; HARMAN 1956).

Caloric restriction (CR), which leads to lifespan extension in different organisms, has an impact on mitochondria and mitochondrial function. In mice, CR leads to better mitochondrial oxidative capacity upon age and it was reported that mitochondrial biogenesis is increased through CR (Lanza et al. 2012; Lopez-Lluch et al. 2006). This suggests that the protective effects of CR on lifespan work at least partly through mitochondrial pathways. CR leads to an increase in mitochondrial respiratory rate and to less generation of oxidants (Lopez-Lluch et al. 2006; Sohal et al. 1994). Additionally NAD^+/NADH can regulate sirtuin activity, which are involved in longevity and also in the lifespan extension through CR (Evans et al. 2010).

In yeast, aging was shown to directly affect the function of the electron transport chain and mtDNA maintenance; mitophagy and oxidant generation increase (Jazwinski 2002). A decrease in the mitochondrial membrane potential, which is also accompanied with aging, triggers the activation of retrograde signaling which leads to an activation of anti-aging genes and also to a crosstalk with Tor1 (target of rapamycin). Those genes control metabolism of proteins or carbohydrates, fatty acid oxidation, and also stabilize genes in the nucleus (Amigo et al. 2016; Jazwinski 2002, 2013).

1.6 Flavonoids

Flavonoids are a subgroup of phytochemicals from the polyphenol family and are secondary metabolites in plants. Although they are not essential for development and growth in the plants, they are ubiquitous and most species contain high concentrations of flavonoids. At the moment, more than 8000 different flavonoids are known to occur naturally in different plants (Del Rio et al. 2013). These flavonoids have various ecological functions for the plant, including protection from UV, microbial infections, and stressors. In addition, they can attract pollinators and are fundamental for symbiotic or pathologic interactions with bacteria and fungi. They are also thought to be involved in signaling processes, among others in nitrogen-fixing (Del Rio et al. 2013; Kennedy 2014).

Due to their ubiquitous occurrence in plants, flavonoids and other phenolic compounds are naturally part of the human diet, and it is thought that they are partly responsible for the beneficial effects of fruits and vegetables. Diets rich in polyphenols (for example the Mediterranean diet) are shown to lead to better human health (Anderson und Nieman 2016; Kennedy 2014). There is increasing evidence that long-term intake of flavonoids can affect life- and healthspan, and also reduce the incidence of age-related diseases including cancer, chronic diseases and neurological diseases (Del Rio et al. 2013). Bioactive flavonoids include anti-oxidative, anti-aging, anti-inflammatory, anti-microbial, anti-cancer, neuroprotective and cardioprotective properties, and seem to be able to modulate brain functions (Li und Vederas 2009; Ly et al. 2015; Kennedy 2014).

Chemically, flavonoids contain a skeleton of 15 carbons which consists out of two aromatic rings with a three carbon bridge, which can form a third heterocyclic ring. Flavonoids can again be subdivided into flavones, flavonols, isoflavones, flavanones and anthocyanidins and chalcones. The flavonoid backbone can have different substituents (Del Rio et al. 2013).

1.6.1 2-hydroxychalcone

The subgroup of the chalcones has received increasing attention due to its biological activities. Different derivatives of chalcones are present in various edible plants like liquorice, citrus fruits, apples and others, but also in vegetables like tomatoes and bean sprouts. Plants containing chalcones have widely been used in traditional medicine (Di Carlo et al. 1999).

Chalcones have been described to show anti-cancer properties and are handled as potential anti-cancer drugs. In endothelial cells some chalcones showed the possibility to activate the transcription of STAT3 and NF-κ, mediators of inflammatory diseases and cancer. Others reported activities that promote initiation of apoptosis in human cell culture and regulation of cyclins and cyclin-dependent kinases (Cdks), therefore suggesting an interaction with cell cycle progression (Orlikova et al. 2011).

Hydroxychalcones count to the derivatives of chalcones with their hydroxy-substitutes being at different positions, e.g. 2 hydroxychalcone, 2'hydroxychalonce, 4-hydroxychalone, 4'-hydroxychalcone and different dihydroxychalcones.

Like all the chalcones, 2-hydroxychalone (2-HC) contains two aromatic rings connected by three carbons; in addition, it bears a hydroxyl group at the second position of one ring. With a structural formula of $C_5H_{12}O_2$, its molecular weight is 224.25 g/mol. In the resin of the plant *Dracaena cinnabari*, which grows in tropical areas like the African east coast, 2-HC can be found together with other polyphenols and other chalcones. This plant has been used in the traditional medicine against different maladies, but so far no studies about the biological properties of the single flavonoid 2-HC have been conducted (Khare 2015).

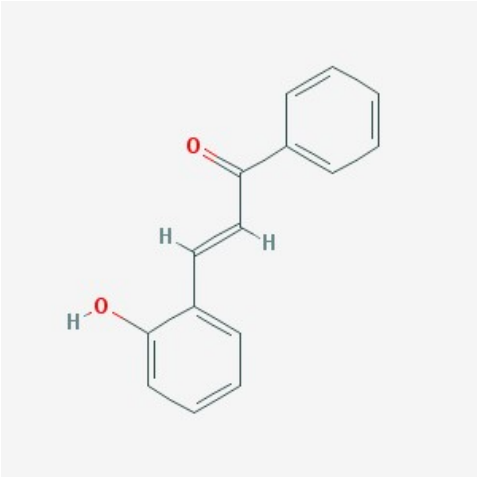

Figure 2 Chemical structure of 2-hydroxychalcone. From: https://pubchem.ncbi.nlm.nih.gov

1.7 Previous work leading to this study

In our laboratory, a high-throughput screen with *Saccharomyces cerevisiae* was performed, testing a library of polyphenols on its effects on lifespan in diverse scenarios (Zimmermann 2012). 2-hydroxychalcone emerged among the hits and was further tested (Hofer 2016). Interestingly, a lifespan extension and a better survival in wildtype yeast cells could be observed upon treatment with 2-HC. An optimum concentration of 75 µM 2-HC was chosen to be best for showing a significant reduction of PI-stained cells compared to the control, which implies less cell death (*Figure 3*A). PI analysis and clonogenicity assays at different time points showed a reduction of dead cells and an increase of survival, respectively (*Figure 3*B+C).

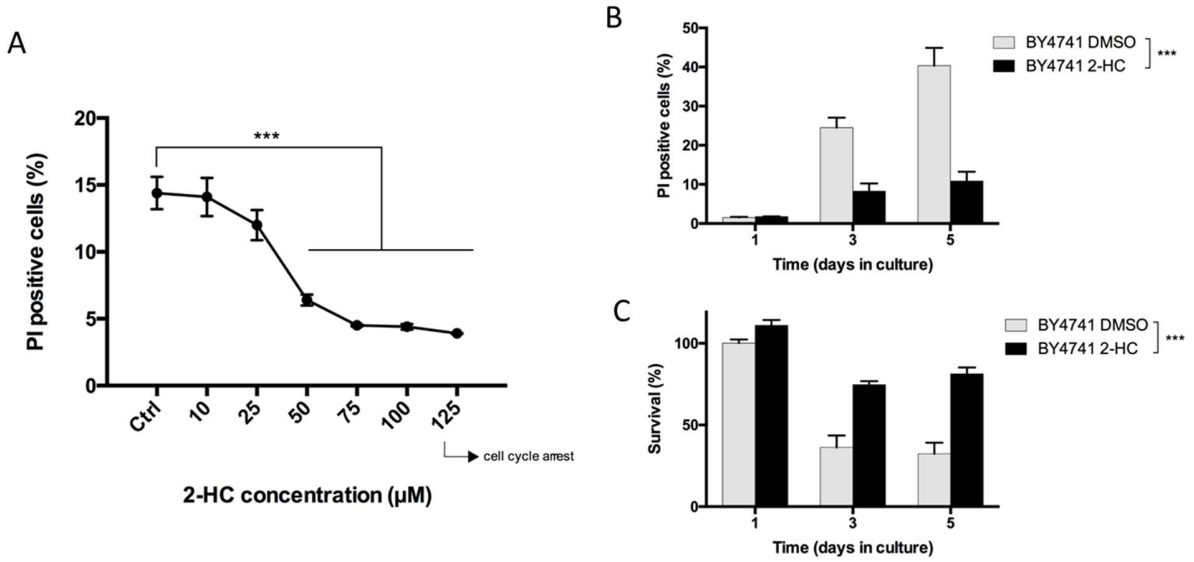

Figure 3 2-hydroxychalcone shows cytoprotective effects in wildtype BY4741 cells. BY4741 yeast cells were grown in SMG and treated with 2-HC at an OD_{600} of 0.2. (A) Samples were stained with propidium iodide (PI) and analyzed with flow cytometry (FACS) on day 3. 30000 cells were analyzed. Cell count was measured with CASY, showing a cell cycle arrest for concentrations higher than 125 µM. (B) Cells were treated with 75 µM 2-HC and analyzed at indicated time points. Samples were stained with PI and 30000 cells were analyzed with FACS. (C) Cells were treated with 75 µM 2-HC and analyzed at indicated time points. Colony forming units after plating of 500 cells on YPD agar plates. Figures and legend adapted from (Hofer 2016)

1.8 Aim of this work

In this work the goal was to further characterize the cytoprotective properties of 2-HC in *Saccharomyces cerevisiae* and to test its effects on the fruit fly *Drosophila melanogaster*. We could transfer the lifespan-extending effect of 2-HC to the fruit fly, indicating an evolutionarily conserved mechanism. Furthermore it could be shown, that 2-HC treatment in yeast leads to the upregulation of a high number of mitochondrial proteins. 2-HC preconditions cells for growth on respiratory stress and the respiratory rate is significantly increased. This suggests a mitochondrial involvement in the cytoprotective activity of 2-HC. It could be shown that flavonoids can specifically modulate different pathways and target cell functions. This makes natural substances like flavonoids promising candidates for the use as therapeutics for healthier aging. Data obtained in this work are the basis for future application of 2-HC in humans.

2 Materials

2.1 Strains

All yeast strains used in this work are derivatives of *Saccharomyces cerevisiae* BY4741 and are listed in Table 1.

In all fly experiments *Drosophila melanogaster* wildtype w^{1118} from the laboratory of Ronald Kühnlein (Max Planck Institute for Biophysical Chemistry, Germany) was used.

Table 1 Yeast strains used in this work including their genotype and origin

STRAIN	GENOTYPE	ORIGIN
BY4741 (wildtype)	Mat a; his3Δ1, leu2Δ0, met14Δ0, ura3Δ0	Euroscarf
pTN9	BY4741 Pho8ΔN60::URA	Sabrina Schröder
Δatg1	*BY4741 atg1::KanMX*	Patrick Rockenfeller
Δatg5	*BY4741 atg5::KanMX*	Sebastian Hofer
Δatg7	*BY4741 atg7::HIS*	Christoph Ruckenstuhl
Δatg8	*BY4741 atg8::HIS*	Christoph Ruckenstuhl
Δatg11	*BY4741 atg11::KanMX*	Patrick Rockenfeller
Δatg32	*BY4741 atg32::KanMX*	Esther Fröhlich
Δslt2	*BY4741 slt2::KanMX*	Euroscarf
Δmdm38	*BY4741 mdm38::KanMX*	Euroscarf
Δuth1	*BY4741 uth1::KanMX*	Euroscarf
Δaup1	*BY4741 aup1::KanMX*	Julia Ring
Δvtc1	*BY4741 Δvtc1::hphNT1*	This work
Δvtc2	*BY4741 Δvtc2::hphNT1*	This work
Δvtc3	*BY4741 Δvtc3::hphNT1*	This work
Δvtc4	*BY4741 Δvtc4::hphNT1*	This work
Δego1	*BY4741 Δego1::hphNT1*	This work
Δgtr2	*BY4741 Δgtr2::hphNT1*	This work
Δego3	*BY4741 Δego3::hphNT1*	This work

pCC4	*BY4741 with pCC4*	Katharina Kainz
pCC5	*BY4741 with pCC5*	Katharina Kainz
MLS-dsRED	BY4741 with *DsRed Su1-69*	Sabrina Büttner

2.2 Plasmids

For the generation of deletion strains, the plasmid pFA6a with a hygromycin cassette (hphNT1) was used as a template. All plasmids used in this work are shown in Table 2.

Table 2 Plasmids used in this work

PLASMID	DESCRIPTION	INSERT	SOURCE
pCC4	HIS3 marker	pho8ΔN60+MLS	Campbell, 1998
pRS313	Corresponding empty vector to pCC4/5		Campbell, 1998
pFA6a–hphNT1	Hygromycin B resistance cassette		Janke, 2004

2.3 Primers

All primers used in this work were obtained from Eurofins Genomics (Germany) and are listed in Table 3. Lyophilized primers were dissolved in water to a concentration of 100 pmol/µg and stored at -20°C. For the verification of all the knock-outs a control PCR with the gene specific control primer and the cassette specific reverse primer were performed.

Table 3 Primers used for generation and control of deletion strains and

GENE	TYPE	SEQUENCE
VTC1	S1	CTACATTATCGAATACGATTAAACACTACGCCAGATTTCCACAA TATGCGTACGCTGCAGGTCGAC
	S2	GAAAATACAGTTTGTGCGTAACCCACGCTTACGATATTGGAATT ACAATTTCAATCGATGAATTCGAGCTCG
	ctrl	GCTTGTTACGGTCGGTGATAC
VTC2	S1	GAA AAAAAAGTAGAAAGAACGACTACACCTCAACATAACGACA CTTTTTTGACATGCGTACGCTGCAGGTCGAC

	S2	CATTACAAACATAAAAACACATGGTCTCAGTAGATAGAGTACAT ATTCTA ATCGATGAATTCGAGCTCG
	ctrl	GACCTCAAAAAAGGCAGGC
VTC3	S1	GGCTATTAGA GCGAACAGCA GAATTTGTCC TTGGTTTTCA GAGTTTGAAA ATG CGTACGCTGCAGGTCGAC
	S2	GTAACTGGTACTTGTGTAATATATGTGTATATAAAAAATATACAT GTTCTTA ATCGATGAATTCGAGCTCG
	ctrl	CCAGAGAGCGGCTTACATC
VTC4	S1	GCTAACAATCAAATCGGCCAATAAAAGAGCATAACAAGGC AGGAACAGCTATGCGTACGCTGCAGGTCGAC
	S2	CTAATATGATTATTACTTAATTATACAGTAAAAAAAACACGCTGT GTATTCAATCGATGAATTCGAGCTCG
	ctrl	CACTTTTTCACACACCGCAC
EGO1	S1	CCAGTATAGCGGTAGTGGATACAACGACAGATTTAAGTCGTAA AAATGCGTACGCTGCAGGTCGAC
	S2	CATTTATTTATAAAGCTTGTATACCAATGATGTTATACAAAAAGT TCATTAATCGATGAATTCGAGCTCG
	ctrl	GTCAGCTACCCGCCTCAC
GTR2	S1	CAGATTAACAAAACTCCAGGACAACGGTACTAATA CACATACAACATGCGTACGCTGCAGGTCGAC
	S2	CAATATGTATCTATATACCCTAATATTTTCATGCCTTACGTCTTT CAATCGATGAATTCGAGCTCG
	ctrl	GACGGCTTTAACTTCATATTTCG
EGO3	S1	GGCAGTTATCAGCAGCAAACGGTATCCAAAATATTGAAGCAAT ATGCGTACGCTGCAGGTCGAC
	S2	GAGACGCATGAAAAGGTGTGGCCTCGATACATATTGTTAAACC CATCAATCGATGAATTCGAGCTCG
	ctrl	CGGTAATTCGCAGATTTTACG
HYGRO CASSE TTE	Ctrl rev	GTCGACCTGCAGCGTACG

2.4 Growth media

2.4.1 Yeast growth media

For the synthetic minimal growth media amino acids, carbon sources and YNB (yeast nitrogen base + ammonium sulfate) were prepared as 10x stock and separately autoclaved at 121° for 25 minutes in an autoclave sterilizer or a certoclave. The stocks were added to autoclaved ddH2O after sterilization and below 60°C.
For solid plates 2% agar was added to the double distilled water before sterilization. Glucose and galactose were obtained from AppliChem and glycerol from VWR chemicals.

For transformations full media (YPD) was used, which was sterilized after mixing of all ingredients (*Table 4)* and antibiotics were added below 60°C.

Table 4 Composition of the media used in yeast experiments

MEDIA	***COMPOSITION***
SMG (synthetic minimal galactose media)	0,17% yeast nitrogen base (BD) 0,5% ammonium sulfate (Roth) 2% galactose 80 mg/l histidine 200 mg/l leucine 300 mg/l uracil 30 mg/l adenine 30 mg/l all other amino acids
SMD/SMGly agar plates	SM media as above + 2% Agar 2% dextrose/glycerol instead of galactose
Selective SMG-His media	As above lacking histidine
YPD (yeast peptone dextrose) full medium	1% Yeast extract (BD) 2% Bacto peptone (BD) 4% Glucose
YPD agar plates	As above + 2% agar
Hygromycin B YPD agar plates	As above + 250mg/l Hygromycin B

2.4.2 Fly media

Table 5 Composition of fly media used in this work

MEDIA	*COMPOSITION*
1x SYA	100 g baker's yeast (Lesaffre, France) 50 g sucrose (Roth, Germany) 10 g agar 3 ml propionic acid 3g nipagin (in 30 ml 96% EtOH) add ddH2O to above to make 1 liter
1,5x SYA (0,6% agar)	as above except: 150g baker's yeast 6g agar
Standard Bloomington media with minor modifications	4,2 g agar 85,3 g sugar beet syrup 7,5 g baker's yeast 8,3 g soy meal 67 g corn meal 1,3 g nipagin (in 4,2 ml 96% EtOH) 5,25 ml propionic acid add ddH2O to above to make 1 liter

2.5 2-hydroxychalcone

The flavonoid 2-hydroxychalcone used in this work was obtained by ABCR (Germany). It was dissolved in DMSO (Roth, Germany ≥ 99.8% purity) as 500x stock for yeast and 1000x stock for fly experiments. Stocks were stored at -20°C for up to one month.

2.6 Buffers and Solutions

2.6.1 PI staining

PBS was prepared as 10x stock and stored at room temperature, PI was prepared as 1000x stock and stored at -20°C.

Table 6 Solutions used for PI staining

Solution	Components
Phosphate buffered saline (PBS)	25mM potassium phosphate buffer 0,9% (w/v) NaCl, pH 7,0
1000x propidium iodide (PI)	100 µg/ml in ddH2O, Sigma-Aldrich (USA)

2.6.2 Alkaline phosphatase based (ALP) assay

Table 7 Solutions used for ALP assay

Solution	Components
Assay buffer	250 mM Tris/HCl (pH 9,0) 10 mM magnesium sulfate 10 µM zinc sulfate
BSA	1 mg/ml in ddH2O
α-naphtylphosphate	55 mM α-naphtylphosphate diluted in assay buffer
Glycine NaOH buffer	2 M glycine/NaOH (pH 11,0)
BioRad reagent	Biorad diluted 1:5 in ddH2O

2.6.3 Yeast knock-out transformation

Table 8 Solutions used for yeast transformation

Solution	Components
LiAc/TE solution	0,1 M lithium acetate 10 mM Tris-HCl pH=7,5 1 mM EDTA pH=8 ddH_2O
PEG solution	0,1 M lithium acetate 10 mM Tris-HCL pH=7,5 1 mM EDTA pH=8 40% PEG ddH_2O
Carrier DNA	Herringsperm-DNA

2.6.4 DNA extraction from a single colony

Table 9 Solution used for DNA extraction from colonies

Solution	Components
LiAc-SDS solution	200 mM lithium acetate 1% SDS

2.6.5 Agarose gel electrophoresis

Table 10 Solutions used for agarose gel electrophoresis

Solution	Components
TRIS-Acetate-EDTA-Buffer (TAE)	40 mM Tris/acetic acid (pH 8,0) 1 mM EDTA
Agarose gel	1% (w/v) agarose 0,001% (w/v) ethidium bromide (Fermentas)
6x loading dye	87% glycerine with brominephenol blue

2.6.6 Cell count measurement (CASY cell counter)

Table 11 Solutions used for measurement at the CASY cell counter

Solution	Components
CASYton™	0.9% NaCl 0.1 mM EDTA

2.6.7 SDS-PAGE and western blot analysis

Table 12 Solutions used for SDS PAGE and western blot analysis

Solution	Components
Electrophoresis buffer	25 mM Tris/HCl 192 mM glycine 0,2% SDS, pH 8,3
Stacking gel	250 mM Tris/HCl, pH 6,8 0,2% SDS 5% acrylamide 0,13% N,N'-methylenebisacrylamide 0,1% ammonium peroxide sulfate (APS) 0,01% TEMED

Running gel	250 mM Tris/HCl, pH 8,8 0,2% SDS 12,5% acrylamide 0,13% N,N'-methylenebisacrylamide 0,1% ammonium peroxide sulfate (APS) 0,01% TEMED
Blotting buffer	20 mM Tris 150 mM glycine 0,05% SDS 20% methanol
TBS(-T)	10 mM Tris/HCl 150 mM NaCl, pH 7,6 (0,02% Triton-X 100)
Blocking solution	milk powder in 5x TBS or 1x in TBS-T
ECL detection mix	1:1 mix of solution 1 and 2 (GE Healthcare-Amersham Biosciences)

2.6.8 Cells lysis with glass beads

Table 13 Solutions used for cell lysis

Solution	Components
Lysis buffer P+	50 mM Tris/HCl, pH 7.4 150 mM NaCl 1% Triton x100 1 mM EDTA 1mM PMSF 1x Complete
Glass beads	

2.7 Chemicals, enzymes and standards

For all primary antibodies α-rabbit was used as secondary antibody. According to the dilution media of the primary antibody the secondary antibody was diluted either in TBS-T + 1% milk powder or TBS + 5% milk powder (*Table 14*).

Table 14 Antibodies used in this work

ANTIBODY	DILUTION
Nde1	1:1000 in TBS-T + 1% milk powder
Ndi1	1:1000 in TBS-T + 1% milk powder
Gapdh	1:40000 in TBS-T + 1% milk powder
Rip1	1:1000 in TBS-T + 1% milk powder
Kar2	1:10000 in TBS-T + 1% milk powder
Sec61	1:3000 in TBS-T + 1% milk powder
Sdh4	1:250 in TBS + 5% milk powder
Sdh1	1:600 in TBS + 5% milk powder
Por1	1:1000 in TBS-T + 1% milk powder
Cox2	1:1000 in TBS-T + 1% milk powder
Ssc1	1:2000 in TBS-T + 1% milk powder
OM45	
α-rabbit	1:10000 in TBS-T + 1% milk powder
α-rabbit	1:10000 in TBS + 5% milk powder

Table 15 Chemicals and enzymes used in this work

Chemical/Enzyme	Source
Taq polymerase	Fermentas
10x Taq polymerase buffer	Fermentas
Phusion® High Fidelity DNA Polymerase	Finnzymes
10x Buffer 2	
dNTPs	Fermentas

Table 16 Standards used in this work

Standard	Source
PageRulerTM prestained protein ladder	Fermentas
Lambda DNA/EcoRI+HindIII Marker	Fermentas
100 bp DNA ladder	Fermentas

2.8 Instruments and expendable materials

Table 17 Instruments and expendables used in this work

Instrument	Source
Analytical balance	Sartorius and KERN ABJ
Agarose gel documentation system	Biozym Diagnostic
Autoclave	Systec
BD FACS Aria$_{TM}$ Flow Cytomerter	BD Biosciences
Binocular system for fly work	Stemi 2000 (Zeiss)
Camera for RING assay	Canon EOS 50D
Cell counter CASY$_{TM}$	Schärfe Systems
Centrifuge for 96-well plates	Sigma 3-18K
ChemiDoc$_{TM}$ Touch	Bio-Rad Laboratories GesmbH
CO_2 control system for fly work	Gunther Tietsch
Colony counter	LemnaTec
Electroblotting power supply	Biorad Power Pac300
Electrophoresis chamber	Thermo EC
Firesting O_2 Pyroscience	Pyroscience
Fluorescence microscope	Zeiss
Fluorescence plate reader	Tecan GeniosPro
Fly incubator	HPP 750 (Memmert)
Freezer (-80°C)	Thermo Forma Scientific, Sanyo
Frige / Freezer (-20°C)	Liebherr
Incubator	Unitron
Laminar flow cabinet	CleanAir
Magnetic stirrer	Heidolph
Mini-BeadBeater$_{TM}$	Biospec Products
Multi-pipettes	Eppendorf, Gilson and Matrix (Thermo Scientific)
pH-meter	Methrom
Photometer	Genesys 10 UV (Thermo Spectronic)
Pipettes	Eppendorf
RING apparatus	Intralaboratory work
Shaker incubator for 96-deep well plates	Microtron and Multitron Pro (Infors HD)
Shaker incubator for flasks	Multitron Pro (Infors HD)
Thermocycler	GeneAmp PCR Systems
Thermomixer Compact	Eppendorf
UV transilluminator	UVP
Vortex	Vortex Genie 2 (Scientific Industries)
Water bath	GFL GmbH
Water Dest 2208	GFL GmbH

Expendables	Source
96 deep well plates	VWR
15ml/50ml flasks	Sarstedt
FACS plates	Greiner
Reaction tubes	Eppendorf
Tips	Eppendorf
Vials for fly aging	K-TK (Germany)
Vials for RING assay	VWR

3 Methods

3.1 Yeast methods

Yeast strains were stored for long periods at -80°C in a 1:1 mixture of overnight culture (ONC) and 50% glycerol.

3.1.1 Aging experiments and biochemical assays

3.1.1.1 Chronological aging

For all aging experiments in this work galactose containing synthetic media (SMG) was used. 10-50 ml media was inoculated with an ONC to an OD_{600} of 0.05 and incubated at 28°C under constant shaking. When an OD_{600} of 0.2 was reached (after 5 – 5.5 hours) 2-HC was added. 2-HC was dissolved as 500x stock in DMSO and added to the media to reach a final DMSO concentration of 0.2%. The control group was treated with 0.2% DMSO. In all yeast experiment a final 2-HC concentration of 75 µM was used, since this was determined as the optimum concentration for wildtype cells (Hofer 2016).

At the same time when the flavonoid was added, the culture was aliquoted into 96-deep well plates (DWP) with a volume of 500 µl per well. Remaining wells were filled with the same volume of ddH2O and the whole plates were sealed with gas permeable foils. The plates were placed in an incubator shaker at 28°C with 1000 rpm.

All experiments were performed on different time points of this chronological aging (flavonoid addition is t_0).

3.1.1.2 PI staining and flow cytometer

On different time points 10 µl of cell suspension was transferred to 96-well FACS plates and resuspended in 100 µl PI solution (in 1xPBS). After an incubation time of 5 minutes in the dark, the plates were centrifuged at 4500 rpm for 5 minutes and the pellet was resuspended in 100 µl 1x PBS.

At the flow cytometer FACS 30,000 cells of each well were analyzed with a flow rate of 2 µl/sec and with 30 µl sample volume. Mixing was set to a number of 2 with a volume of 50 µl and a mixing speed of 180 µl/sec. Washing was performed with 400 µl.

PI measurement was conducted with the PerCP-Cy5.5 channel (excitation wavelength 488 nm and emission 695 nm).

3.1.1.3 Alkaline phosphatase (ALP) assay

For the ALP assay genetically modified strains (pho8Δ60) were used. Those strains express an alkaline phosphatase Pho8 without the membrane anchor, therefore the Pho8 is in the cytosol. Pho8 is not activated until transport into the vacuole and processing through vacuolar proteases. This delivery can only occur through unselective macroautophagy, where parts of the cytoplasm are packed into autophagosomes. With a biochemical colorimetric assay the activity of Pho8 can be measured through the reaction of α-naphtylphosphate to fluorescent naphthalene. The phosphatase activity of the corresponding wildtype strain has to be subtracted as background.

Cell lysis was performed as following: 1 ml of cell culture was harvested from the DWPs, washed and resuspended in 200 µl cold assay buffer. Then this suspension was transferred into 1.5 ml tubes containing 100 µl washed glass beads and subsequently lysed during shaking in a Mini-BeadBeater. The shaking was repeated 3 times, the duration was increased from 45 seconds to 2 minutes in later time points. In between a pause of 1-2 minutes on -20°C was made. After lysis the cells were centrifuged at 4°C with 10,000 g for 10 minutes and the supernatant transferred into fresh tubes.

A Bradford assay was performed to determine the protein concentration, a standard curve was made with BSA in a range from 0 to 12 µg. The measurement was done in 96-well plates, with 300 µl 1x BioRad solution and after 10 minutes of incubation, with a TECAN platereader at 600 nm.

After the determination of the protein concentration, 1.5 µg protein was used for the ALP assay. It was performed in DWPs and to the protein extract assay buffer was added to 550 µl. With 50 µl α-naphtylphosphate the reaction was started and after incubation for 20 minutes at 28°C the reaction was stopped with 200 µl glycine NaOH buffer. Blanks without protein sample were treated the same way.
After mixing and transfer of 100 µl to 96-well microtiter plates with black bottom, the fluorescence was measured with a TECAN platereader with an excitation wavelength of 340 nm and emission at 485 nm.
The resulting fluorescence corresponds to the ALP activity and therefore the rate of autophagy. The background has to be subtracted to eliminate the activity of other phosphatases.

The ALP assay for detecting mitophagy is the exact same procedure. Compared to the before mentioned assay, here the Pho8 contains not only the 60 amino acid deletion but also a mitochondrial localization sequence (MLS). The Pho8 is therefore present in the mitochondria and gets transported into the vacuole through mitophagy. Additionally, the modified Pho8 is expressed from a plasmid (pCC4) and not stable integrated into the genome. The background is adjusted with the empty vector pRS313.

3.1.1.4 Clonogenicity assay

In the clonogenicity assay 500 cells of a chronologically aging yeast culture were plated onto agar plates to determine their survival. The cell counts were determined with a CASY instrument, diluting the aliquots first 1:100 in ddH2O and then further 1:1000 in CASYton. Two measurement cycles with 200 µl were performed with a detection limit of 1.5-15 µm and a background signal lower than two powers under the measured yeast cells. For plating a 1:10,000 dilution in ddH2O was used (out of the 1:100 dilution used for measurement at the CASY). SMD plates were incubated for two days, SMGly for four days at 28°C. Living cells were counted as colony forming units.

3.1.1.5 Respiration measurement

The respiration of yeast cells was measured through the determination of the oxygen consumption with a FirestingO_2 system. Before every measurement the system has to be calibrated with freshly prepared 1% sodium sulfide and ddH2O for the values 0% and 100% respectively and for every channel used. Tubes were filled with 1 ml cell culture and the rest ddH2O to close it bubble free with paraffin film. During the measurement the temperature was kept at 28°C and the samples were constantly stirred. Before the measurement, dead cells were determined through PI staining and the cell counts in the tube was measured with a CASY. This allowed a determination of the oxygen consumption per living cell, excluding all dead cells. O_2 consumption rate was calculated in excel from all runs the trendline was $R^2 > 0.9$.

3.1.1.6 Fluorescence microscopy

Cells expressing a DsRed Su1-69 protein, encoded by the vector pYX142, were used to visualize mitochondria. 100 µl cells were harvested after 16 hours and analyzed with a confocal fluorescence microscope. Exposure time was 100 ms for differential interference contrast (DIC) pictures and 1000 ms for pictures with the dsRED filter to detect mitochondria.

3.1.2 SILAC

3.1.2.1 Labeling of the samples

For the SILAC experiment the DMSO control was labeled with heavy isotopes (13C), 2-HC samples with light isotopes (12C). Cells were aged as above, except for the use of media containing heavy Lys-4 and Arg-10 for the control samples. During growth in this media all the synthesized proteins incorporated the different isotopes of the amino acids.

Two independent replicate experiments were performed, harvesting 20 OD_{600} units of cell culture on days 1 and 3.

3.1.2.2 Protein extraction and quantification

The harvested cells were washed and then disrupted on ice to extract the proteins. Therefore the pellets were resuspended in 150 µl Lysis buffer P+. The suspension was then transferred to a pre-cooled cryo tube with 200 µl glass beads and shaken 3 times for 1 minute (with 1 minute intermediate on ice) with the Mini-BeadBeater. Afterwards, samples were centrifuged at 10,000 rpm for 10 minutes at 4°C and supernatant was transferred into fresh Eppendorf test tubes. The pellet was then again diluted in 70 µl lysis buffer, centrifuged and the supernatants were pooled.

The protein concentration was determined using BioRad protein assay with BSA as a standard.

The protein lysates of the DMSO control and the 2-HC treated cells were mixed in equal volumes and analyzed with mass spectrometry by Jörn Dengjel (University of Fribourg). The arising frameshift due to the isotopes permits a differentiation between the two conditions and can show differences in the protein expression profile.

3.1.3 Immunoblotting

3.1.3.1 Protein extraction

20 OD_{600} units cell culture were harvested 20 and 30 hours after addition of 2-HC/DMSO at 3,500 rpm. After washing with ddH_2O, pellets were shock frozen with N_2 and stored at -80°C.

Protein extraction was performed with cell lysis based on glass beads and the protein concentration was determined with BioRad protein assay (as described above for the SILAC samples).

3.1.3.2 SDS-PAGE

Separation of the proteins according to their size was performed with a denaturized polyacrylamide gel. Due to binding to sodium dodecyl sulfate (SDS) proteins get negatively charged and migrate through the gel depending on their molecular weight. 10 µg protein extract was loaded on the gel. Proteins are focused in the upper stacking gel with a concentration of 5% acrylamide and separated in the running gel containing 12.5%

acrylamide. The electrophoresis took place in Tris-Glycin-SDS running buffer. PageRuler Prestained protein ladder was used as standard.

3.1.3.3 Western Blot analysis

After the SDS-PAGE proteins were transferred to a PVDF membrane. After activation in methanol and washing in ddH2O the transfer was made for 60 minutes at 230 mA in a tank-blot-system of BioRad and with CAPS transfer buffer.

Afterwards membranes were cut and blocked with 1% milk powder overnight. Each cut part was incubated with the corresponding primary and secondary antibodies for 1 hour under shaking conditions at room temperature. Three washing steps of 5 minutes with TBS-T were performed between each step.

Prior to detection at the Chemidoc, membranes were incubated with the ECL reaction mixture for five minute.

3.1.4 Generation of knock-out strains

3.1.4.1 PCR for gene knock-outs

The creation of knock-outs was performed according to Janke et al. 2004 with the plasmid pFA6a–hphNT1 and the designed S1 and S2 primer (Table 18).

The PCR program was as followed: denaturation at 97°C, 10 cycles of 97°C for 1 min, 54°C for 30 sec, 68°C for 2 min 40 sec and subsequently 20 cycles with an extension of the elongation time for 20 seconds in every cycle. Due to the hot start protocol, the Phusion Polymerase was added after the denaturation step of 3 minutes.

Table 18 Composition of the PCR to create a knock-out

Volume	Component
0,5 µl	Phusion polymerase (5u/µl)
5 µl	Buffer 2 (10x)
8,75 µl	dNTP (5 mM)
3,2 µl	S1 primer (1:10 diluted)
3,2 µl	S2 primer (1:10 diluted)
28,5 µl	ddH2O
1 µl	pFA6a–hphNT1

3.1.4.2 Agarose gel electrophoresis to confirm the deletion PCR

Gel electrophoresis was performed with 1% agarose gels. Agarose powder was melted in TAE in the microwave and after cooling down to about 50 °C 0,001 % ethidium bromide was added in the gel tray before polymerization. PCR products were stained with one sixth of the volume of 6x DNA loading dye, the standard Lambda/Eco/HindIII was used to determine the length of the DNA fragments. The electrophoresis took place with about 100 Volt and with 0,5x TAE as running buffer, the detection followed with a UV trans-illuminator.

3.1.4.3 Yeast transformation

Yeast transformation was done referring to the lithium acetate/PEG method. With an ONC, 50 ml YPD were inoculated to an OD_{600} of 0.2. When an OD_{600} of 0.6 was reached, cells were harvested for 5 minutes at 3500 rpm and washed with 10 ml ddH2O and then with 10 ml LiAc/TE solution. The pellet was then resuspended in 200µl LiAc/TE solution and incubated for 30 minutes at 28 °C. After this incubation time cells were ready for transformation and 50 µl aliquots of the cells were mixed with 300 µl PEG solution, 10 µl herring sperm DNA (freshly denaturized for 5 minutes on 95°C, then placed on ice) and the rest of the PCR product. This mixture was incubated at 28°C for 30 minutes and consequently heat-shocked at 42 °C for 20 minutes. After this time cells were harvested, diluted in 70 µl ddH2O and plated on YPD plates. After one day, they were stamped on selective plates containing hygromycin and incubated for 3 days at 28°C.

3.1.4.4 Verification of the knock-outs

To confirm the knock-out clones with a control PCR, the DNA had to be extracted from the clones.

A single yeast colony was suspended in 100 µl 200 mM LiAC-SDS solution, vortexed and incubated for 5 minutes at 70 °C. 300 µM 96% ethanol was added for DNA precipitation, samples were vortexed and DNA was collected by centrifugation for 3 minutes at 15,000 g. The supernatant was removed, the pellet washed with 500 µM 70% ethanol and air dried. Then the DNA was dissolved in 100 µl ddH2O, the debris was removed by short centrifugation and 1 µl supernatant was used for the PCR.

The control PCR was performed with the taq polymerase according to the following composition *(Table 19*). The program starts with a DNA denaturation of 94 °C for 3 minutes, followed by 35 cycles of: 30 seconds 94 °C denaturation, 45 seconds 54 °C (depending on the Tm of the primer) primer annealing, 45 seconds 68 °C elongation. After the last cycle the 68 °C was hold for 10 minutes for a final extension step. To verify the integration of the cassette, the gene specific control primer (fwd) and a hygromycin cassette specific S1 control primer (rev) were used.

Table 19 Composition for the PCR to verify the knock-out and the right integration of the cassette

Volume	***Component***
0,1 µl	Taq polymerase (5u/µl)
2 µl	Buffer (10x)
2 µl	dNTP (5 mM)
0,4 µl	each primer (1:10 diluted)
14,1 µl	ddH2O
1 µl	DNA

3.2 Fly methods

3.2.1 Aging conditions

The *Drosophila melanogaster* wildtypes were reared in large plastic vials (ø 48 mm, height 104 mm, volume 170 ml) with foam plugs. After hatching of three days they were transferred to a new vial and allowed to mate for 24 hours. Then flies were collected and sorted into portions of 20 flies in small vials (ø 28 mm, height 66 mm, volume 30 ml) with males and females apart from each other. The average age of the flies was 2 days when sorted and this was also the start of the flavonoid treatment. The sorting occurred under CO_2 anesthesia for maximum 6 minutes.

During the aging experiments flies were kept at 25 °C with 70% humidity and with a light/dark cycle of 12 hours. The flies were transferred to fresh food every 2-3 days, accidentally escaped flies were censored.

Stocks were kept in small vials at 18°C on standard Bloomington media.

3.2.2 Food preparation

For all the experiments we aged the flies on 1x SYA media with 1% agar. This media contains only agar, baker's yeast and sugar, which were added to ddH2O one after the other, each of them brought to boil before adding the next one. After reaching a temperature of 60 °C propionic acid and nipagin were added and the 2-HC was added at a temperature below 45 °C to exclude inactivation through heat. The flavonoid was prepared as 1000x stocks in DMSO with a final concentration of 0.1% DMSO. The control group was DMSO in the same concentration.

After the addition of 2-HC, the food was poured in vials, filled with approximately 2-5 ml media, and dried at room temperature overnight. Storage was at 4°C for a maximum of 2 weeks.

3.2.3 Climbing assays to determine locomotor function

The locomotor function was determined with a rapid iterative negative geotaxis (RING) assay (Gargano et al. 2005). The aging of the flies for this assay was exactly like the agings previously mentioned, except the number of flies per vial was 10. For the assay the flies were transferred into long plastic vials (ø 25 mm, height 93 mm). After an acclimatization time of 20 minutes in the dark, flies were rapped to the bottom of the vial. Then the climbing to the top was recorded on video. Disturbing due to light sources was eliminated through red light conditions. The procedure was repeated four times, the first time was not used for evaluation. After 5 seconds a screen shot was taken and the climbed distance was calculated with the Fiji image software. A mean for all flies of the same concentration in different vials was determined.

4 Results

4.1 2-hydroxychalcone has conserved cytoprotective activities

4.1.1 2-HC treatment leads to increased viability in *Saccharomyces cerevisiae*

Based on the previously performed experiments leading up to this work *(Figure 3*), we further examined the effect of 2-hydroxychalcone (2-HC) on *Saccharomyces cerevisiae*, a unicellular eukaryote, which is successfully used to model – among others - the aging process (Botstein und Fink 2011). Galactose-containing synthetic media (SMG) was used because there the cytoprotective activity was shown to be higher than in glucose media (Hofer 2016). Compared to glucose media, where *S. cerevisiae* exclusively performs fermentation for metabolization of the glucose, on galactose media yeast undergoes fermentation as well as respiration (Fendt und Sauer 2010; Ostergaard et al. 2000).

We examined survival via propidium iodide (PI), which stains dead cells due to their perforated cell membrane, and confirmed the previously obtained results. 2-HC treatment (with 75µM) showed a protective effect on cells, leading to a significant reduction of PI-stained cells compared to the solvent (0.2% DMSO) control (*Figure 4*).

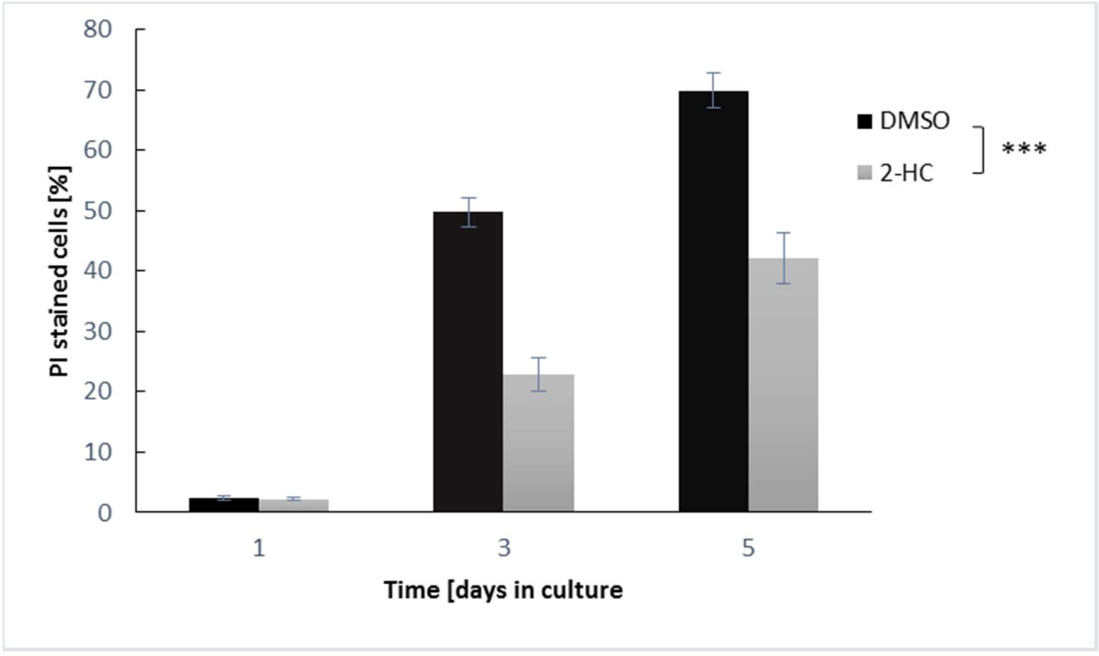

Figure 4. 2-HC leads to a reduction in PI staining in BY4741. BY4741 yeast cells were grown in SMG and treated with 75µM 2-HC at an OD_{600} of 0.2. After 1, 3 and 5 days' samples were PI stained and 30000 cells were analyzed with FACS. Data represent mean ± S.E.M., n=16, differences were significant with *** $p < 0{,}001$

4.1.2 2-HC treatment increases lifespan in *Drosophila melanogaster*

To investigate whether the beneficial effects of 2-HC are transferable to higher model organisms we treated wildtype w^{1118} fruit flies with 2-HC. Previous experiments had demonstrated that 2-HC can be added during the food preparation at a temperature under 40 °C. Flies with an average lifetime of 2 days were sorted into female and male and transferred onto the 2-HC supplemented food. Concentrations in the range of 1 µM up to 15 µM were tested and a concentration-dependent, lifespan-expanding effect could be observed in comparison to the DMSO control in both female and male flies (*Figure 5, Figure 17*). The concentration optimums differed between the sexes. In females, lower concentrations of 5 µM to 10 µM 2-HC showed the best effect, whereas in males the 15 µM concentration led to the longest mean survival (*Figure 5*). It should be noted that male flies generally tend to have a shorter lifespan than females (Tower und Arbeitman 2009).

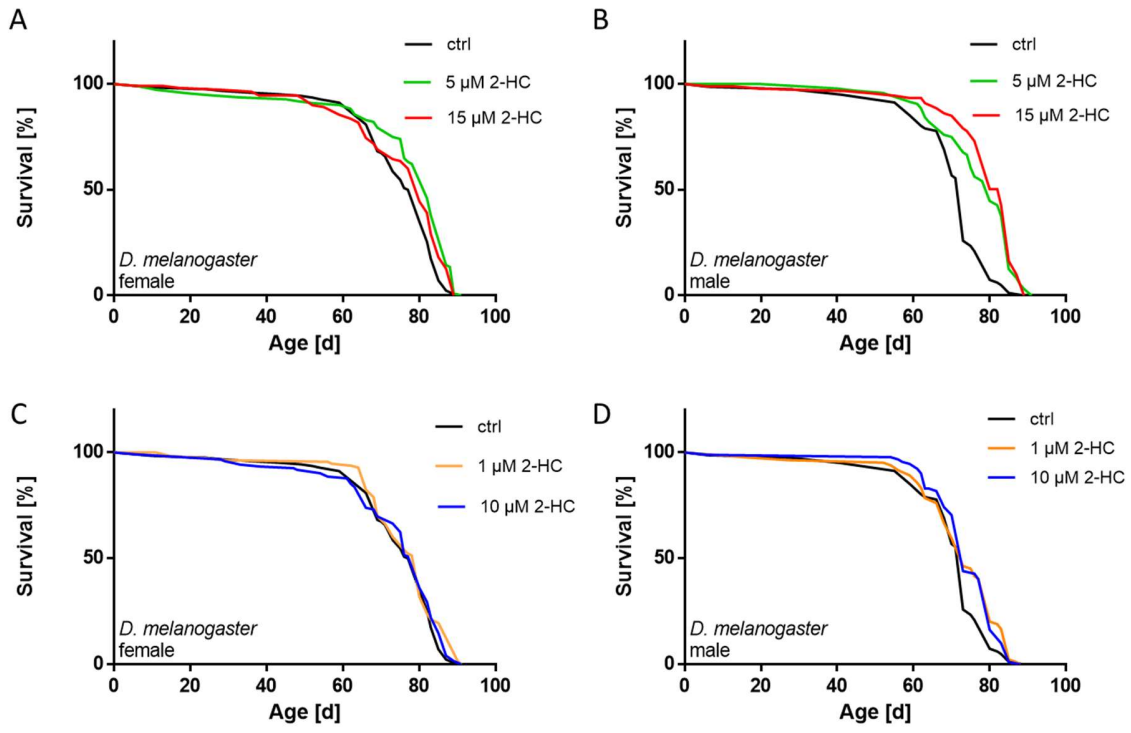

Figure 5 Lifespan extension in fruit flies upon 2-HC treatment. Flies were transferred to 2-HC supplemented SYA food or control food (SYA with DMSO) at an average age of 2 days and they were transferred on fresh food every 2-3 days. (A+C) Female w^{1118} wildtype flies. Mean lifespan 80 days with DMSO, 1µM, 10µM, 15µM and 82 days with 5µM (B+D) Male w^{1118} wildtype flies. Mean lifespan 73 days with DMSO, 1µM, 10µM, 80 days with 5µM and 83 days with 15µM.
Control is the same in A+C and B+D and experiments were conducted simultaneously. male n (flies) = 81-96, female n (flies) = 110-126.

We also performed an experiment where the flies were first pre-aged on untreated SYA and then shifted onto 2-HC or DMSO after two weeks. Within this other experiment we still observed the lifespan extending effects of 2-HC (*Figure 6).*

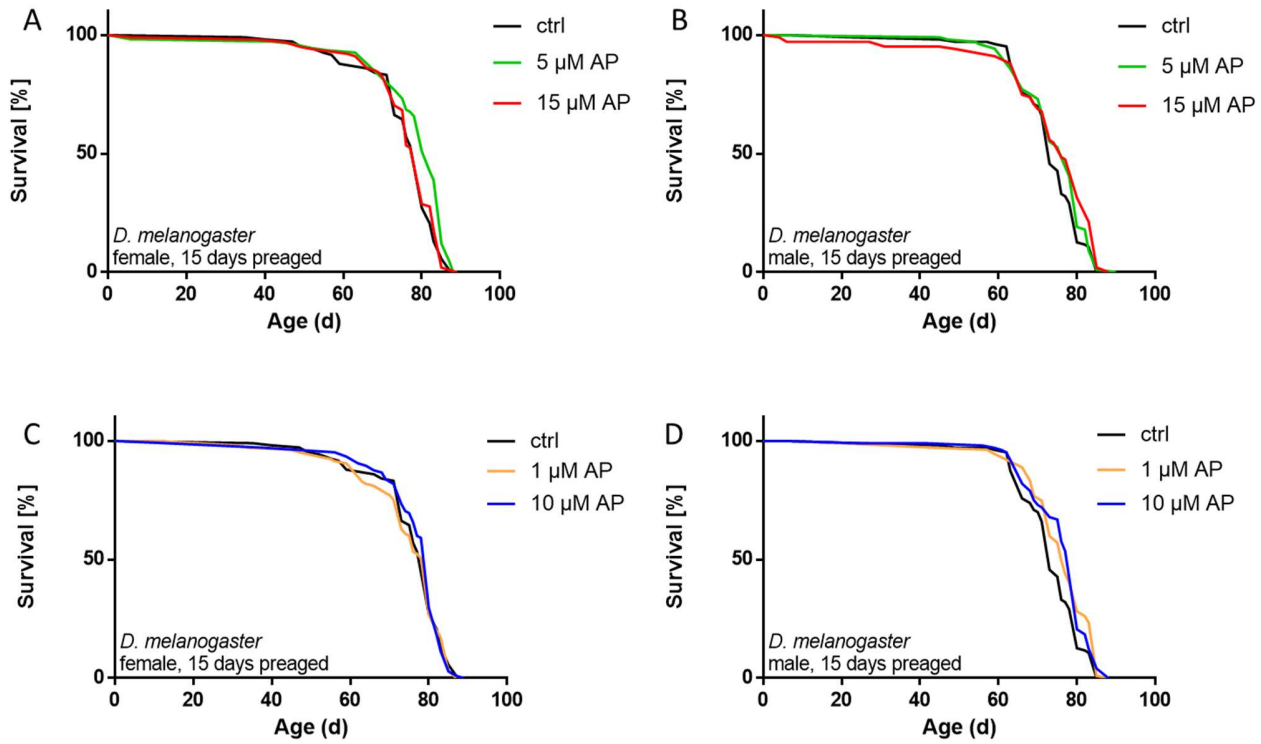

Figure 6 Lifespan extension in pre-aged fruit flies upon 2-HC treatment. Flies were aged on SYA food and were put on 2-HC supplemented food at an average age of 15 days. They were transferred on fresh food every 2-3 days. (A+C) Female w^{1118} wildtype flies. Mean lifespan 80 days with DMSO, 1µM, 10µM, 15µM and 82 days with 5µM (B+D) Male w^{1118} wildtype flies Mean lifespan 73 days with DMSO, 76 days with 1µM, 5µM, 15µM and 80 days with 10µM. Control is the same in A+C and B+D and experiments were conducted simultaneously. male n (flies) = 81-96, female n (flies) = 93-107

It must be noted that the lifespan-extending effect was not equally prominent within all experiments or with all concentrations in further experiments performed. In fact, in one experiment, 2-HC showed no beneficial effect in males (*Figure 17*). However, in this specific experiment, the 15 µM concentration (which showed the best results in the other aging experiments) wasn't tested.

4.2 Unselective macroautophagy does not underlie the lifespan-extending effect of 2-HC

Macroautophagy is a main cytoprotective cellular route. It removes accumulating waste as misfolded proteins or damaged organelles inside the cell, and at the same time provides the cell with energy and nutrients. Autophagy is decreasing with age and low rates of autophagy could be related to different age-associated diseases (Melendez et al. 2008; Hansen et al. 2008; Del Roso et al. 2003). We thus tested if 2-HC can modulate unselective macroautophagy in yeast.

4.2.1 The rescuing effect of 2-HC is maintained in macroautophagy-deficient strains

Different knock-out strains of autophagy-related genes (ATGs) were treated with 2-HC to monitor the effect of the flavonoid in autophagy-deficient yeast cells. These knock-out strains didn't show an inhibition of the beneficial effects of 2-HC. Compared to the control sample (treated with DMSO), a decrease in PI-stained cells was observed in all knock-out as well as in the wildtype cells treated with 2-HC (*Figure 7*). While an alteration in the survival rates was present in some deletion strains compared to the wildtype, the rescuing effect was indeed maintained in all strains.

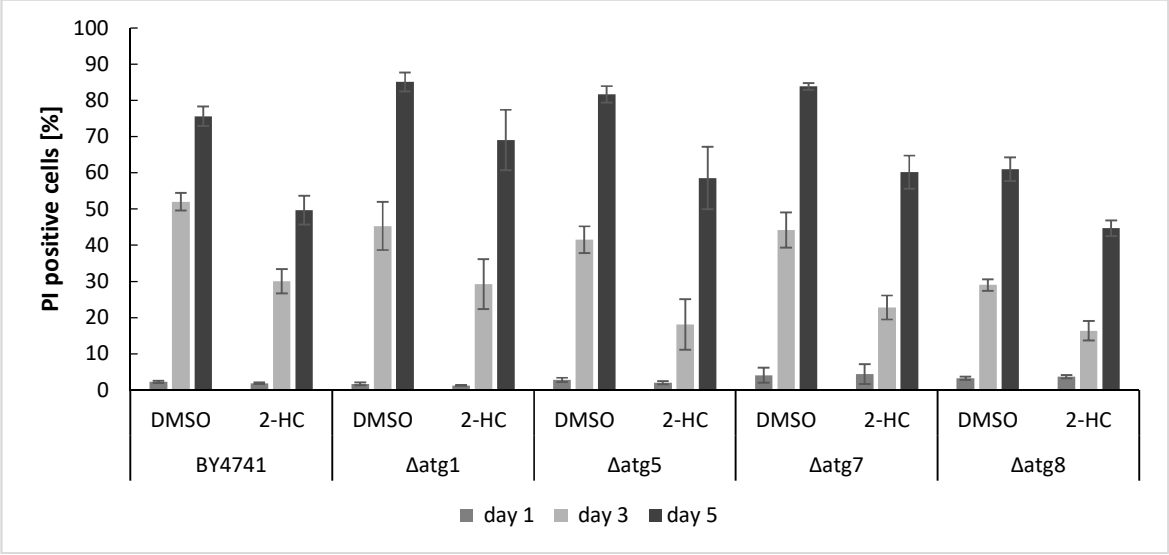

Figure 7 In autophagy-deficient strains the rescuing effect of 2-HC is not inhibited. BY4741 and its atg knock-out strains were grown in SMG and treated with 75µM 2-HC at an OD_{600} of 0.2. Analyzing of 30,000 cells by FACS after PI staining on day 1, 3 and 5 of treatment. Data represent mean ± S.E.M., n = 3-6

4.2.2 Macroautophagic activity is decreased upon 2-HC treatment

To verify those results and to detect macroautophagy, an alkaline phosphatase-based (ALP) assay was performed in yeast. Using a modified strain with a truncated Pho8 phosphatase, we explored if the rates of macroautophagy are altered upon treatment with 2-HC.

Interestingly, we could observe a decrease in ALP activity and therefore in the levels of macroautophagy in the treated cells compared to the DMSO-treated control. On day 1, the macroautophagic rate was reduced by 40% while on day 3 and 5 a minor decrease was observed *(Figure 8*).

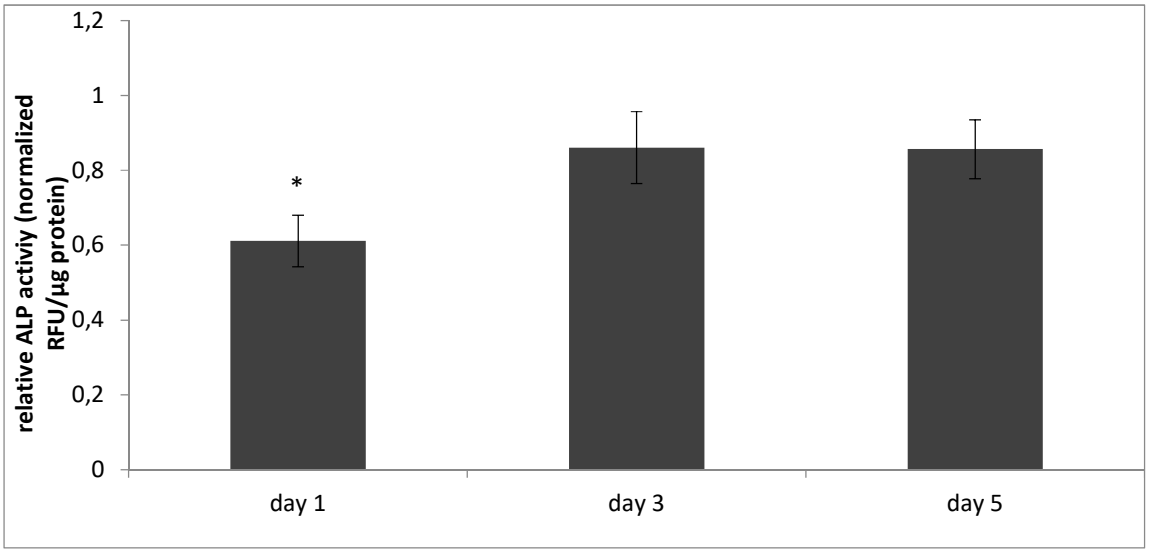

Figure 8 2-HC treatment leads to a decrease in ALP activity. BY4741 yeast cells were grown in SMG and treated with 75 µM 2-HC at an OD_{600} of 0.2. On days 1, 3 and 5 the ALP-activity was measured. Results are shown relative to the DMSO control. Data represent mean ± S.E.M, n = 3, * $p < 0.05$

Altogether, these experiments suggest that macroautophagy is not involved in the 2-HC-mediated cytoprotective effects.

4.3 Proteomic analysis shows an increase in the expression of multiple mitochondrial proteins

To inquire by which mechanistic routes 2-HC might modulate cytoprotection, we next used a systematic approach and analyzed the whole cell proteomes of 2-HC-treated yeast cells

and the respective DMSO control using SILAC (stable isotope labelling by amino acids in cell culture). SILAC can show differences in the protein expression through isotopic labeling and subsequent analyzation by mass spectrometry.

All proteins that were identified to show a log2 ratio DMSO/2-HC of more than ±1 in the two independently performed experiments were further analyzed with bioinformatics tools. Using this threshold at ±1, 445 proteins were found to be upregulated on day 1, no proteins were downregulated. To cluster the proteins, gene ontology was used to illustrate the annotation into different components. An Overrepresentation Test (PANTHER, GO Ontology database Released 2016-10-27) with the Annotation Data Set “GO cellular component complete” was performed and each protein was mapped into one or more categories. Based on the *S. cerevisiae* genome, it could be calculated, which was the expected value for a protein to be in a category (number of *S. cerevisiae* proteins that map to annotation data category / total number of *S. cerevisiae* input list * number of proteins to map). Dividing the number of proteins that were upregulated through the expected number delivered the fold enrichment of the proteins in this category (*Table 21*). Interestingly, many cellular components/proteins showing a threefold enrichment or more compared to the expected value ($p < 0.05$) were mitochondrial, including the ATP-synthase complex, the mitochondrial nucleoid, the mitochondrial respiratory chain, the oxidoreductase complex, the mitochondrial protein complex, inner and outer mitochondrial membrane and the mitochondrial envelope (*Figure 9, Table 20).*

. Similarly, taking a less stringent change, 13 out of 39 cellular components that showed an enrichment of more than twofold were associated with mitochondria, which makes one third of the upregulated components mitochondrial. However, the greatest upregulation was shown for the Arp2/3 protein complex, which plays a major role in the regulation of the actin cytoskeleton, and the chaperonin-containing T-complex, which mediates protein folding in the cytosol. Other upregulated components include the proton-transporting two-sector ATPase complex that catalyzes ATP hydrolysis or synthesis and a number of ribosomal and proteasomal proteins (*Table 21*).

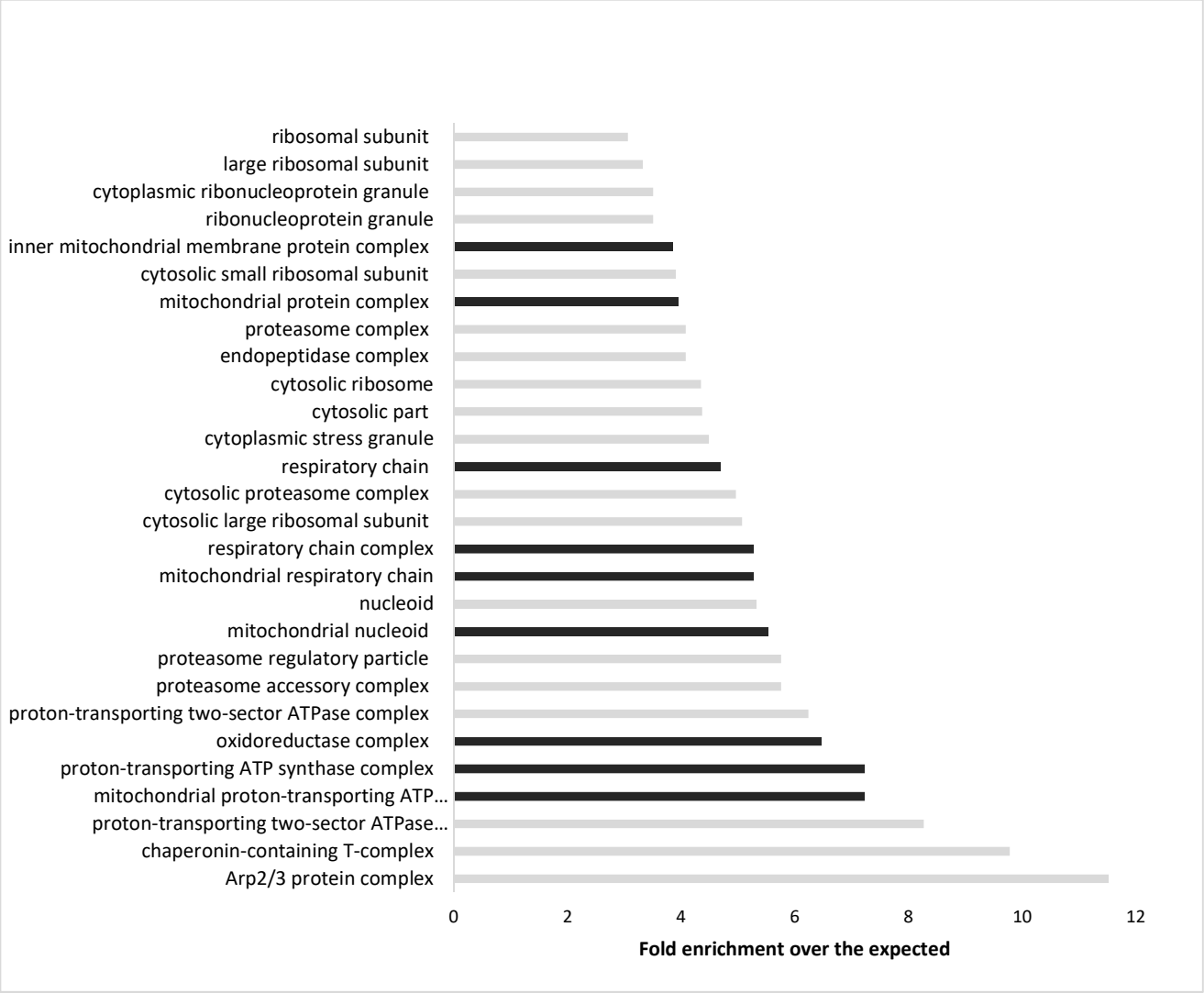

Figure 9 Cellular components of yeast cells upregulated upon 2-HC treatment. Proteins with a 2-log ratio DMSO/2-HC < -1 in both clones on day 1 were mapped into categories with the overrepresentation test from PANTHER (GO Ontology database Released 2016-10-27). Annotation Data Set was "GO cellular component complete" and only cellular components that show a fold enrichment of more than 3 compared to the expected value are shown if $p < 0.05$. In black all components associated with mitochondria.

On day 3, 356 proteins were found to be upregulated and 5 were downregulated. Mapping the corresponding proteins to components resulted in a majority of ribosomal origin. The cytosol and the cell wall also showed a fold increase of more than 2 in relation to the expected abundance. On day 3, mitochondrial components showed a minor upregulation of 1.71 fold compared to the expected value (*Table 22*).

4.4 Mitophagy is not involved in the cytoprotective effects of 2-HC

Given the prominent upregulation of mitochondrial proteins upon 2-HC treatment, we wanted to analyze, if more mitochondria were present in the treated samples. This we did with fluorescence microscopy, using the mitochondrial marker DsRed Su1-69, but no differences in mitochondrial mass could be observed with the used confocal microscope after 16 hours (*Figure 18*).

The SILAC results could also be explained due to renewed mitochondria, this is why we tested for a specific degradation of mitochondria through mitophagy. Damaged mitochondria lead to impaired fusion and can result in apoptosis through the release of pro-apoptotic proteins. Mitophagy can degrade damaged or old mitochondria and prevent apoptosis (Kim et al. 2007; Maiuri et al. 2007). We argued that general macroautophagy, which seems to be reduced in our scenario (see chapter 4.2), could be downregulated as a sign of cellular health due to mitochondrial renewal.

4.4.1 Selective macromitophagy is not increased upon 2-HC treatment

Different strains with knock-outs of genes that are involved in mitophagy were treated with 2-HC and PI-stained. Among the tested genes was *atg11*, which codes for a scaffold protein that is involved in all kinds of selective macroautophagy and that can bind to the gene product of *atg32*, a mitophagy-specific transmembrane protein (Mao et al. 2013; Okamoto et al. 2009). The other gene deletions regarded proteins that were described to modulate mitophagy in some part: the mitochondrial outer membrane protein Uth1 (Kissova et al. 2004), the mitochondrial protein phosphatase Aup1 (Tal et al. 2007), the mitogen-activated protein kinase Slt2 (Mao et al. 2011) and the component of the mitochondrial K+/H+ exchange system Mdm38 (Nowikovsky et al. 2007). These knock-out strains are not able to perform mitophagy or at least lead to a decrease in the macromitophagic rate (reviewed in Muller et al. 2015).

PI staining revealed that the cytoprotective effect of 2-HC is still present in all these knock-outs, suggesting that macromitophagy is not involved in the beneficial properties of 2-HC *(Figure 10).*

Of note, some of the knock-outs behave differently than the wildtype, leading to more (Δslt2) or less (Δuth1) cell death and also the rescue effect of 2-HC varies between the strains. In general, the variability of the 2-HC effect was rather extensive. Nevertheless, the rescuing effect was maintained in all strains and all experiments.

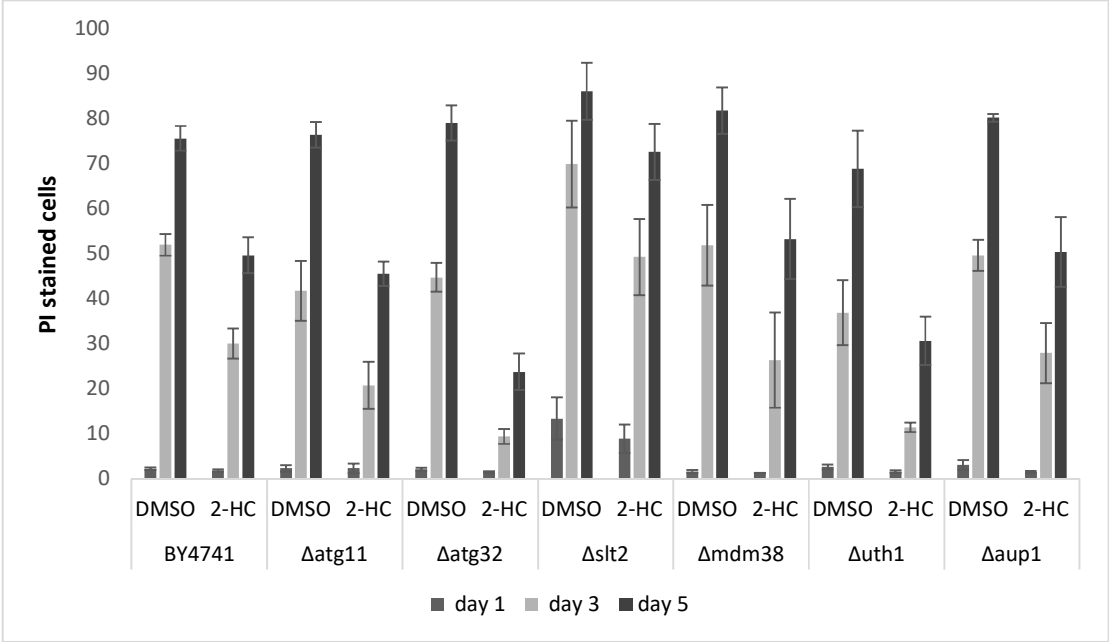

Figure 10 Mitophagy-deficient strains show a cytoprotective effect upon 2-HC treatment. BY4741 and the different knock-out strains which can't perform mitophagy were grown in SMG and treated with 75µM 2-HC at an OD_{600} of 0.2. Analyzing of 30,000 cells by FACS after PI staining on day 1, 3 and 5 of treatment. n = 3-6, Data represent mean ± S.E.M.

To further examine macromitophagy, an alkaline-phosphate based assay specifically for macromitophagy was performed (Campbell und Thorsness 1998). Compared to the previously described ALP assay for unselective macroautophagy, in this assay, the truncated *PHO8ΔN60* contained a mitochondrial localization sequence and is expressed from a plasmid (pCC4). The ALP activity was reduced at all measured time points upon 2-HC treatment as compared to the DMSO treated control *(Figure 11)*

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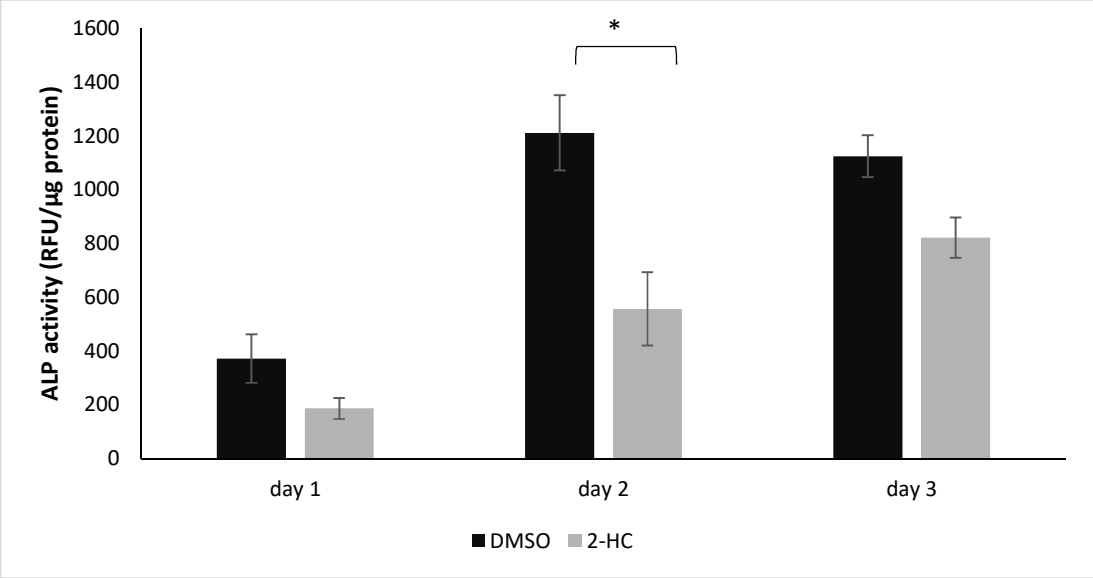

Figure 11 Macromitophagy is decreased upon 2-HC treatment on the different time points. BY4741 yeast cells with a pCC4 plasmid were grown in SMG and treated with 75 µM 2-HC at an OD_{600} of 0.2. On days 1-3 the ALP-activity was measured. Data represent mean ± S.E.M, n = 3-6, * $p < 0.05$

Altogether, macromitophagy doesn't seem to underlie the lifespan-extending effect of 2-HC. The rescuing effect is maintained in mitophagy-deficient strains and the macromitophagic rate even seems to be decreased upon 2-HC treatment.

4.4.2 Deletion strains of genes related to microautophagy still show the beneficial effect of 2-HC

Another autophagic process is microautopahgy, where in contrast to macroautophagy no autophagosome is built and the lysosome directly takes up the cytosolic components through an invagination of the membrane (Reggiori et al. 2012). The process of microautophagy is not very well characterized and the existence of micromitophagy in yeast is still not clear (Muller et al. 2015; Shutt und McBride 2013), however, in electron microscopy studies it could be shown that the vacuole membrane can sequester mitochondria (Kissova und Camougrand 2009).

In yeast, two complexes are thought to be involved in microautophagy, the VTC and the EGO complex (Uttenweiler et al. 2007; Uttenweiler und Mayer 2008). We therefore generated knock-outs of the members of these two complexes to analyze, if the rescuing

properties of 2-HC are abolished in those strains. Subsequently, PI staining of the cells at different time points was conducted and there could still be seen a rescue effect of 2-HC in all the knock-out strains (*Figure 12*). This indicates that the cytoprotective properties of 2-HC are not based on microautophagy, at least based on the single-knockout analysis.

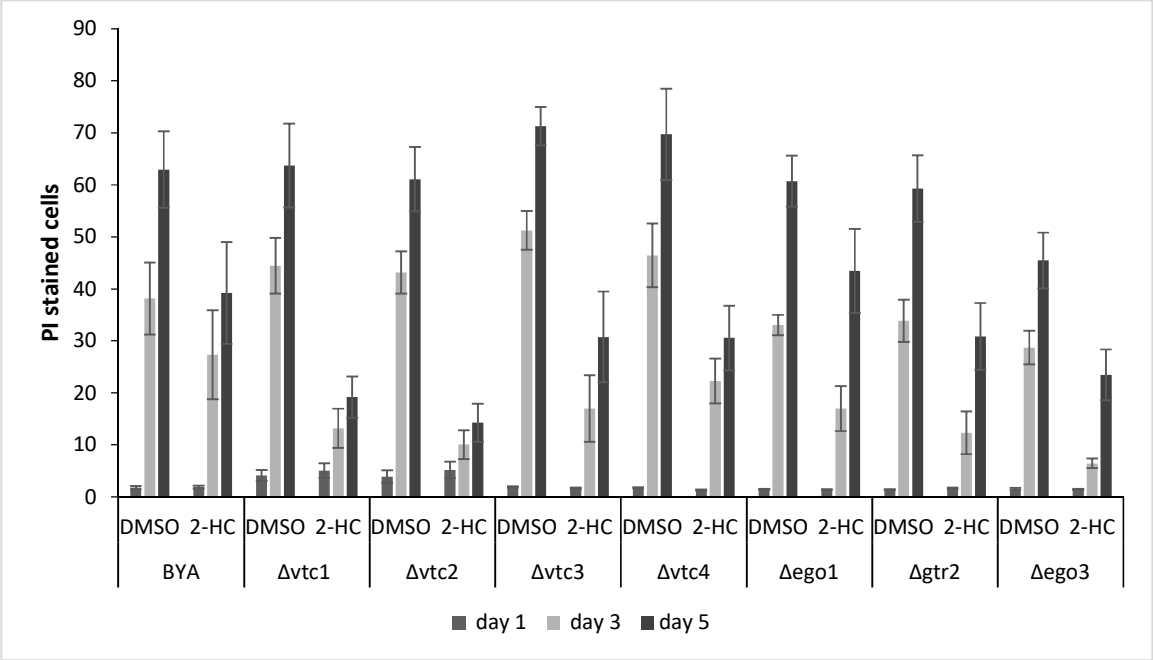

Figure 12 2-HC treatment shows cytoprotective effects in microautophagy-deficient strains. BY4741 and different strains with knock-outs of genes that are essential for microautophagy were grown in SMG and treated with 75µM 2-HC at an OD_{600} of 0.2. Analyzing of 30,000 cells by FACS after PI staining on day 1, 3 and 5 of treatment. n = 8. Data represent mean ± S.E.M.

4.5 2-HC-treated cells show a modulation of respiration

4.5.1 The respiratory rate is increased in 2-HC treated cells

As one of the main functions of mitochondria is respiration, we wanted to check whether 2-HC has an impact on the respiratory capacity. Therefore, we measured the oxygen consumption of 2-HC-treated cells at different time points during aging. To determine the oxygen consumption at the single-cell level, cell counts of the sample were measured using a CASY cell counter and PI staining was performed to exclude dead cells. Interestingly, the respiratory rate per living cell was increased significantly in 2-HC-treated cells compared to the DMSO control (Figure 13). The oxygen consumption between the

samples and different plates varied, but an increase compared to the DMSO control was observed in all experiments. On both, day 1 and day 2, an average increase of the respiratory rate of 3.5 fold was observed in the treated samples.

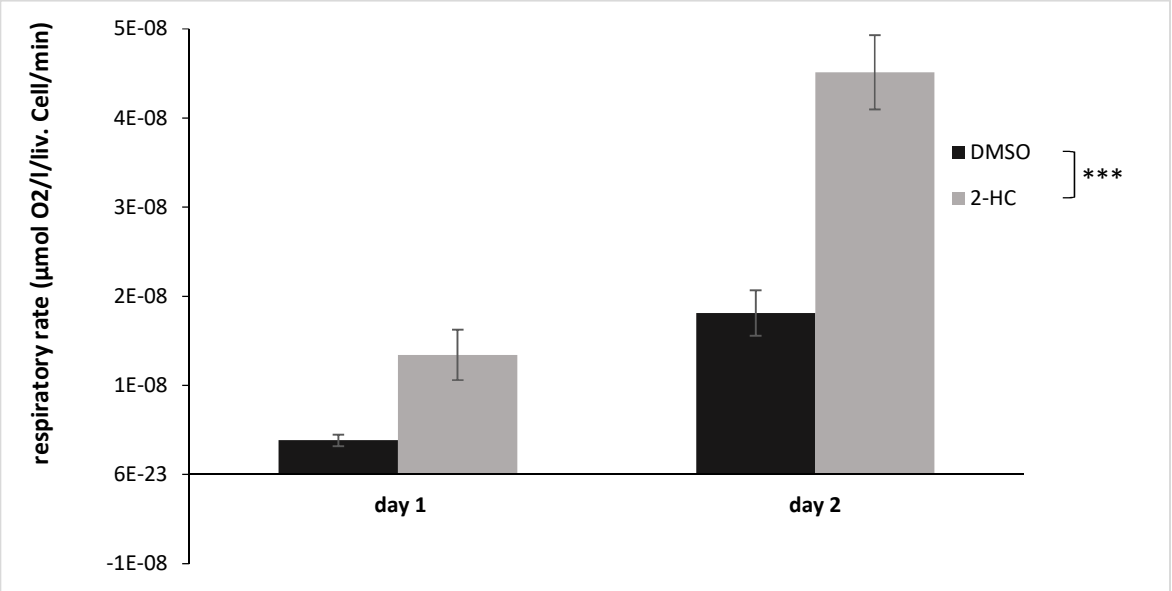

Figure 13 Respiratory rate is increased upon 2-HC treatment. BY4741 cells were grown in SMG and treated with 75µM 2-HC at an OD_{600} of 0.2. Oxygen consumption was measured in pooled samples with FirestingO2. Respiratory rate for a single living cells was determined by excluding dead cells with cell count measurement at CASY and PI staining at FACS. Data represent mean ± S.E.M., n = 9 Differences were significant with *** $p < 0.001$

4.5.2 Treatment with 2-HC preconditions cells for growth on respiratory media

To confirm this mitochondrial involvement through respiration, clonogenicity assays were performed on different media: 2-HC treated cells and the corresponding DMSO controls were plated simultaneously on glucose media (SMD) and glycerol media (SMGly). Glycerol is not fermentable and forces the cells to obtain energy through respiration. Before plating, the cells were grown in galactose-containing SMG media, where cells can undergo fermentation and respiration (Fendt und Sauer 2010; Ostergaard et al. 2000). Respiration is the preferred metabolization route on galactose, therefore mitochondrial mass is elevated and mitochondrial proteins are more accumulated compared to growth

in glucose media (Renvoise et al. 2014). However, a shift on respiration-only media provokes a respiration stress for the cells.

On day 1, more than 90% of the DMSO control cells were viable on SMD but only 29.7% on SMGly. In contrast, 2-HC treated cells showed more than 90% viability on both, glucose and glycerol (*Figure 14*). Thus, 2-HC seems to precondition cells for growth upon respiration stress. On the following days, the control cells – according to the growing age – succumbed also upon plating on glucose media. 2-HC treatment could improve survival on both SMD and SMGly plates (*Figure 14*).

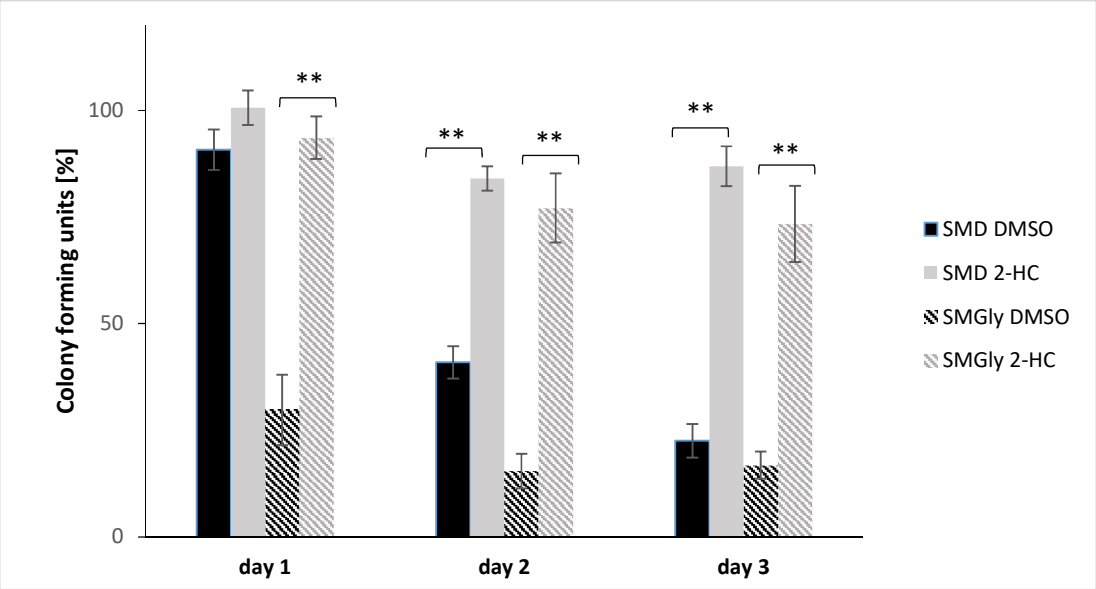

Figure 14 2-HC preconditions cells for growth on respiratory media. BY4741 yeast cells were grown in SMG and treated with 75 µM 2-HC at an OD_{600} of 0.2. After counting the cells with CASY 500 cells were plated on agar plates with glucose (SMD) and glycerol (SMGly). Living cells are determined as colony forming units. Data represent mean ± S.E.M., n = 3, ** $p < 0.01$

4.5.3 Altered expression of proteins of the respiratory chain

These data suggest an involvement of respiration in the cytoprotective effect of 2-HC. We thus more specifically analyzed the obtained SILAC results presented above (*chapter 4.3; Table 20*) for an alteration in the expression of proteins of the respiratory chain. Interestingly, out of the 23 genes of the respiratory chain found in the SILAC experiment, almost all of them were upregulated upon 2-HC treatment on both days (no threshold set).

Table 20 Modulation in the expression of genes of the respiratory chain upon the SILAC approach. The log2 ratio DMSO/2-HC was obtained from the SILAC experiment. Listed are all genes involved in the respiratory chain and ratios of two independent experiments are shown for day 1 and day 3.

Negative numbers show an upregulation in 2-HC treated samples. NaN = not a number; color code: Ratio DMSO/2-HC < -1; Ratio DMSO/2-HC between -1 and 0; Ratio DMSO/2-HC > 0

		Ratio DMSO/2-HC day 1		Ratio DMSO/2-HC day 3	
Complex I	NDI1	-3,418010	-3,469098	-3,028911	-3,157528
	NDE1	-1,693528	-2,662277	-0,401940	-2,035106
Complex II	SDH3	-4,999723	-4,884828	-4,693201	-3,267427
	SDH2	-1,522551	-1,107648	0,031254	-0,079264
	SDH4	NaN	NaN	NaN	-4,541971
	SDH1	-1,348721	-1,837635	0,197614	0,129085
Complex III	RIP1	-4,680308	-3,287853	-4,437745	-4,001524
	COR1	-1,625133	-1,956795	-0,405088	-0,415345
	QCR2	-1,630573	-2,329232	-1,157689	-3,005782
	QCR7	-1,845443	-2,206029	-0,390718	-0,406235
	QCR8	-4,325626	NaN	NaN	-3,842304
	QCR10	NaN	-2,829408	NaN	-1,966743
	CYT1	-1,333989	-2,195682	-0,261863	-0,448390
Complex IV	COX1	NaN	-4,091135	-3,148545	-3,390018
	COX2	-1,622066	-2,553850	-0,916644	-1,406101
	COX4	-3,658756	NaN	-2,975851	-3,120545
	COX5A	-3,654136	NaN	NaN	-3,889375
	COX6	-1,615641	-2,572008	0,553950	-0,966968
	COX7	-3,693220	-0,385111	NaN	-4,332818
	COX8	NaN	-3,374614	NaN	-4,540426
	COX9	-3,675858	-1,011181	-0,354630	-0,132357
	COX12	-1,493906	-0,501811	NaN	-0,047398
	COX13	-2,592223	-0,992231	NaN	-1,605069
	COX15	-1,702609	-1,957748	-0,467833	-0,949115

In order to validate these results, we checked the protein expression levels with a western blot using different antibodies against a set of the identified proteins. We chose early time points and harvested the cells after 20 and 30 hours.

Surprisingly, we couldn't see an upregulation in any of the mitochondrial proteins or proteins of the respiratory chain identified in the SILAC experiment. Only Cox2, a protein of the complex IV of the respiratory chain, was significantly upregulated in this targeted

analysis. Other proteins of the respiratory chain, like Nde1 and Ndi1 of complex I, Sdh1 and Sdh4 of complex II or Rip1 of complex III showed no differences in the expression levels compared to the DMSO control. Also other mitochondrial proteins like Por1 or Ssc1 did not show any alteration. As a control the ER proteins Kar2 and Sec61 were also tested, but no differences in expression levels were found (*Figure 15)*. Interestingly, OM45, a protein of the mitochondrial outer membrane was significantly upregulated after 30 hours but not after 20 hours (*Figure 15*). This shows a very tight time window with rapid changes in the expression depending on different time points. However, these data are very preliminary and more experiments need to be done since they represent only one experiment with 4 independent samples without loading controls.

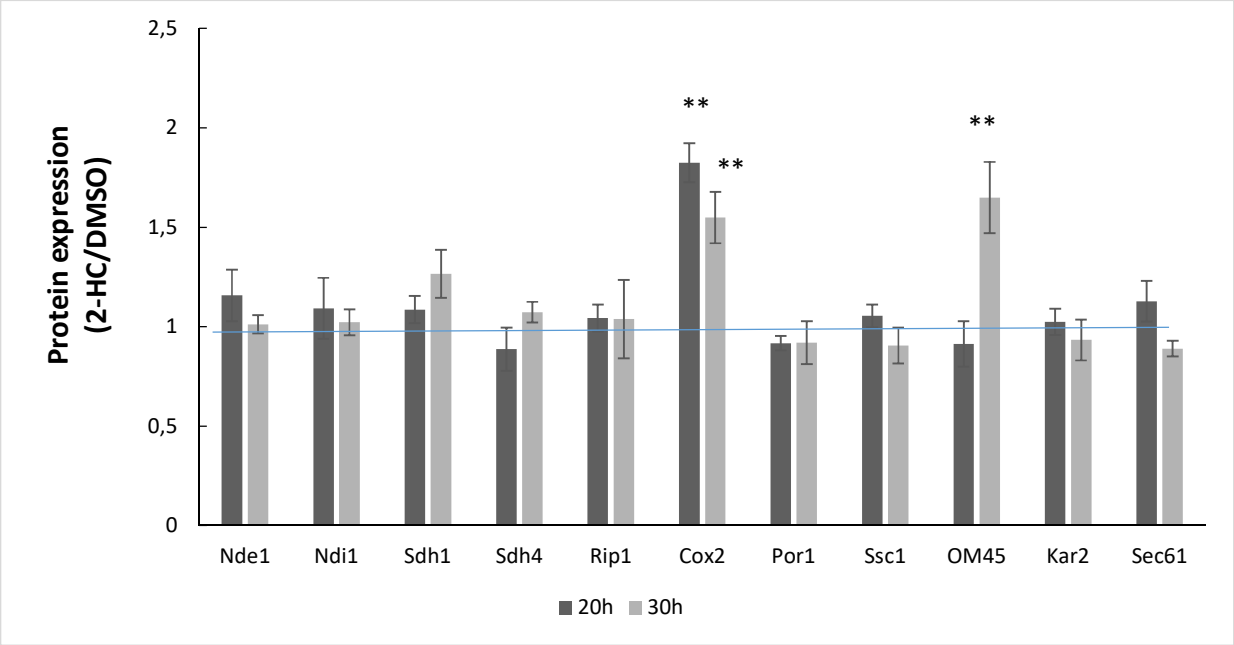

Figure 15 2-HC induced alteration of protein expression upon western blot analysis. BY4741 yeast cells were grown in SMG and treated with 75 µM 2-HC at an OD_{600} of 0.2. Cells were harvested after 20 and 30 hours of treatment. After chemical lysis equal amounts of samples were loaded on SDS gels and western blot analysis was performed. Proteins were detected with respective antibodies and quantified densitometric. Data represents mean ± S.E.M., n = 4, * $p < 0.05$, *** $p < 0.001$

4.6 Locomotor activity of flies improves upon treatment with 2-HC

Increased mitochondrial function leads to more ATP and could therefore result in increased locomotor activity in *Drosophila melanogaster*. We thus measured this with a RING assay using negative geotaxis (Gargano et al. 2005). During this experiment flies

were tapped onto the bottom of large vials and monitored while they instinctively tried to climb to the top.

The locomotor activity was assessed in male and female flies at 10, 20 and 30 days of age, measuring the climbed distance in five seconds. In females, we observed an increase of the climbing activity upon 2-HC treatment at all measured time points, although differences were more prominent at earlier time points. In males, the climbing activity was not altered at days 10 and 20, but on day 30 a significant increase in the climbing distance could be observed in 2-HC-treated flies (*Figure 16*). This experiment has to be repeated to confirm these results.

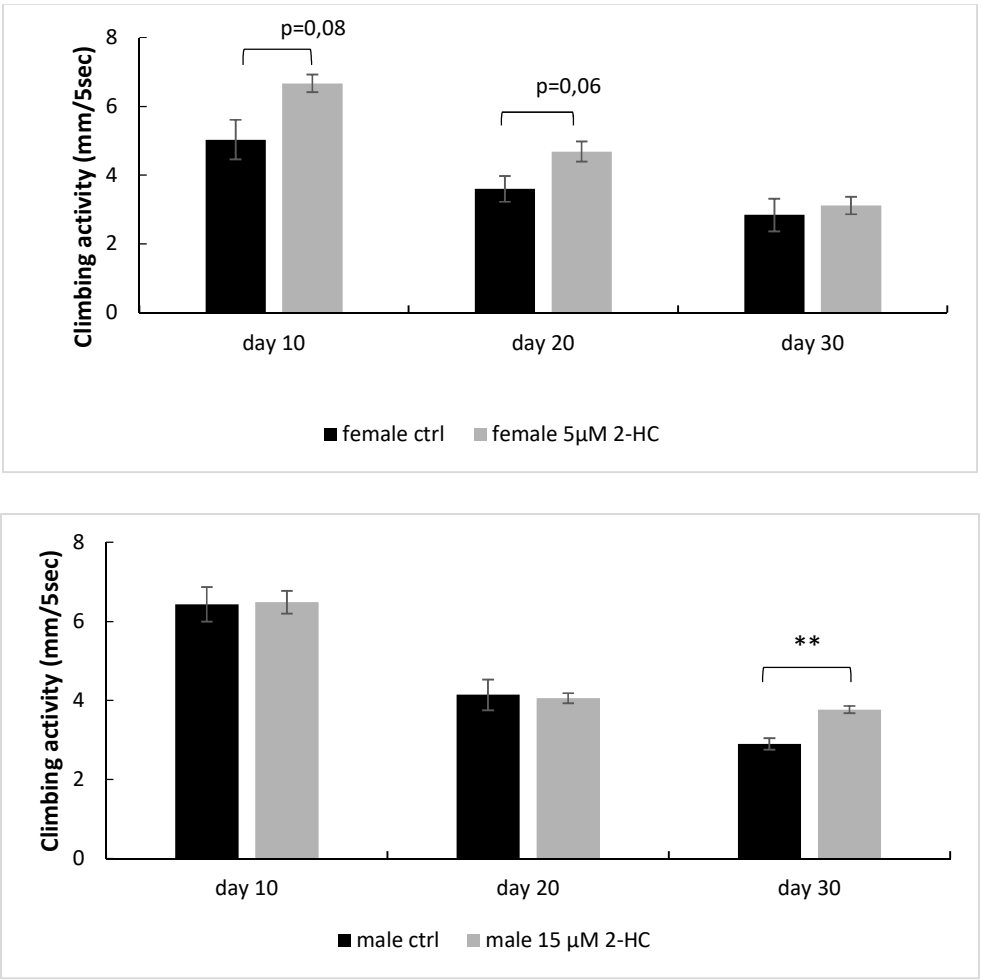

Figure 16 Increased climbing activity in fruit flies upon 2-HC treatment. w^{1118} flies were put onto 2-HC supplemented SYA food or control food at an average age of 2 days and were transferred on fresh food every 2-3 days. The climbing assay was performed at red light conditions with a ring assay. Flies were tapped to the ground 4 times and climbing to the top was monitored. After 5 seconds a picture was taken and the climbed distance of each fly was calculated using Fiji. Data represent mean ± S.E.M., n (flies) = 40. ** $p < 0.01$

5 Discussion

Aging is always accompanied with physical and psychological changes, leading to a loss of function and senescence. It is the greatest risk factor for developing diseases and this makes it to one of the greatest challenges in today's society, constantly increasing costs of the health systems (Christensen et al. 2009).

While human lifespan has been increasing constantly during the last years, our healthspan hasn't been increasing at the same speed. Aging research should therefore not lead to increased numbers of elderly people, but to a healthier population, living longer without severe disabilities (Longo et al. 2015).

Interestingly, caloric restriction is known to lead to a major lifespan extension in different organisms from yeast to human, and is accompanied with an increase in healthspan, protecting against various diseases and age-related loss of function (Colman et al. 2014). Also several anti-aging substances like spermidine, rapamycin or resveratrol have been found, which are able to extend lifespan in different organisms; some of them mimicking caloric restriction (Bjedov et al. 2010; Ehninger et al. 2014; Eisenberg et al. 2009). Still, the quest for further pharmacological interventions continues, especially of natural substances with such effects. Polyphenols are promising candidates for their use as anti-aging drugs due to their numerous beneficial properties and their occurrence in a normal human diet. Described properties of flavonoids range from anti-oxidative, anti-inflammatory, anti-microbial, anti-cancer, neuroprotective to cardioprotective functions (Li und Vederas 2009; Ly et al. 2015; Kennedy 2014). However, few studies have evaluated the role of specific flavonoids, since mostly whole plants or plant extracts were investigated, which contain a mixture of numerous flavonoids and other compounds. Furthermore, the specific mode of action and cellular targets of specific flavonoids are unknown.

Previous work of our group could already show the cytoprotective effects of a flavonoid, 2-hydroxychalcone (2-HC), in yeast and evaluate a concentration optimum. In this study we could confirm those results and show that this finding is transferable to the fruit fly *Drosophila melanogaster,* a higher model organism. This suggests an evolutionary conserved protective effect of 2-HC. In addition, we could show that in 2-HC treated cells mitochondrial function, or more specifically respiration, is increased. Indeed, for potential

applications as a drug in the future and to eliminate possible side effects, the revelation of the working mechanism of the beneficial cytoprotective effects of 2-HC is essential. Moreover, the uncovering of a mechanism that can extend lifespan in different organisms can lead to a better understanding of the aging process itself and its key pathways.

5.1 2-HC treatment has age-protective effects on *Drosophila melanogaster*

Testing different concentrations of 2-HC in fruit flies, we could show a better survival compared to the control. Male and female animals show different concentration optima: lower concentrations led to the best outcome in female while higher concentrations of 15 µM showed best results in male. In addition to these experiments, where flies were kept on 2-HC supplemented food during the entire aging, we also tried to pre-age flies before the start of the treatment. It is known that events early in life can delay the onset of aging but on the other hand, it is possible that the flavonoid only causes beneficial effects in aged flies and is even harmful for younger and fitter flies. The observed effects were the same when treatment started at an age of 15 days: 2-HC treatment led to an increase in mean lifespan in male and female groups, and the concentration optima occurred in the same range. In one of the aging experiments, no lifespan extension in males could be observed, however, in this experiment only 5 µM and 10 µM were used whereas 15 µM was determined as the best concentration for male. Thus, both, 2-HC treatment early in age and late in age seem to have beneficial effects on the lifespan of the fruit fly.
More aging experiments have to be performed with flies and are ongoing at the moment. Additionally, it will be very important to measure the food uptake in the treated and untreated flies to exclude a positive effect on survival through caloric restriction.

In another fly experiment, we compared the locomotor activity between 2-HC treated flies and control flies with a RING (rapid iterative negative geotaxis) assay. The locomotor activity can be seen as a parameter for healthspan, assessing mobility but also cognitive function (Iliadi und Boulianne 2010). The negative geotaxis, when flies instinctively start climbing to the top after being tapped onto the bottom, decreases with ongoing age as

well as with neurodegeneration (Iliadi und Boulianne 2010; Gargano et al. 2005). We could observe a better climbing activity in treated flies compared to the control. The climbing activity could only be determined until day 30, since after that locomotor activity decreases rapidly. In females on day 10 and 20, an increase could be observed, whereas in males a significant increase was seen at day 30. This difference could be explained due to the fact that female flies are bigger and fatter than male flies, leading to decreased motility which helps noticing differences earlier. Generally, the climbing ability seems to improve with 2-HC treatment, which could be traced back to more ATP and better mitochondrial function.

5.2 Mitochondria are involved in 2-HC-induced cytoprotection

Isotope labeling with SILAC and subsequent mass spectrometry has emerged as an easy and powerful method. The in-vivo labeling has created the possibility to measure alterations of protein levels between different conditions. This allowed us to search for differences in the gene expression of 2-HC treated yeast cells compared to the DMSO control. Annotation of the upregulated genes from day 1 into cellular components showed an upregulation of components associated with mitochondria. One third of the cellular components that showed an enrichment of more than twofold were mitochondrial, thus suggesting an involvement of mitochondria in the beneficial effects of 2-HC. Mitochondria play a crucial role in a lot of cellular processes: first of all, generation of energy, but also regulation of cellular metabolism, signaling pathways and programmed cell death (Muller et al. 2015). Additionally, in animal aging, a decline in mitochondrial mass as well as function can be observed, suggesting that mitochondria play a role in healthy aging (Amigo et al. 2016; Preston et al. 2008; Ferguson et al. 2005; Navarro und Boveris 2007). However, the component that showed the highest enrichment was the Arp2/3 complex, which is essential in the regulation of the actin cytoskeleton. Still, this result could be connected with mitochondria since the movement of mitochondria in yeast seems to be driven through actin polymerization which requires the Arp2/3 complex (Boldogh et al. 2001).

These results suggest that either the total number of mitochondria is increased or the mitochondrial function itself is more efficient. In aging both, the mitochondrial mass and

the function are decreased (Navarro und Boveris 2007; Muller-Hocker et al. 1997; Bratic und Trifunovic 2010). A study in rats showed that a variety of mitochondrial proteins in the heart are downregulated with age, the majority of them (39 of 94 proteins) are involved in oxidative phosphorylation, most of the others play a role in functional processes like substrate metabolism or tricarboxylic acid cycle (Preston et al. 2008). Our SILAC results show an increase in different mitochondrial components, but by far the highest enrichment was found in the respiratory chain and the ATP synthase.

The remarkable upregulation of mitochondrial compounds led to further experiments in yeast to explore the possibility that mitochondrial mass might be enhanced. Fluorescence microscopic analysis of a strain with a dsRED mitochondria marker didn't show a major difference in the dsRED signal, and therefore in the mitochondrial mass, between the different conditions. However, changes might be only detectable with more sophisticated microscopic tools than those employed in this work, where a proper quantification of the signal wasn't possible. .

The SILAC results might also be explained due to renewed mitochondria. Although macroautophagy didn't seem to be involved in the cytoprotection of 2-HC, this mitochondrial upregulation made us to test for a specific degradation of mitochondria through macromitophagy. Macromitophagy has been shown to define yeast longevity, degrading dysfunctional or excessive mitochondria which could lead to a younger and more efficient population of mitochondria (Richard et al. 2013).

Our data suggests that macromitophagy is not involved, since deletion strains of macromitophagy-related genes still showed the rescuing effect of 2-HC and the mitophagy rate was not increased. The macromitophagy rate, evaluated with an ALP assay, even seemed to be significantly decreased upon 2-HC treatment. However, these data have to be regarded with caution. Especially at later days, the protein assay to determine the total concentration after cell disruption showed highly increased protein levels in the 2-HC treated samples compared to the DMSO control, which resulted in the use of smaller sample volumes for the ALP assay. It seems like the cell disruption of DMSO-treated cells was less effective. This use of up to five times more sample volume of the control sample could lead to alterations of the results, leading to higher activity in the control samples.

Nevertheless, together with the results of the deletion strains, our data suggests that increased macromitophagy is not the case in 2-HC treated cells. In fact, it rather seems that the effect is decreased macromitophagy. A decrease could be explained if mitochondria are generally in a better state upon a 2-HC treatment, resulting in less dysfunctional mitochondria which need to be degraded.

There is still another possibility for degradation/renewal of mitochondria, namely mitophagy based on a microautophagic process. The existence of this so-called micromitophagy in yeast is still not sure and the process hasn't been well characterized, but electron microscopy studies suggest that mitochondria can be sequestered directly by the vacuole (Kissova und Camougrand 2009). Generated knock-outs of genes of the two complexes that are thought to be involved in micromitophagy didn't inhibit the cytoprotective effects of 2-HC. In micromitophagy, mitochondria would have to be transported to the vacuole and as previously mentioned, the Arp2/3 complex is involved in the movement of mitochondria, which is (according to the SILAC experiment) highly upregulated upon 2-HC treatment. However, no indication of the involvement of microauto(mito)phagy has been found. Still, it can't be excluded that this is the case, since very little is known about this process or possible functional overlaps that may need a multiple deletion strategy.

The production of ATP through aerobic respiration is one of the most prominent roles of mitochondria, as reflected by the high number of proteins for this purpose (Bratic und Trifunovic 2010). Measuring the respiratory rate per living cell we could observe significantly higher rates in 2-HC treated samples compared to the DMSO control. This goes along with the results of the SILAC experiment, which show that on day 1 all detected proteins involved in the respiratory chain complexes have been upregulated. Additionally, we performed a clonogenicity assay, plating the cells on glucose and glycerol media. The non-fermentable glycerol media forces cells to get energy through aerobic respiration, whereby this leads to increased respiration stress. While the DMSO treated cells could hardly manage this stress on day 1 with only 30% of cells surviving, in 2-HC treated cells more than 90% could form colonies on the glycerol media. This suggests that the cells

are preconditioned for growth upon respiratory stress, which is also in line with the higher respiratory rate.

However, in this clonogenicity assay, during later days the rescuing properties of 2-HC can also be observed on glucose media, where yeast cells can undergo their preferred way of energy generation, fermentation. This rescuing effect in cells that don't respire indicates that 2-HC doesn't work exclusively through a mitochondrial or respiration-dependent manner. It has to be noted, that 2-HC treatment also leads to an increased viability in yeast cells when cultured in glucose media, where cells don't use aerobic respiration for growth (Hofer 2016). However, upon PI staining, the protecting effect in galactose media is more prominent, leading to a greater reduction of PI stained cells compared to glucose media. This difference could rely on the described alteration in respiration.

At day 3, no upregulation of mitochondrial components could be observed according to the SILAC experiment. This could be due to a few different reasons. First of all, 2-HC was added only once to the media, when the cells were in stationary phase. If cells get an initial boost in respiration or in mitochondrial renewal at the time of addition of the flavonoid this could lead to changes in the cells that result in better survival. Furthermore, the experiment was conducted in galactose media, on which yeast can respire but also undergo fermentation. When all the galactose in the media is consumed, which is the case on day 3, cells have to undergo aerobic respiration for energy generation. Thus, in the control samples mitochondrial genes will be expressed to a higher level and therefore it could be that no further upregulation with 2-HC treatment is seen. In accordance with this, it seems that at day 3, the respiratory rate in treated samples is not increased to such a high rate as on day 1 and 2, however, not enough independent experiments have been conducted to confirm this (data not shown).

Surprisingly, in a western blot analysis with antibodies against different mitochondrial proteins we could only show a significant increase in the complex IV protein Cox2. Other mitochondrial proteins, including other proteins of the respiratory chain, didn't seem to be upregulated upon 2-HC treatment after 20 and 30 hours. Using specific antibodies that directly bind the protein is a quite accurate method for quantification, however, only a

small selection of proteins of the different complexes were tested. Equal amounts of 10 µg protein were loaded on the gel, however, it is essential to confirm this. The most common housekeeping gene Gapdh can't be used due to an upregulation upon 2-HC treatment, therefore another housekeeping gene or a protein assay that stains total protein levels have to be used. Those results were obtained in one single experiment with four independent samples, therefore repetitions of the experiment have to be undertaken to verify if all respiratory proteins are upregulated like the SILAC experiment suggests or if the upregulation is specifically for complex IV. Of all the respiratory chain complexes, complex IV seems to play the most important role in aging, showing a decrease in enzymatic activity with age (Muller-Hocker et al. 1997; Navarro und Boveris 2007; Ferguson et al. 2005). To obtain convincing data, more proteins of each complex have to be analyzed.

Respiration is highly increased upon 2-HC treatment and treated cells can manage respiration stress better. What can't be said for sure is, if this enhanced respiration is due to an improvement of the respiratory function or if it is only a consequence due to a higher number of mitochondria.

5.3 Conclusion and Outlook

In this work we could demonstrate that 2-HC shows great potential for further use as an anti-aging drug. The cytoprotective properties observed in *S. cerevisiae* could be transferred to the higher model organism *D. melanogaster*, indicating evolutionary conservation. Mitochondrial involvement is strongly suggested due to an upregulation of various proteins and components of mitochondria and due to an increased respiration compared to the control. This data indicates improved mitochondrial function upon 2-HC treatment, which goes along with findings that mitochondrial function decreases with age in various organisms. While macroautophagy, unselective as well as selective for mitochondria, could be excluded to be the beneficial mechanism, it is not clear whether the process of micromitophagy could play a role. Too little is known about this process to investigate a modulation by 2-HC.

Based on our data, increased respiration, which leads to more energy through higher ATP generation, is thought to be involved in the working mechanism of 2-HC.
Nevertheless it has to be said that this is probably not the only mode of action since it seems that in yeast there is also a cytoprotective effect when cells grow on media where respiration is repressed. It will be a major task to explore whether this upregulation of respiration is the cause or rather a correlative effect of the lifespan extending action of 2-HC treatment. It has yet to be shown if the lifespan extension would be abolished after blocking mitochondrial targets.

2-HC and flavonoids in general seem to be natural substances with a high potential for therapeutic use. We could show that 2-HC acts in a specific manner, directly influencing the function of cells, organelles and molecular pathways. It can be expected that flavonoids modulate different systems and more than one cellular target will occur. Therefore more experiments have to be conducted to unravel how 2-HC influences mitochondria and respiration and which other pathways are modulated. The SILAC approach disclosed more possible components that could be additionally involved in the mechanism of 2-HC, for example the SILAC experiments suggest an involvement of the proteasomes, and it seems like the cellular membrane is altered upon 2-HC treatment (better membrane disruption). It is crucial to understand every possible interference between 2-HC and the cell for the potential use in humans and for a better understanding of the aging process itself.

6 Supplemental Data

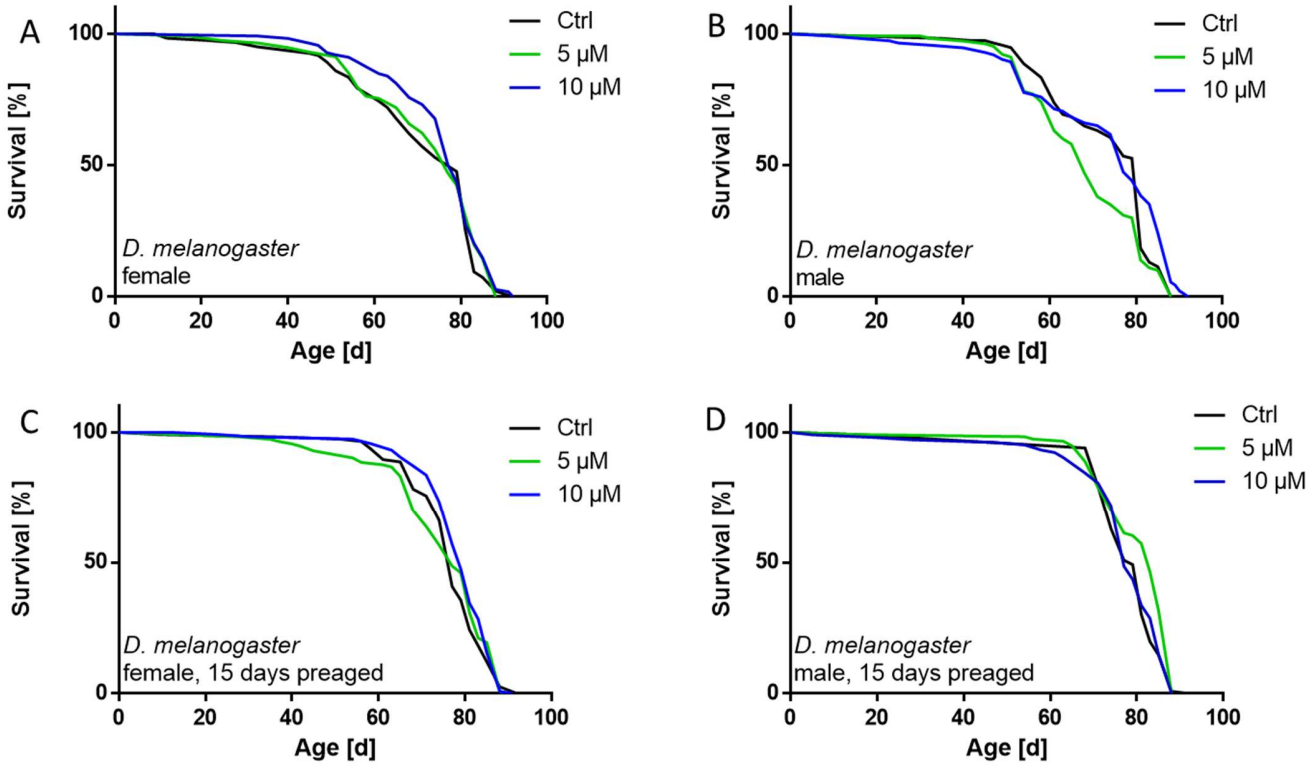

Figure 17 Lifespan extension in fruit flies upon 2-HC treatment. (A + B) Flies were transferred to 2-HC supplemented SYA food or control food at an average age of 2 days and they were transferred on fresh food every 2-3 days. W^{1118} wildtype female flies: mean lifespan 77 days (DMSO, 5 µM) and 79 days (10 µM). W^{1118} wildtype male flies: mean lifespan 81 days (DMSO), 77 days (5 µM), 68 days (10µM). (C+D) Flies were aged on SYA food and were put on 2-HC supplemented food at an average age of 15 days. They were transferred on fresh food every 2-3 days. Preaged w^{1118} wildtype female flies: mean lifespan 77 days (DMSO, 5 µM) and 79 days (10 µM). Preaged w^{1118} wildtype male flies: mean lifespan 79 days (DMSO), 83 days (5 µM), 79 days (10µM). n (flies) = 97 - 116

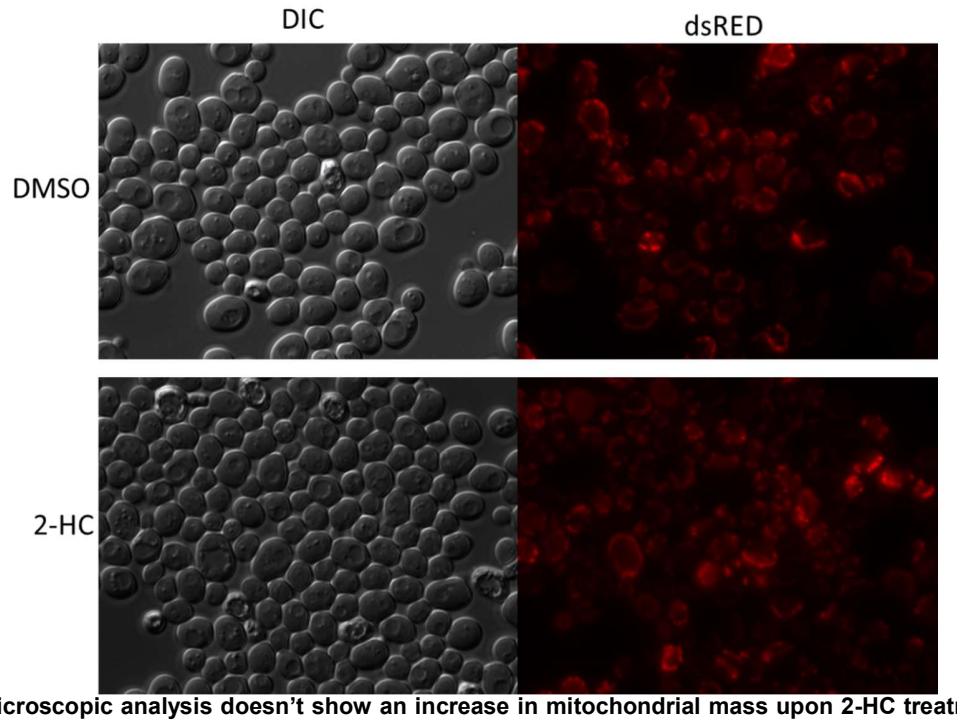

Figure 18 Microscopic analysis doesn't show an increase in mitochondrial mass upon 2-HC treatment. Cells were grown in SMG and treated with DMSO/75 µM 2-HC at an OD_{600} of 0.2. After 16 hours images were taken with dsRed filters, representative images are represented.

Table 21 Cellular components of yeast cells upregulated upon 2-HC treatment. Proteins with a 2log ratio DMSO/2-HC > -1 in both clones on day 1 were mapped into categories with the overrepresentation test from PANTHER (GO Ontology database Released 2016-10-27). Annotation Data Set was "GO cellular component complete" and only cellular components that show a fold enrichment of more than 2 compared to the expected value are shown if $p < 0.05$. (1) number of proteins in reference list S. cerevisiae (6728 proteins) that map to category (2) number of upregulated proteins in SILAC (input list, 438 genes) that map to category (3) number of proteins of input list expected to be in this category based on reference list (4) fold enrichment of proteins observed in the input list over expected (5) p-value, determined by binominal statistic

Annotation data category	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
Arp2/3 protein complex (GO:0005885)	8	6	0,52	11,52	1,17E-02
chaperonin-containing T-complex (GO:0005832)	11	7	0,72	9,78	6,71E-03
proton-transporting two-sector ATPase complex, catalytic domain	13	7	0,85	8,27	1,93E-02
mitochondrial proton-transporting ATP synthase complex	17	8	1,11	7,23	1,36E-02
proton-transporting ATP synthase complex (GO:0045259)	17	8	1,11	7,23	1,36E-02
oxidoreductase complex (GO:1990204)	38	16	2,47	6,47	5,15E-06
proton-transporting two-sector ATPase complex (GO:0016469)	32	13	2,08	6,24	1,95E-04
proteasome accessory complex (GO:0022624)	24	9	1,56	5,76	2,43E-02
proteasome regulatory particle (GO:0005838)	24	9	1,56	5,76	2,43E-02
mitochondrial nucleoid (GO:0042645)	25	9	1,63	5,53	3,32E-02
nucleoid (GO:0009295)	26	9	1,69	5,32	4,46E-02
mitochondrial respiratory chain (GO:0005746)	32	11	2,08	5,28	7,54E-03
respiratory chain complex (GO:0098803)	32	11	2,08	5,28	7,54E-03
cytosolic large ribosomal subunit (GO:0022625)	94	31	6,12	5,07	2,70E-10
cytosolic proteasome complex (GO:0031597)	31	10	2,02	4,96	3,18E-02
respiratory chain (GO:0070469)	36	11	2,34	4,69	2,19E-02
cytoplasmic stress granule (GO:0010494)	89	26	5,79	4,49	2,82E-07
cytosolic part (GO:0044445)	246	70	16,01	4,37	4,78E-22
cytosolic ribosome (GO:0022626)	173	49	11,26	4,35	1,16E-14
endopeptidase complex (GO:1905369)	49	13	3,19	4,08	1,85E-02
proteasome complex (GO:0000502)	49	13	3,19	4,08	1,85E-02
mitochondrial protein complex (GO:0098798)	109	28	7,1	3,95	9,97E-07
cytosolic small ribosomal subunit (GO:0022627)	63	16	4,1	3,9	3,87E-03
inner mitochondrial membrane protein complex (GO:0098800)	84	21	5,47	3,84	1,77E-04
ribonucleoprotein granule (GO:0035770)	127	29	8,27	3,51	6,66E-06
cytoplasmic ribonucleoprotein granule (GO:0036464)	127	29	8,27	3,51	6,66E-06
large ribosomal subunit (GO:0015934)	143	31	9,31	3,33	6,64E-06
ribosomal subunit (GO:0044391)	241	48	15,69	3,06	8,67E-09
organelle outer membrane (GO:0031968)	112	21	7,29	2,88	1,43E-02
mitochondrial outer membrane (GO:0005741)	107	20	6,97	2,87	2,33E-02
outer membrane (GO:0019867)	113	21	7,36	2,85	1,62E-02

ribosome (GO:0005840)	349	61	22,72	2,68	2,60E-09
membrane protein complex (GO:0098796)	247	43	16,08	2,67	5,73E-06
mitochondrial membrane part (GO:0044455)	179	31	11,65	2,66	8,31E-04
cytosol (GO:0005829)	832	129	54,16	2,38	1,31E-18
ribonucleoprotein complex (GO:1990904)	725	108	47,2	2,29	1,21E-13
intracellular ribonucleoprotein complex (GO:0030529)	725	108	47,2	2,29	1,21E-13
mitochondrial membrane (GO:0031966)	395	54	25,71	2,1	1,96E-04
mitochondrial envelope (GO:0005740)	434	59	28,25	2,09	6,23E-05

Table 22 Cellular components of yeast cells upregulated upon 2-HC treatment. Proteins with a 2log ratio DMSO/2-HC > -1 in both clones on day 3 were mapped into categories with the overrepresentation test from PANTHER (GO Ontology database Released 2016-10-27). Annotation Data Set was "GO cellular component complete" and only cellular components that show a fold enrichment of more than 2 compared to the expected value are shown if $p < 0.05$. (1) number of proteins in reference list S. cerevisiae (6728 genes) that map to category (2) number of upregulated proteins in SILAC (input list, 356 genes) that map to category (3) number of proteins of input list expected to be in this category based on reference list (4) fold enrichment of proteins observed in the input list over expected (5) p-value, determined by binominal statistic

Annotation data category	**(1)**	**(2)**	**(3)**	**(4)**	**(5)**
cytosolic large ribosomal subunit (GO:0022625)	94	32	4,97	6,43	1,47E-13
cytosolic ribosome (GO:0022626)	173	47	9,15	5,13	6,60E-17
cytosolic part (GO:0044445)	246	63	13,02	4,84	3,59E-22
large ribosomal subunit (GO:0015934)	143	34	7,57	4,49	4,26E-10
cytoplasmic stress granule (GO:0010494)	89	20	4,71	4,25	6,74E-05
fungal-type cell wall (GO:0009277)	107	21	5,66	3,71	2,90E-04
ribosomal subunit (GO:0044391)	241	47	12,75	3,69	1,67E-11
external encapsulating structure (GO:0030312)	113	21	5,98	3,51	6,85E-04
cell wall (GO:0005618)	113	21	5,98	3,51	6,85E-04
ribonucleoprotein granule (GO:0035770)	127	23	6,72	3,42	3,24E-04
cytoplasmic ribonucleoprotein granule (GO:0036464)	127	23	6,72	3,42	3,24E-04
ribosome (GO:0005840)	349	62	18,47	3,36	5,29E-14
extracellular region (GO:0005576)	113	20	5,98	3,34	2,55E-03
cytosol (GO:0005829)	832	116	44,02	2,63	1,45E-20
preribosome (GO:0030684)	185	24	9,79	2,45	4,48E-02
ribonucleoprotein complex (GO:1990904)	725	93	38,36	2,42	3,06E-13
intracellular ribonucleoprotein complex (GO:0030529)	725	93	38,36	2,42	3,06E-13
plasma membrane (GO:0005886)	523	57	27,67	2,06	1,25E-04

7 Abbreviations

2-HC	2-hydroxychalcone
AIF	Apoptosis-inducing factor
ALP	Alkaline phosphatase
Akt/PKB	RAC-alpha serine/threonine-protein kinase
AMP	Adenosine monophosphate
Atg	Autophagy related gene
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CFU	Colony forming units
CLS	Chronological life span
CMA	Chaperone mediated autophagy
CR	Calorie restriction
Ctrl	Control
Cvt pathway	Cytoplasm to vacuole targeting pathway
D. melanogaster	*Drosophila melanogaster*
ddH2O	Double-distilled water
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
dsRed	*Discosoma sp.* red fluorescent protein
DWP	Deep well plate
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAL4	Yeast derived transcriptional activator
IIS	insulin/insulin-like growth factor-like signaling
KanMX	KanMX cassette (geneticin resistance marker)
mRNA	Messenger ribonucleic acid

mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
NLS	Nuclear localization signal
O2	Oxygen
OD_{600}	Optical density 600 nm
ONC	Over night culture
PAGE	Polyacrylamide gel electrophoresis
PAS	Pre-autophagosomal structure
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PE	phosphatidylethanolamine
PI	Propidium iodide
PI3P	Phosphatidylinositol 3 phosphate
PtdIns	Phosphatidylinositol
RING assay	Rapide iterative negative geotaxis assay
RLS	Replicative life span
RNAi	RNA interference
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SMD	Synthetic minimal medium with glucose
SMG	Synthetic minimal medium with galactose
SMGly	Synthetic minimal medium with glycerol
SYA	Sucrose yeast agar medium
TAE	Tris base-acetic acid-EDTA-buffer
TCA cycle	Tricarboxylic acid – citric acid cycle
TNF receptors	Tumor necrosis factor receptor
TOR	Target of rapamycin
UAS	Upstream activation sequence

YPD	Yeast peptone dextrose medium

8 References

Amigo, Ignacio; da Cunha, Fernanda M.; Forni, Maria Fernanda; Garcia-Neto, Wilson; Kakimoto, Pamela A.; Luevano-Martinez, Luis A. et al. (2016): Mitochondrial form, function and signalling in aging. In: *The Biochemical journal* 473 (20), S. 3421–3449. DOI: 10.1042/BCJ20160451.

Anderson, John J. B.; Nieman, David C. (2016): Diet Quality-The Greeks Had It Right! In: *Nutrients* 8 (10). DOI: 10.3390/nu8100636.

Ashkenazi, A.; Dixit, V. M. (1998): Death receptors: signaling and modulation. In: *Science (New York, N.Y.)* 281 (5381), S. 1305–1308.

Ashrafi, K.; Sinclair, D.; Gordon, J. I.; Guarente, L. (1999): Passage through stationary phase advances replicative aging in Saccharomyces cerevisiae. In: *Proceedings of the National Academy of Sciences of the United States of America* 96 (16), S. 9100–9105.

Beach, David; Durkacz, Barbara; Nurse, Paul (1982): Functionally homologous cell cycle control genes in budding and fission yeast. In: *Nature* 300 (5894), S. 706–709. DOI: 10.1038/300706a0.

Bjedov, Ivana; Toivonen, Janne M.; Kerr, Fiona; Slack, Cathy; Jacobson, Jake; Foley, Andrea; Partridge, Linda (2010): Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. In: *Cell metabolism* 11 (1), S. 35–46. DOI: 10.1016/j.cmet.2009.11.010.

Boldogh, I. R.; Yang, H. C.; Nowakowski, W. D.; Karmon, S. L.; Hays, L. G.; Yates, J. R. 3rd; Pon, L. A. (2001): Arp2/3 complex and actin dynamics are required for actin-based mitochondrial motility in yeast. In: *Proceedings of the National Academy of Sciences of the United States of America* 98 (6), S. 3162–3167. DOI: 10.1073/pnas.051494698.

Botstein, D. (1997): GENETICS. Yeast as a Model Organism. In: *Science* 277 (5330), S. 1259–1260. DOI: 10.1126/science.277.5330.1259.

Botstein, David; Fink, Gerald R. (2011): Yeast: an experimental organism for 21st Century biology. In: *Genetics* 189 (3), S. 695–704. DOI: 10.1534/genetics.111.130765.

Bratic, Ana; Larsson, Nils-Goran (2013): The role of mitochondria in aging. In: *The Journal of clinical investigation* 123 (3), S. 951–957. DOI: 10.1172/JCI64125.

Bratic, Ivana; Trifunovic, Aleksandra (2010): Mitochondrial energy metabolism and ageing. In: *Biochimica et biophysica acta* 1797 (6-7), S. 961–967. DOI: 10.1016/j.bbabio.2010.01.004.

Broker, Linda E.; Kruyt, Frank A. E.; Giaccone, Giuseppe (2005): Cell death independent of caspases: a review. In: *Clinical cancer research : an official journal of the American Association for Cancer Research* 11 (9), S. 3155–3162. DOI: 10.1158/1078-0432.CCR-04-2223.

Buttner, Sabrina; Eisenberg, Tobias; Carmona-Gutierrez, Didac; Ruli, Doris; Knauer, Heide; Ruckenstuhl, Christoph et al. (2007): Endonuclease G regulates budding yeast life and death. In: *Molecular cell* 25 (2), S. 233–246. DOI: 10.1016/j.molcel.2006.12.021.

Buttner, Sabrina; Eisenberg, Tobias; Herker, Eva; Carmona-Gutierrez, Didac; Kroemer, Guido; Madeo, Frank (2006): Why yeast cells can undergo apoptosis: death in times of peace, love, and war. In: *The Journal of cell biology* 175 (4), S. 521–525. DOI: 10.1083/jcb.200608098.

Campbell, C. L.; Thorsness, P. E. (1998): Escape of mitochondrial DNA to the nucleus in yme1 yeast is mediated by vacuolar-dependent turnover of abnormal mitochondrial compartments. In: *Journal of cell science* 111 (Pt 16), S. 2455–2464.

Carroll, Bernadette; Hewitt, Graeme; Korolchuk, Viktor I. (2013): Autophagy and ageing: implications for age-related neurodegenerative diseases. In: *Essays in biochemistry* 55, S. 119–131. DOI: 10.1042/bse0550119.

Chien, Samson; Reiter, Lawrence T.; Bier, Ethan; Gribskov, Michael (2002): Homophila: human disease gene cognates in Drosophila. In: *Nucleic acids research* 30 (1), S. 149–151.

Christensen, Kaare; Doblhammer, Gabriele; Rau, Roland; Vaupel, James W. (2009): Ageing populations. The challenges ahead. In: *The Lancet* 374 (9696), S. 1196–1208. DOI: 10.1016/S0140-6736(09)61460-4.

Clancy, D. J.; Gems, D.; Harshman, L. G.; Oldham, S.; Stocker, H.; Hafen, E. et al. (2001): Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. In: *Science (New York, N.Y.)* 292 (5514), S. 104–106. DOI: 10.1126/science.1057991.

Colman, Ricki J.; Beasley, T. Mark; Kemnitz, Joseph W.; Johnson, Sterling C.; Weindruch, Richard; Anderson, Rozalyn M. (2014): Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. In: *Nature communications* 5, S. 3557. DOI: 10.1038/ncomms4557.

Cory, Suzanne; Adams, Jerry M. (2002): The Bcl2 family: regulators of the cellular life-or-death switch. In: *Nature reviews. Cancer* 2 (9), S. 647–656. DOI: 10.1038/nrc883.

Cuervo, Ana Maria (2008): Autophagy and aging: keeping that old broom working. In: *Trends in genetics : TIG* 24 (12), S. 604–612. DOI: 10.1016/j.tig.2008.10.002.

Darsow, T.; Rieder, S. E.; Emr, S. D. (1997): A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. In: *The Journal of cell biology* 138 (3), S. 517–529.

Del Rio, Daniele; Rodriguez-Mateos, Ana; Spencer, Jeremy P. E.; Tognolini, Massimiliano; Borges, Gina; Crozier, Alan (2013): Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. In: *Antioxidants & redox signaling* 18 (14), S. 1818–1892. DOI: 10.1089/ars.2012.4581.

Del Roso, Alessandra; Vittorini, Simona; Cavallini, Gabriella; Donati, Alessio; Gori, Zina; Masini, Matilde et al. (2003): Ageing-related changes in the in vivo function of rat liver macroautophagy and proteolysis. In: *Experimental gerontology* 38 (5), S. 519–527.

Di Carlo, G.; Mascolo, N.; Izzo, A. A.; Capasso, F. (1999): Flavonoids: old and new aspects of a class of natural therapeutic drugs. In: *Life sciences* 65 (4), S. 337–353.

Dolinski, Kara; Botstein, David (2007): Orthology and functional conservation in eukaryotes. In: *Annual review of genetics* 41, S. 465–507. DOI: 10.1146/annurev.genet.40.110405.090439.

Douzery, Emmanuel J. P.; Snell, Elizabeth A.; Bapteste, Eric; Delsuc, Frederic; Philippe, Herve (2004): The timing of eukaryotic evolution: does a relaxed molecular clock reconcile

proteins and fossils? In: *Proceedings of the National Academy of Sciences of the United States of America* 101 (43), S. 15386–15391. DOI: 10.1073/pnas.0403984101.

Du, C.; Fang, M.; Li, Y.; Li, L.; Wang, X. (2000): Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. In: *Cell* 102 (1), S. 33–42.

Duina, Andrea A.; Miller, Mary E.; Keeney, Jill B. (2014): Budding yeast for budding geneticists: a primer on the Saccharomyces cerevisiae model system. In: *Genetics* 197 (1), S. 33–48. DOI: 10.1534/genetics.114.163188.

Ehninger, Dan; Neff, Frauke; Xie, Kan (2014): Longevity, aging and rapamycin. In: *Cellular and molecular life sciences : CMLS* 71 (22), S. 4325–4346. DOI: 10.1007/s00018-014-1677-1.

Eisenberg, Tobias; Knauer, Heide; Schauer, Alexandra; Buttner, Sabrina; Ruckenstuhl, Christoph; Carmona-Gutierrez, Didac et al. (2009): Induction of autophagy by spermidine promotes longevity. In: *Nature cell biology* 11 (11), S. 1305–1314. DOI: 10.1038/ncb1975.

Elmore, Susan (2007): Apoptosis: a review of programmed cell death. In: *Toxicologic pathology* 35 (4), S. 495–516. DOI: 10.1080/01926230701320337.

Evans, Charles; Bogan, Katrina L.; Song, Peng; Burant, Charles F.; Kennedy, Robert T.; Brenner, Charles (2010): NAD+ metabolite levels as a function of vitamins and calorie restriction: evidence for different mechanisms of longevity. In: *BMC chemical biology* 10, S. 2. DOI: 10.1186/1472-6769-10-2.

Fabrizio, P.; Pozza, F.; Pletcher, S. D.; Gendron, C. M.; Longo, V. D. (2001): Regulation of longevity and stress resistance by Sch9 in yeast. In: *Science (New York, N.Y.)* 292 (5515), S. 288–290. DOI: 10.1126/science.1059497.

Fabrizio, Paola; Longo, Valter D. (2003): The chronological life span of Saccharomyces cerevisiae. In: *Aging cell* 2 (2), S. 73–81.

Fendt, Sarah-Maria; Sauer, Uwe (2010): Transcriptional regulation of respiration in yeast metabolizing differently repressive carbon substrates. In: *BMC systems biology* 4, S. 12. DOI: 10.1186/1752-0509-4-12.

Ferguson, Melissa; Mockett, Robin J.; Shen, Yi; Orr, William C.; Sohal, Rajindar S. (2005): Age-associated decline in mitochondrial respiration and electron transport in Drosophila melanogaster. In: *The Biochemical journal* 390 (Pt 2), S. 501–511. DOI: 10.1042/BJ20042130.

Fontana, Luigi; Partridge, Linda; Longo, Valter D. (2010): Extending healthy life span--from yeast to humans. In: *Science (New York, N.Y.)* 328 (5976), S. 321–326. DOI: 10.1126/science.1172539.

Freitas, Alex A.; Magalhaes, Joao Pedro de (2011): A review and appraisal of the DNA damage theory of ageing. In: *Mutation research* 728 (1-2), S. 12–22. DOI: 10.1016/j.mrrev.2011.05.001.

Gargano, Julia Warner; Martin, Ian; Bhandari, Poonam; Grotewiel, Michael S. (2005): Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in Drosophila. In: *Experimental gerontology* 40 (5), S. 386–395. DOI: 10.1016/j.exger.2005.02.005.

Geng, Jiefei; Klionsky, Daniel J. (2008): The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. In: *EMBO reports* 9 (9), S. 859–864. DOI: 10.1038/embor.2008.163.

Gensler, Helen L.; Bernstein, Harris (1981): DNA Damage as the Primary Cause of Aging. In: *The Quarterly Review of Biology* 56 (3), S. 279–303. DOI: 10.1086/412317.

Goffeau, A.; Barrell, B. G.; Bussey, H.; Davis, R. W.; Dujon, B.; Feldmann, H. et al. (1996): Life with 6000 Genes. In: *Science* 274 (5287), S. 546–567. DOI: 10.1126/science.274.5287.546.

Grunewald, Anne; Voges, Lisa; Rakovic, Aleksandar; Kasten, Meike; Vandebona, Himesha; Hemmelmann, Claudia et al. (2010): Mutant Parkin impairs mitochondrial function and morphology in human fibroblasts. In: *PloS one* 5 (9), e12962. DOI: 10.1371/journal.pone.0012962.

Han, Jiahuai; Zhong, Chuan-Qi; Zhang, Duan-Wu (2011): Programmed necrosis: backup to and competitor with apoptosis in the immune system. In: *Nature immunology* 12 (12), S. 1143–1149. DOI: 10.1038/ni.2159.

Hansen, Malene; Chandra, Abha; Mitic, Laura L.; Onken, Brian; Driscoll, Monica; Kenyon, Cynthia (2008): A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. In: *PLoS genetics* 4 (2), e24. DOI: 10.1371/journal.pgen.0040024.

HARMAN, D. (1956): Aging: a theory based on free radical and radiation chemistry. In: *Journal of gerontology* 11 (3), S. 298–300.

Harshman; Hoffmann; Clark (1999): Selection for starvation resistance in Drosophila melanogaster. Physiological correlates, enzyme activities and multiple stress responses. In: *J Evolution Biol* 12 (2), S. 370–379. DOI: 10.1046/j.1420-9101.1999.00024.x.

Herbener, G. H. (1976): A morphometric study of age-dependent changes in mitochondrial population of mouse liver and heart. In: *Journal of gerontology* 31 (1), S. 8–12.

Hill, Michelle M.; Adrain, Colin; Duriez, Patrick J.; Creagh, Emma M.; Martin, Seamus J. (2004): Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. In: *The EMBO journal* 23 (10), S. 2134–2145. DOI: 10.1038/sj.emboj.7600210.

Hofer, Sebastian (2016): Characterization of flavonoid-mediated cytoprotective effects in different models of Huntington's disease. Master thesis.

Iliadi, Konstantin G.; Boulianne, Gabrielle L. (2010): Age-related behavioral changes in Drosophila. In: *Annals of the New York Academy of Sciences* 1197, S. 9–18. DOI: 10.1111/j.1749-6632.2009.05372.x.

Ishihara, N.; Hamasaki, M.; Yokota, S.; Suzuki, K.; Kamada, Y.; Kihara, A. et al. (2001): Autophagosome requires specific early Sec proteins for its formation and NSF/SNARE for vacuolar fusion. In: *Molecular biology of the cell* 12 (11), S. 3690–3702.

Janke, Carsten; Magiera, Maria M.; Rathfelder, Nicole; Taxis, Christof; Reber, Simone; Maekawa, Hiromi et al. (2004): A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. In: *Yeast (Chichester, England)* 21 (11), S. 947–962. DOI: 10.1002/yea.1142.

Jazwinski, S. Michal (2002): Growing old: metabolic control and yeast aging. In: *Annual review of microbiology* 56, S. 769–792. DOI: 10.1146/annurev.micro.56.012302.160830.

Jazwinski, S. Michal (2013): The retrograde response: when mitochondrial quality control is not enough. In: *Biochimica et biophysica acta* 1833 (2), S. 400–409. DOI: 10.1016/j.bbamcr.2012.02.010.

Jennings, Barbara H. (2011): Drosophila – a versatile model in biology & medicine. In: *Materials Today* 14 (5), S. 190–195. DOI: 10.1016/S1369-7021(11)70113-4.

Johnson, Simon C.; Rabinovitch, Peter S.; Kaeberlein, Matt (2013): mTOR is a key modulator of ageing and age-related disease. In: *Nature* 493 (7432), S. 338–345. DOI: 10.1038/nature11861.

Joza, N.; Susin, S. A.; Daugas, E.; Stanford, W. L.; Cho, S. K.; Li, C. Y. et al. (2001): Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. In: *Nature* 410 (6828), S. 549–554. DOI: 10.1038/35069004.

Juhasz, Gabor; Erdi, Balazs; Sass, Miklos; Neufeld, Thomas P. (2007): Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila. In: *Genes & development* 21 (23), S. 3061–3066. DOI: 10.1101/gad.1600707.

Junnila, Riia K.; List, Edward O.; Berryman, Darlene E.; Murrey, John W.; Kopchick, John J. (2013): The GH/IGF-1 axis in ageing and longevity. In: *Nature reviews. Endocrinology* 9 (6), S. 366–376. DOI: 10.1038/nrendo.2013.67.

Kachroo, Aashiq H.; Laurent, Jon M.; Yellman, Christopher M.; Meyer, Austin G.; Wilke, Claus O.; Marcotte, Edward M. (2015): Evolution. Systematic humanization of yeast genes reveals conserved functions and genetic modularity. In: *Science (New York, N.Y.)* 348 (6237), S. 921–925. DOI: 10.1126/science.aaa0769.

Kamada, Y.; Funakoshi, T.; Shintani, T.; Nagano, K.; Ohsumi, M.; Ohsumi, Y. (2000): Tor-mediated induction of autophagy via an Apg1 protein kinase complex. In: *The Journal of cell biology* 150 (6), S. 1507–1513.

Kanki, Tomotake; Wang, Ke; Cao, Yang; Baba, Misuzu; Klionsky, Daniel J. (2009): Atg32 is a mitochondrial protein that confers selectivity during mitophagy. In: *Developmental cell* 17 (1), S. 98–109. DOI: 10.1016/j.devcel.2009.06.014.

Kaushik, Susmita; Cuervo, Ana Maria (2012): Chaperone-mediated autophagy: a unique way to enter the lysosome world. In: *Trends in cell biology* 22 (8), S. 407–417. DOI: 10.1016/j.tcb.2012.05.006.

Kawamata, Tomoko; Kamada, Yoshiaki; Kabeya, Yukiko; Sekito, Takayuki; Ohsumi, Yoshinori (2008): Organization of the pre-autophagosomal structure responsible for autophagosome formation. In: *Molecular biology of the cell* 19 (5), S. 2039–2050. DOI: 10.1091/mbc.E07-10-1048.

Kennedy, D. O. (2014): Polyphenols and the Human Brain. Plant "Secondary Metabolite" Ecologic Roles and Endogenous Signaling Functions Drive Benefits. In: *Advances in Nutrition: An International Review Journal* 5 (5), S. 515–533. DOI: 10.3945/an.114.006320.

Khare, C. P. (2015): Ayurvedic Pharmacopoeial Plant Drugs. Expanded Therapeutics. s.l.: CRC Press. Online verfügbar unter http://gbv.eblib.com/patron/FullRecord.aspx?p=4067502.

Kihara, A.; Noda, T.; Ishihara, N.; Ohsumi, Y. (2001): Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. In: *The Journal of cell biology* 152 (3), S. 519–530.

Kim, Insil; Rodriguez-Enriquez, Sara; Lemasters, John J. (2007): Selective degradation of mitochondria by mitophagy. In: *Archives of biochemistry and biophysics* 462 (2), S. 245–253. DOI: 10.1016/j.abb.2007.03.034.

Kischkel, F. C.; Hellbardt, S.; Behrmann, I.; Germer, M.; Pawlita, M.; Krammer, P. H.; Peter, M. E. (1995): Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. In: *The EMBO journal* 14 (22), S. 5579–5588.

Kissova, Ingrid; Deffieu, Maika; Manon, Stephen; Camougrand, Nadine (2004): Uth1p is involved in the autophagic degradation of mitochondria. In: *The Journal of biological chemistry* 279 (37), S. 39068–39074. DOI: 10.1074/jbc.M406960200.

Kissova, Ingrid Bhatia; Camougrand, Nadine (2009): Glutathione participates in the regulation of mitophagy in yeast. In: *Autophagy* 5 (6), S. 872–873.

Knott, Andrew B.; Perkins, Guy; Schwarzenbacher, Robert; Bossy-Wetzel, Ella (2008): Mitochondrial fragmentation in neurodegeneration. In: *Nature reviews. Neuroscience* 9 (7), S. 505–518. DOI: 10.1038/nrn2417.

Lamb, Christopher A.; Yoshimori, Tamotsu; Tooze, Sharon A. (2013): The autophagosome: origins unknown, biogenesis complex. In: *Nature reviews. Molecular cell biology* 14 (12), S. 759–774. DOI: 10.1038/nrm3696.

Lanza, Ian R.; Zabielski, Piotrek; Klaus, Katherine A.; Morse, Dawn M.; Heppelmann, Carrie J.; Bergen, H. Robert 3rd et al. (2012): Chronic caloric restriction preserves mitochondrial function in senescence without increasing mitochondrial biogenesis. In: *Cell metabolism* 16 (6), S. 777–788. DOI: 10.1016/j.cmet.2012.11.003.

Laun, Peter; Rinnerthaler, Mark; Bogengruber, Edith; Heeren, Gino; Breitenbach, Michael (2006): Yeast as a model for chronological and reproductive aging - a comparison. In: *Experimental gerontology* 41 (12), S. 1208–1212. DOI: 10.1016/j.exger.2006.11.001.

Li, Jesse W-H; Vederas, John C. (2009): Drug discovery and natural products: end of an era or an endless frontier? In: *Science (New York, N.Y.)* 325 (5937), S. 161–165. DOI: 10.1126/science.1168243.

Locksley, R. M.; Killeen, N.; Lenardo, M. J. (2001): The TNF and TNF receptor superfamilies: integrating mammalian biology. In: *Cell* 104 (4), S. 487–501.

Longo, Valter D.; Antebi, Adam; Bartke, Andrzej; Barzilai, Nir; Brown-Borg, Holly M.; Caruso, Calogero et al. (2015): Interventions to Slow Aging in Humans: Are We Ready? In: *Aging cell* 14 (4), S. 497–510. DOI: 10.1111/acel.12338.

Longo, Valter D.; Fabrizio, Paola (2012): Chronological aging in Saccharomyces cerevisiae. In: *Sub-cellular biochemistry* 57, S. 101–121. DOI: 10.1007/978-94-007-2561-4_5.

Lopez-Lluch, G.; Hunt, N.; Jones, B.; Zhu, M.; Jamieson, H.; Hilmer, S. et al. (2006): Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. In: *Proceedings of the National Academy of Sciences of the United States of America* 103 (6), S. 1768–1773. DOI: 10.1073/pnas.0510452103.

Ly, Christina; Yockell-Lelievre, Julien; Ferraro, Zachary M.; Arnason, John T.; Ferrier, Jonathan; Gruslin, Andree (2015): The effects of dietary polyphenols on reproductive health and early development. In: *Human reproduction update* 21 (2), S. 228–248. DOI: 10.1093/humupd/dmu058.

Madeo, F.; Frohlich, E.; Frohlich, K. U. (1997): A yeast mutant showing diagnostic markers of early and late apoptosis. In: *The Journal of cell biology* 139 (3), S. 729–734.

Madeo, Frank; Herker, Eva; Maldener, Corinna; Wissing, Silke; Lachelt, Stephan; Herlan, Mark et al. (2002): A caspase-related protease regulates apoptosis in yeast. In: *Molecular cell* 9 (4), S. 911–917.

Maiuri, M. Chiara; Zalckvar, Einat; Kimchi, Adi; Kroemer, Guido (2007): Self-eating and self-killing: crosstalk between autophagy and apoptosis. In: *Nature reviews. Molecular cell biology* 8 (9), S. 741–752. DOI: 10.1038/nrm2239.

Mannella, Carmen A. (2008): Structural diversity of mitochondria: functional implications. In: *Annals of the New York Academy of Sciences* 1147, S. 171–179. DOI: 10.1196/annals.1427.020.

Mao, Kai; Wang, Ke; Liu, Xu; Klionsky, Daniel J. (2013): The scaffold protein Atg11 recruits fission machinery to drive selective mitochondria degradation by autophagy. In: *Developmental cell* 26 (1), S. 9–18. DOI: 10.1016/j.devcel.2013.05.024.

Mao, Kai; Wang, Ke; Zhao, Mantong; Xu, Tao; Klionsky, Daniel J. (2011): Two MAPK-signaling pathways are required for mitophagy in Saccharomyces cerevisiae. In: *The Journal of cell biology* 193 (4), S. 755–767. DOI: 10.1083/jcb.201102092.

Mari, Muriel; Griffith, Janice; Rieter, Ester; Krishnappa, Lakshmi; Klionsky, Daniel J.; Reggiori, Fulvio (2010): An Atg9-containing compartment that functions in the early steps of autophagosome biogenesis. In: *The Journal of cell biology* 190 (6), S. 1005–1022. DOI: 10.1083/jcb.200912089.

May, Alexander I.; Devenish, Rodney J.; Prescott, Mark (2012): The many faces of mitochondrial autophagy: making sense of contrasting observations in recent research. In: *International journal of cell biology* 2012, S. 431684. DOI: 10.1155/2012/431684.

Mayer, Andreas (2008): Chapter 11 Cell-Free Reconstitution of Microautophagy in Yeast. In: Autophagy: Lower Eukaryotes and Non-Mammalian Systems, Part A, Bd. 451: Elsevier (Methods in Enzymology), S. 151–162.

McBride, Heidi M.; Neuspiel, Margaret; Wasiak, Sylwia (2006): Mitochondria: more than just a powerhouse. In: *Current biology : CB* 16 (14), R551-60. DOI: 10.1016/j.cub.2006.06.054.

Medawar, P. B. (1955): The Definition and Measurement of Senescence. In: G. E. W. Wolstenholme und Margaret P. Cameron (Hg.): Ciba Foundation Symposium - General Aspects (Colloquia on Ageing, Vol, 1). Chichester, UK: John Wiley & Sons, Ltd (Novartis Foundation Symposia), S. 4–15.

Melendez, Alicia; Hall, David H.; Hansen, Malene (2008): Monitoring the role of autophagy in C. elegans aging. In: *Methods in enzymology* 451, S. 493–520. DOI: 10.1016/S0076-6879(08)03229-1.

Melendez, Alicia; Neufeld, Thomas P. (2008): The cell biology of autophagy in metazoans: a developing story. In: *Development (Cambridge, England)* 135 (14), S. 2347–2360. DOI: 10.1242/dev.016105.

Mortiboys, Heather; Thomas, Kelly Jean; Koopman, Werner J. H.; Klaffke, Stefanie; Abou-Sleiman, Patrick; Olpin, Simon et al. (2008): Mitochondrial function and morphology are impaired in parkin-mutant fibroblasts. In: *Annals of neurology* 64 (5), S. 555–565. DOI: 10.1002/ana.21492.

Muller, Matthias; Lu, Kaihui; Reichert, Andreas S. (2015): Mitophagy and mitochondrial dynamics in Saccharomyces cerevisiae. In: *Biochimica et biophysica acta* 1853 (10 Pt B), S. 2766–2774. DOI: 10.1016/j.bbamcr.2015.02.024.

Muller-Hocker, J.; Aust, D.; Rohrbach, H.; Napiwotzky, J.; Reith, A.; Link, T. A. et al. (1997): Defects of the respiratory chain in the normal human liver and in cirrhosis during aging. In: *Hepatology (Baltimore, Md.)* 26 (3), S. 709–719. DOI: 10.1002/hep.510260324.

Navarro, Ana; Boveris, Alberto (2007): The mitochondrial energy transduction system and the aging process. In: *American journal of physiology. Cell physiology* 292 (2), C670-86. DOI: 10.1152/ajpcell.00213.2006.

Nixon, Ralph A. (2013): The role of autophagy in neurodegenerative disease. In: *Nature medicine* 19 (8), S. 983–997. DOI: 10.1038/nm.3232.

Noda, T.; Kim, J.; Huang, W. P.; Baba, M.; Tokunaga, C.; Ohsumi, Y.; Klionsky, D. J. (2000): Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. In: *The Journal of cell biology* 148 (3), S. 465–480.

Nowikovsky, K.; Reipert, S.; Devenish, R. J.; Schweyen, R. J. (2007): Mdm38 protein depletion causes loss of mitochondrial K+/H+ exchange activity, osmotic swelling and mitophagy. In: *Cell death and differentiation* 14 (9), S. 1647–1656. DOI: 10.1038/sj.cdd.4402167.

Okamoto, Koji; Kondo-Okamoto, Noriko; Ohsumi, Yoshinori (2009): Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. In: *Developmental cell* 17 (1), S. 87–97. DOI: 10.1016/j.devcel.2009.06.013.

Orlikova, Barbora; Tasdemir, Deniz; Golais, Frantisek; Dicato, Mario; Diederich, Marc (2011): Dietary chalcones with chemopreventive and chemotherapeutic potential. In: *Genes & nutrition* 6 (2), S. 125–147. DOI: 10.1007/s12263-011-0210-5.

Ostergaard, S.; Olsson, L.; Johnston, M.; Nielsen, J. (2000): Increasing galactose consumption by Saccharomyces cerevisiae through metabolic engineering of the GAL gene regulatory network. In: *Nature biotechnology* 18 (12), S. 1283–1286. DOI: 10.1038/82400.

Pallauf, Kathrin; Rimbach, Gerald (2013): Autophagy, polyphenols and healthy ageing. In: *Ageing research reviews* 12 (1), S. 237–252. DOI: 10.1016/j.arr.2012.03.008.

Preston, Claudia C.; Oberlin, Andrew S.; Holmuhamedov, Ekhson L.; Gupta, Anu; Sagar, Sandeep; Syed, Rashad H. Khazi et al. (2008): Aging-induced alterations in gene transcripts and functional activity of mitochondrial oxidative phosphorylation complexes in the heart. In: *Mechanisms of ageing and development* 129 (6), S. 304–312. DOI: 10.1016/j.mad.2008.02.010.

Reggiori, Fulvio; Klionsky, Daniel J. (2013): Autophagic processes in yeast: mechanism, machinery and regulation. In: *Genetics* 194 (2), S. 341–361. DOI: 10.1534/genetics.112.149013.

Reggiori, Fulvio; Komatsu, Masaaki; Finley, Kim; Simonsen, Anne (2012): Selective types of autophagy. In: *International journal of cell biology* 2012, S. 156272. DOI: 10.1155/2012/156272.

Renehan, A. G. (2001): What is apoptosis, and why is it important? In: *BMJ* 322 (7301), S. 1536–1538. DOI: 10.1136/bmj.322.7301.1536.

Renvoise, Margaux; Bonhomme, Ludovic; Davanture, Marlene; Valot, Benoit; Zivy, Michel; Lemaire, Claire (2014): Quantitative variations of the mitochondrial proteome and phosphoproteome during fermentative and respiratory growth in Saccharomyces cerevisiae. In: *Journal of proteomics* 106, S. 140–150. DOI: 10.1016/j.jprot.2014.04.022.

Richard, Vincent R.; Leonov, Anna; Beach, Adam; Burstein, Michelle T.; Koupaki, Olivia; Gomez-Perez, Alejandra et al. (2013): Macromitophagy is a longevity assurance process that in chronologically aging yeast limited in calorie supply sustains functional mitochondria and maintains cellular lipid homeostasis. In: *Aging* 5 (4), S. 234–269. DOI: 10.18632/aging.100547.

Rose, M. R.; Vu, L. N.; Park, S. U.; Graves, J. L., JR (1992): Selection on stress resistance increases longevity in Drosophila melanogaster. In: *Experimental gerontology* 27 (2), S. 241–250.

Rose, Michael R. (2010): Evolutionary biology of aging. New York: Oxford University Press. Online verfügbar unter http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=151230.

Scott, Ryan C.; Schuldiner, Oren; Neufeld, Thomas P. (2004): Role and regulation of starvation-induced autophagy in the Drosophila fat body. In: *Developmental cell* 7 (2), S. 167–178. DOI: 10.1016/j.devcel.2004.07.009.

Shutt, Timothy E.; McBride, Heidi M. (2013): Staying cool in difficult times: mitochondrial dynamics, quality control and the stress response. In: *Biochimica et biophysica acta* 1833 (2), S. 417–424. DOI: 10.1016/j.bbamcr.2012.05.024.

Slee, E. A.; Adrain, C.; Martin, S. J. (2001): Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. In: *The Journal of biological chemistry* 276 (10), S. 7320–7326. DOI: 10.1074/jbc.M008363200.

Sohal, Rajindar S.; Ku, Hung-Hai; Agarwal, Sanjiv; Forster, Michael J.; Lal, Harbans (1994): Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. In: *Mechanisms of ageing and development* 74 (1-2), S. 121–133. DOI: 10.1016/0047-6374(94)90104-X.

Steinkraus, K. A.; Kaeberlein, M.; Kennedy, B. K. (2008): Replicative aging in yeast: the means to the end. In: *Annual review of cell and developmental biology* 24, S. 29–54. DOI: 10.1146/annurev.cellbio.23.090506.123509.

Stocco, D. M.; Hutson, J. C. (1978): Quantitation of mitochondrial DNA and protein in the liver of Fischer 344 rats during aging. In: *Journal of gerontology* 33 (6), S. 802–809.

Sun, Yaning; Yolitz, Jason; Wang, Cecilia; Spangler, Edward; Zhan, Ming; Zou, Sige (2013): Aging studies in Drosophila melanogaster. In: *Methods in molecular biology (Clifton, N.J.)* 1048, S. 77–93. DOI: 10.1007/978-1-62703-556-9_7.

Tait, Stephen W. G.; Green, Douglas R. (2013): Mitochondrial regulation of cell death. In: *Cold Spring Harbor perspectives in biology* 5 (9). DOI: 10.1101/cshperspect.a008706.

Tal, Ruth; Winter, Gal; Ecker, Nitai; Klionsky, Daniel J.; Abeliovich, Hagai (2007): Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. In: *The Journal of biological chemistry* 282 (8), S. 5617–5624. DOI: 10.1074/jbc.M605940200.

Tower, John; Arbeitman, Michelle (2009): The genetics of gender and life span. In: *Journal of biology* 8 (4), S. 38. DOI: 10.1186/jbiol141.

Uttenweiler, Andreas; Mayer, Andreas (2008): Microautophagy in the yeast Saccharomyces cerevisiae. In: *Methods in molecular biology (Clifton, N.J.)* 445, S. 245–259. DOI: 10.1007/978-1-59745-157-4_16.

Uttenweiler, Andreas; Schwarz, Heinz; Neumann, Heinz; Mayer, Andreas (2007): The vacuolar transporter chaperone (VTC) complex is required for microautophagy. In: *Molecular biology of the cell* 18 (1), S. 166–175. DOI: 10.1091/mbc.E06-08-0664.

Wei, Min; Fabrizio, Paola; Hu, Jia; Ge, Huanying; Cheng, Chao; Li, Lei; Longo, Valter D. (2008): Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. In: *PLoS genetics* 4 (1), e13. DOI: 10.1371/journal.pgen.0040013.

Williams, George C. (1957): Pleiotropy, Natural Selection, and the Evolution of Senescence. In: *Evolution* 11 (4), S. 398. DOI: 10.2307/2406060.

Wissing, Silke; Ludovico, Paula; Herker, Eva; Buttner, Sabrina; Engelhardt, Silvia M.; Decker, Thorsten et al. (2004): An AIF orthologue regulates apoptosis in yeast. In: *The Journal of cell biology* 166 (7), S. 969–974. DOI: 10.1083/jcb.200404138.

Yang, Zhifen; Huang, Ju; Geng, Jiefei; Nair, Usha; Klionsky, Daniel J. (2006): Atg22 recycles amino acids to link the degradative and recycling functions of autophagy. In: *Molecular biology of the cell* 17 (12), S. 5094–5104. DOI: 10.1091/mbc.E06-06-0479.

Yen, T. C.; Chen, Y. S.; King, K. L.; Yeh, S. H.; Wei, Y. H. (1989): Liver mitochondrial respiratory functions decline with age. In: *Biochemical and biophysical research communications* 165 (3), S. 944–1003.

Yuan, Yiyuan; Hakimi, Parvin; Kao, Clara; Kao, Allison; Liu, Ruifu; Janocha, Allison et al. (2016): Reciprocal Changes in Phosphoenolpyruvate Carboxykinase and Pyruvate Kinase with Age Are a Determinant of Aging in Caenorhabditis elegans. In: *The Journal of biological chemistry* 291 (3), S. 1307–1319. DOI: 10.1074/jbc.M115.691766.

Zimmermann, Andreas (2012): Flavonoids affect yeast lifespan in a context-dependent manner. Master thesis.

Zong, Wei-Xing; Thompson, Craig B. (2006): Necrotic death as a cell fate. In: *Genes & development* 20 (1), S. 1–15. DOI: 10.1101/gad.1376506.