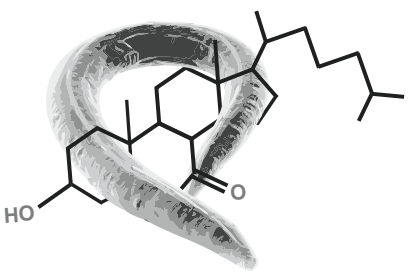
Small Molecule Modulators of Dauer Formation and Longevity in *Caenorhabditis Elegans*

by Ben Becker



Small Molecule Modulators of Dauer Formation and Longevity in *Caenorhabditis Elegans*

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Table of contents

Table of Abbreviations

AICAR	50-aminoimidazole-4-car-boxamide-1-b-D-ribofuranosid
°C	degree celsius
7-KC	7-ketocholesterol
7-keto-DA	7-keto-dafachronic acid
AMP	adenosine monophosphate
AMPK	AMP kinase
ANOVA	analysis of variance
AMPK	adenosine triphosphate
C	centi
cDNA	complementary DNA
CR	caloric restriction
CYP	cytochrom p450
DA	dafachronic acid
Daf-c	dauer formation constituive
Daf-d	dauer formation defective
DNA	deoxyribonucleic acid
DBD	DNA binding domain
DGLA	dihomo-γ-linolenic acid
DNase	deoxyribonuclease
DR	dietary restrictions
EMS	ethylmethansulfonat
ER	endoplasmatic reticulum
ETC	electron transport chain
EtOH	Ethanol
FXR	farneosid x receptor
g	gram
GC	gas chromatograph
GFP	green fluorescent protein
h	hours
HEK	human embryonic kidney
Hz	hertz
IGF-1	insulin-like growth factor
IIS	insulin/IGF-1 like signaling
InR	insulin receptor
J	Joules
l	liter
LB	Lysogene Broth
LBD	ligand binding domain
LXR	liver x receptor

m	milli
M	molar
MeOH	Methanol
MRM	multiple reaction monitoring
mRNA	messenger RNA
MS	mass spectrometer
mt	mitochondrial
n	nano
NAC	N-acetylcystein
NAD	nicotinamide adenine dinucleotide
NAE	N-acyethanolamines
NDA	national drug administration
NGM	nematode growth medium
NHR	nuclear hormone receptor
NIA	national institute of aging
NP40	Nonylphenolethoxylat MO=40
OP50	*Echeria Coli* strain OP50
PCR	polymerase chain reaction
PUFA	polyunsaturated fatty acid
qPCR	quantitavie PCR
RNA	ribonucleid acid
RNAi	RNA interference
RNase	ribonuclease
RNAseq	RNA sequencing
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid cycle
TGF-b	transforming growth factor b
TOR	target of rapamycin
UPR	unfolded protein response
UV	ultra violet
VDR	vitamin D receptor
Α	alpha
Β	beta
Γ	Gamma
Δ	Delta
Λ	Lambda
Μ	Micro
Ω	Omega

Abstract

7-ketocholesterol modulates dauer formation and longevity via DAF-12

Ageing is the inevitable fate of most living organisms, and is the greatest risk factor for many diseases such as diabetes, cancer, cardiovascular disorders and neurodegeneration. Deceleration of ageing delays the onset of such diseases and improves health into old age. On the cellular level, several signaling pathways that integrate the nutrient sensors insulin/IGF-1-like signaling (IIS) and target of rapamycin (TOR) play an important role in the modulation of lifespan. Downstream effectors of these pathways include broad cellular functions such as protein translation, mitochondrial activity, autophagy and protein homeostasis, affecting youthfulness of cells and the whole organism. Some of these lifespan modulators were found to be direct targets of small molecules such as glucose and rapamycin. In this study, we asked how might endogenous small molecule metabolites including components of the diet such as sugars, amino acids and fatty acids modulate lifespan? What signaling pathways are involved? How can they be used to alter the onset of age-related diseases and potentially help to develop drugs or even prevent them?

Interestingly, many pathways and signals regulating lifespan in *C. elegans* were originally discovered for their role in regulating the dauer decision. *C. elegans* arrest at the stress-resistant long-lived dauer stage in response to harsh environmental conditions such as high temperature, food scarcity, or high population density. In brief, inhibition the IIS pathway, and resulting transcriptional activity of phosphorylated DAF-16/FOXO activates a hormone biosynthetic pathway that converts cholesterol into bile acid like steroids called the dafachronic acids (DA). DAs are endogenous ligands of the steroid receptor DAF-12, a homolog of mammalian LXR/FXR/VDR, and key determinant of dauer formation. Liganded DAF-12 promotes reproductive development and normal life. Conversely, when these pathways are down-regulated, the unliganded DAF-12 represses these programs and promotes dauer formation and long life.

To identify novel signaling molecules regulating life span, we performed a screen for small molecule metabolites that modulate dauer formation in the insulin receptor mutant *daf-2(e1368)* background. *daf-2(e1368)* displays a temperature sensitive constitutive dauer formation (Daf-c) phenotype that can be enhanced or suppressed. Our premise was that molecules identified as dauer modulators may also be good candidates for modulating lifespan. We supplemented small molecules involved in energy homeostasis and metabolism

such as sugars, amino acids, fatty acids and steroids, and measured dauer formation at a semi-permissive temperature as first readout.

From such screens we identified sugars (glucose, galactose, trehalose), amino acids (tryptophan, glycine) and a fatty acid (arachidonic acid) that reduce dauer formation, suggesting they could activate reproductive programs. Interestingly, we also identified 7-ketocholesterol (7-KC) as potent synergistic enhancer of *daf-2* dauer formation and focused on this molecule as a potential modulator of longevity. Although 7-KC had little effect on wild type dauer formation, it enhanced dauer formation of various Daf-c mutants of the dauer signaling pathways including *daf-2*/InsR, *daf-7*/TGFβ, the Niemann Pick Type C1 homologs, as well as several mutants involved in DA production such as *daf-36*/Rieske oxygenase. Using a biochemical GC-MS approach, we found that 7-KC altered sterol profiles and exhibited an increase in whole body cholesterol but a decrease in 7-dehydrocholesterol, suggesting possible effects on the first step in Δ^7-DA synthesis. Moreover, 7-KC induced hypodermal *daf-9/CYP27A1* expression, a marker of mild DA depletion in wild type animals. Importantly, 7-KC was found to extend median lifespan of wild type animals by 20%, in a manner independent of DAF-16/FOXO but dependent on DAF-12/FXR, as well as on the hormone biosynthetic enzyme DAF-9/CYP27A1.

In vivo mRNA analyses revealed that 7-KC modestly interferes with expression of DAF-12 target genes (*mir-84*, *mir-48* and *mir-241*). Accordingly, competition assays suggest that 7-KC thwarts DAF-12 transcriptional activity in cell culture. Whole transcriptome analyses (RNAseq) in *C. elegans* revealed that 7-KC induces changes in gene expression consistent with regulatory effects on DAF-12 as well as the DAF-12 related receptor, NHR-8. The inferred changes in gene expression suggests that 7-KC opposes known DAF-12 target gene expression and in addition potentially drives a different subset of genes. Taken together, we hypothesize that 7-KC might be converted to an alternative DA-like molecule, 7-keto-DA by DAF-9. In this view, 7-keto-DA might be an alternate DAF-12 ligand, mediating the observed phenotypes.

7-KC is found in all living organisms including humans, where it may be involved in the regulation of bile acid and *de novo* cholesterol synthesis. Moreover 7-KC was shown to be involved in the formation of age related atherosclerotic cardiovascular disease. Our findings might lead to identification of novel direct mammalian 7-KC target genes and might provide a first step to clarify if 7-KC plays a causative role in atherosclerosis.

II Zusammenfassung

7-Ketocholesterol moduliert die Dauer Ausprägung und Langlebigkeit via DAF-12

Altern ist das unausweichliche Schicksal der meisten lebenden Organismen und es ist der größte Risikofaktor für zahlreiche Krankheiten wie Diabetes, Krebs, Herzgefäß- und Neurodegenerative-Erkrankungen. Verlangsamung des Alterungsprozesses zögert den Ausbruch solcher Krankheiten hinaus und stärkt die gesundheitliche Verfassung bis ins hohe Alter. Auf zellulärer Ebene spielen viele kanonische Signalwege wie die Nährstoff-sensitiven Stoffwechselwege IIS und TOR eine entscheidende Rolle bei der Modulierung der Lebensspanne. Über diese Signalwege werden zelluläre Funktionen wie Translation, Mitochondrien-Aktivität, Autophagie und Proteinhomöostasis angesteuert, die ihrerseits die Jugendlichkeit der Zelle und des gesamten Organismus fördern. Einige dieser Signalwege sind direkte Ziele von kleinen Molekülen wie Glukose oder Rapamycin. Grundlage dieser Studie sind die folgenden Fragen: Wie können kleine endogene Metabolite einschließlich solcher, die Bestandteil der Nahrung sind, wie Zucker, Aminosäuren, Fettsäuren und Steroide die Lebensspanne beeinflussen? Welche Signalwege werden durch sie reguliert? Wie können sie genutzt werden um den Ausbruch altersbedingter Krankheiten hinaus zu zögern und bei der Entwicklung von Medikamenten zu helfen? Interessanterweise wurden viele Stoffwechsel- und Signalwege, welche die Lebensspanne in *C. elegans* regulieren, ursprünglich als Regulatoren der Dauer Entscheidung identifiziert. Unter schlechten Umweltbedingungen, wie hoher Temperatur, Nahrungsmangel oder hoher Populationsdichte, unterbricht *C. elegans* die reproduktive Entwicklung und tritt in einen stressresistenten und langlebigen Hibernationszustand ein (Dauer). Kurzgefasst, führt die Aktivierung des Insulin/IGF-1like signaling pathway (IIS) und die daraus resultierende Phosphorylierung von DAF-16/FOXO zu der Aktivierung eines Hormon Biosyntheseweges, der Cholesterin in Gallensäure ähnliche Steroide namens dafachronic acids (DA) umsetzt. DAs wiederum sind endogene Liganden des Steroid Rezeptors DAF-12 - Homolog von LXR/FXR/VDR und das Schlüsselprotein der Dauer Ausprägung. Ligandengebundener DAF-12 fördert genetische Programme, welche die reproduktive Entwicklung und eine reguläre Lebensspanne ermöglichen. Sind im Gegenzug diese Signalwege herunterreguliert unterdrückt ungebundener DAF-12 jene Programme und fördert die Dauer Ausprägung und Langlebigkeit.

Um neue Moleküle zu identifizieren, welche die Lebensspanne regulieren haben wir mehrere Metaboliten zunächst hinsichtlich ihrer Fähigkeit gescreent den Dauer Eintritt der Insulin-Rezeptor Mutante *daf-2(e1368)* zu beeinflussen. *daf-2(e1368)* ist eine temperaturabhängige, konstitutive Dauer Mutante (Daf-c). Dieser Daf-c Phänotyp kann unterdrückt oder verstärkt werden. Unsere Prämisse für diesen Screen: Moleküle die Dauer Ausprägung modulieren sind ebenfalls gute Kandidaten um die Lebensspanne zu beeinflussen. Wir haben kleine Moleküle des Energiehaushaltes und des Metabolismus, z.B. Zucker, Aminosäuren, Fettsäuren und Steroide an *C. elegans* gefüttert. Mit diesem Ansatz identifizierten wir einige Zucker (Glukose, Galaktose, Trehalose), Aminosäuren (Tryptophan, Glycin) und Fettsäuren (Arachidonsäure), welche vermutlich reproduktive Programme aktivieren und den Eintritt in Dauer verringern. Interessanterweise haben wir darüberhinaus 7-Ketocholesterin (7-KC) als einen wirksamen synergistischen Dauer-Verstärker identifiziert. Obwohl 7-KC nur geringfügigen Einfluss auf Dauer im Wild Typ hatte so konnte es doch die Dauer Ausprägung zahlreicher Daf-c Mutanten des Dauer Signalweges verstärken - einschließlich *daf-2*/InsR, *daf-7*/TFG-β, den Niemann Pick Typ C1 Homolog sowie zahlreichen Mutanten involviert in DA- Synthese, unter anderem den von *daf-36*/Rieske Oxygenase. 7-KC veränderte das Sterolprofil und führte zu mehr Cholesterin und weniger 7-Dehydrocholesterin, dies legt eine Inhibierung des ersten Schrittes in der DA –Synthese nahe. Darüberhinaus Induzierte 7-KC hypodermale daf-9/CYP27A1 Expression ein Marker schwachen DA-Schwundes in Wild Typ. Vor allem aber verlängerte 7-KC die Lebensspanne von Wild Typ signifikant (20%) und unabhängig von daf-16/FOXO aber abhängig von DAF-12/FXR und dem biosynthetisch aktiven Enzym daf-9/CYP27A1.In vivo mRNA-Analysen bestätigten, dass 7-KC die Expression der DAF-12 Zielgene (*mir-84*, *mir-48* und *mir-241*) herunter reguliert. Kompetitive Untersuchungen deuten darauf hin, dass 7-KC die Aktivität von DAF-12 in Zellkultur unterdrückt. Transkriptom-Analysen (RNAseq) konnten zeigen, dass 7-KC induzierte Veränderungen mit denen von DAF-12 überlagern und zudem mit denen des DAF-12 verwanden Rezeptor, NHR-8. Grundsätzlich scheinen diese Veränderungen genau entgegengesetzt zu den Regulationen von DAF-12 zu sein und ein zusätzlich Set an Genen zu regulieren. Wir vermuten, dass 7-Ketocholesterol von DAF-9 in ein alternatives DA-Molekül, 7-keto-DA umgesetzt wird. 7-keto-DA könnte ein neuer Ligand für DAF-12 sein und so die Phänotypen induzieren.7-KC kommt in allen lebenden Organsimen. In Menschen könnte es in der Regulation der Gallsäuren-und Cholesterin *de novo* - Synthese beteiligt sein. 7-KC wurde in Zusammenhang mit einer altersbedingten arteriosklerotischen Herzgefäßerkrankung

gebracht. Unsere Resultate könnten zur Entdeckung neuer direkter Targets von 7-KC in Säugetieren führen und einen ersten Schritt zur Klärung der Frage darstellen ob 7-KC tatsächlich eine kausale Rolle in Arteriosklerose spielt.

Introduction

1 Ageing and ageing research

1.1 What is the aim of ageing research?

Ageing is, with few exceptions, the inevitable fate of all living organisms. Since antiquity, humankind has expressed a strong desire to vanquish or postpone the inescapability of death. But discovering the fountain of youth is not the motivation driving the ageing research field. Considerably more compelling is the realization of a link between the age of an organism and the onset of many diseases such as diabetes, cancer, cardiovascular disorders and neurodegenerative diseases (Kenyon, 2010; López-Otín et al., 2013). By understanding fundamental mechanisms underlying ageing, we hope to develop novel approaches toward understanding and treating many age-related diseases. For a long time comparatively little was understood of the mechanisms underlying ageing at the genetic, metabolic and transcriptional level. But during the last decades significant insights have been made to understand the molecular basis of ageing and to unravel how ageing makes organisms susceptible to age-related diseases.

The first multicellular organism used to genetically dissect the molecular basis of ageing was *C. elegans*. The free living nematode was developed by Sydney Brenner for laboratory use in the 1970s (Brenner, 1974). Later, single gene mutations in *age-1* and *daf-2* were among the first mutations discovered that extended lifespan in *C. elegans*.(Friedman and Johnson, 1988b; 1988a; Kenyon et al., 1993; Klass and Hirsh, 1976) Since then, many laboratories have sought to understand ageing and age related diseases in different model organisms. Many interventions and regimes were found conserved in other model organisms including *Drosophila melanogaster* and *Mus Musculus*. In brief, the aim of ageing research is to unravel conserved underlying mechanisms of ageing and get a better understanding of human age related diseases.

1.2 Why do organisms age ?

1.2.1 What is the molecular basis of ageing?

To characterize ageing as a phenotype, recently nine hallmarks of ageing have been suggested (López-Otín et al., 2013). All of them reflect increase of entropy and resulting decline of performance: Genomic instability, telomere attrition, epigenetic alterations, loss of protein homeostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. This decline in performance is a function of time and it used to be a longtime dogma that it is completely independent of genetic regulation. However investigations in model organisms revealed a role of genetic pathways. The current understanding of ageing is the failure of genetic programs promoting cellular functionality and therefore organismal health and youthfulness (Gems and Partridge, 2013; Kenyon, 2010). Up to date, many pathways have been identified in this context, including highly conserved pathways such as the nutrient sensing insulin signaling and target of rapamycin (TOR) pathways, revealing first insights into a complex network. But not only these canonical pathways but also mitochondrial activity (Bratic and Larsson, 2013), and quality of cell clearance (Dillin and Cohen, 2011) were identified as important lifespan modulators. Age-associated dysfunction in these highly orchestrated networks leads to failure of molecular maintenance mechanisms, cellular dysfunction, deteriorated tissue performance and death of the organism. Most notably, it is exactly this decline in orchestrated functionality that makes organisms more susceptible for age related diseases (López-Otín et al., 2013). Although the described genetics play a prominent role in ageing, there are other factors decelerating or accelerating organismal age by impinging on these longevity pathways.

1.2.2 Which other factors can influence ageing?

One of these factors is the ambient condition, including variables like temperature, population density, oxygen availability and quantity as well as quality of diet. In particular, dietary composition of small molecules was shown to influence ageing. Note that for this thesis the term “small molecules” is defined as all endogenous non-protein and non nucleic acid molecules, as well as pharmaceuticals and defined molecules in the diet that have the potential to modulate metabolic processes. The scope of this thesis excludes complex plant extracts that were found to modulate lifespan, like blueberry (Wilson et al., 2006)and ginkgo extracts (Kampkötter et al., 2007). In the diet, especially small molecules like sugars, amino acids, and fatty acids can not only function as energy sources and molecular building blocks

but also as signaling molecules. As such, they reflect environmental conditions to modulate and feedback regulate complex genetic networks.

The most noted lifespan determining intervention linked to diet is caloric restriction (CR). CR is defined as the restriction in caloric intake without malnutrition. Closely related is dietary restriction, which is defined as reduced intake of total food without malnutrition. CR is one of the most intensively studied longevity regimes. It was shown to extend lifespan in yeast (Guarente, 2005), *C. elegans* (Houthoofd and Vanfleteren, 2006; Klass, 1977), *Drosophila melanogaster* (Partridge et al., 2005), *Mus Musculus* and other rodents (McDonald and Ramsey, 2010; Ramsey et al., 2000) and in one study even in non-human primates (Colman et al., 2009), whereas another study showed no effect (Redman and Ravussin, 2011). Nevertheless it was shown in both studies that it delayed onset of metabolic disorders such as diabetes and cancer. Still it remains to be determined if CR really extends health- and lifespan in primates. Human studies measuring lifespan are not very feasible, however beneficial metabolic effect of caloric restriction have been also observed in humans (Cava and Fontana, 2013). In line with CR improving health it is known that high fat and high glycemic diets influence insulin signaling, one of the major longevity pathway, thereby promote obesity, diabetes and even cardiovascular diseases (Aston, 2006; Venn and Green, 2007). Understanding the complex role of nutrition and small molecules might be of particular relevance to understand human ageing. In particular since small molecules provide high potential to develop drugs against age-related diseases. Up to date, genetic manipulations in humans are not feasible; therefore pharmaceutical interventions using small molecules are the only way to treat age related diseases. Accordingly there is an emerging interest in the identification of novel small molecule modulators of longevity.

1.3 How to Study ageing

1.3.1 How is ageing approached statistically?

To understand how ageing can be studied, first an appropriate definition of age is required. Chronological and biological age must be distinguished.

Chronological age can be measured in time units (e.g. hours, days and years). For a population, chronological age is described by statistical values such as median and maximum lifespan. Whereas the median lifespan describes the time-point at which 50% of a population is dead and the maximum lifespan describes the time-point when the last animal of a

population has died. Median and maximum lifespan of manipulated and wild type population are compared to state longevity or shortevity.

Biological age reflects a certain state of physiological health independent of chronological age. It cannot be primary described with statistical values but is only comparative. To state biological age of a given organism, certain values of physiological conditions are compared between manipulated and wild type organisms at a given chronological time-point. Among such markers are proteasomal, autophagic and mitochondrial activity (ROS production, energy and O_2 consumption). Moreover, in *C. elegans* it is known that life-extending treatments often lead to increased stress resistance, altered pharyngeal pumping rate, and shift or extension of the reproductive phase. Therefore these markers are common read-outs to define biological age (Collins et al., 2008). Biological age indicates the health-span, the time span of healthy ageing of an organism. Delayed onset of age-related diseases is defined as extended health-span and is the primary aim of ageing research.

1.3.2 What makes C. elegans *an outstanding organism for ageing studies?*

C. elegans has multiple advantages as a model organism for ageing research. Certain *C. elegans* longevity pathways, like insulin signaling and the TOR pathway as well as cellular downstream functions involved in ageing are conserved to higher organisms. Compared to other multicellular organisms, *C. elegans* has a short lifespan and a short generation time, it can be cultured on agar plates and fed with *E. coli* (Brenner, 1974). Usually, *C. elegans* populations consist of hermaphrodite animals each giving rise to about 250-300 genetically identical progeny through self-fertilization. This makes this organism useful for genetic manipulation. Males (XO) can be induced with heat stress to induce nondisjunction events and then crossed to hermaphrodites to investigate epistatic relations. Moreover, genetic manipulation can be achieved by feeding RNAi expressing bacteria or treatment with chemical mutagens like ethylmethansulfonat (EMS). Pharmaceutical approaches can be carried out by simple feeding assay.

Hermaphrodite *C. elegans* rapidly develop from egg to reproductive adult animals via four larval stages (L1, L2, L3 and L4) to adult animals. Larval stages are separated by molts. Sexually mature animals give rise to progeny for approximately a week and live up to another 2-3 weeks post-reproductively. Notably, this life history schedule is only seen under favorable environmental conditions. As with other organisms, *C. elegans* has the ability to sense its

environment and adopt developmental and maturational timing according to circumstances. Major factors that can influence these timing events are temperature, population density and food availability (Golden and Riddle, 1984). It is known that for example a modest increase in temperature will accelerate these processes. At 15°C development from egg to sexual maturation takes six days whereas at 25 °C it takes only three days. Notably lifespan is inversely related to temperatures, with animals living longer median in terms of median (m) lifespan at 15 °C (m=32d), than at 20 °C (m=22d) or 25 °C (m =16d).

1.3.3 What can we learn from C. elegans *about human ageing?*

Work with model organisms is important to understand general principles of ageing. For practical, ethical and legal reasons, it is not possible to conduct human ageing studies by introducing certain mutations or by pharmaceutical interventions. The intention to manipulate human lifespan has no medical indication, since ageing is not defined as a disease. Even if it was possible the time of human lifespan makes such studies impracticable. Thus we rely on the research in model organisms. Leaving the questions open: How well are findings in model organisms conserved, and might they actually help to improve healthy ageing in human?

Certainly transferability of basic ageing research to humans has its limitations and not all mechanisms might be conserved. But bioinformatics systems approaches provide strong power to validate data in humans by conducting studies of cohorts with people living significant longer than average. In a cohort of Jewish centenarians a significant enrichment in a certain allele of FOXO transcription factor the downstream transcription factor of insulin signaling was shown (Suh et al., 2008). The longevity of a Japanese cohort was linked to certain variants in the insulin receptor (Kojima et al., 2004). These are two examples of many findings indicating that human lifespan determination is regulated by similar genetic mechanisms as in lower organisms. Moreover with insulin signaling and TOR two nutrient sensing pathways are established as potent lifespan modulators in lower and higher organisms. Thus, they might provide targets to modulate healthy ageing in humans with the aim to cure or even prevent age-related diseases. Up to date small molecules provide the only feasible possibility to conduct such interventions. Thus it is of particular interest to understand their role in model organisms and how the interact with longevity pathways.

2 Small molecules interact with the genetics of ageing

With the understanding, that ageing is modulated by genetic networks emerging interest in finding small molecules that modulate such networks to manipulate mammalian lifespan arose. In 2008 the National Institute on Aging (NIA) established the so-called "NIA Interventions Testing Program" to test compounds for their lifespan extending effects in mice. Their concept is to feed compounds to otherwise regular fed wild type mice in parallel to three different cohorts at three different locations. With that they gain unbiased and statistical evaluable data. So far, rapamycin, aspirin, and nordihydroguaiaretic acid (males only) were identified to extend lifespan in genetically heterogeneous mice (Miller et al., 2013; Strong et al., 2008), and other compounds like resveratrol and simavastin were shown to have no effect under these conditions (Miller et al., 2011). Multiple molecules that are tested by this program are suggested based on their lifespan modulating capacity in other organisms. In particular over the past ten years, many of such molecules have been identified, especially in *C. elegans* and *Drosophila melanogaster* (Lucanic et al., 2013). Some of them could clearly be linked to one of the known longevity pathways, whereas the role of others remains elusive. Many of these compounds have been identified in screens, where small molecules were simply fed to model organisms. This feeding approach is prone to certain problems, though. First, it needs to be considered that the uptake of molecules varies according to their chemical properties. Second, bacteria and yeast, which are used as food sources for *C. elegans* and *Drosophila*, respectively are living organisms. They might metabolize fed compounds and change amount or even quality of the tested small molecule.

The following paragraphs summarize a) the genetic underlying ageing pathways, b) how small molecules can influence such pathways and c) how ageing pathways are connected to build a complex network. It is graphically summarized in ***Figure 3*** at the end of this section.

2.1 Insulin/IGF-1 Signaling (IIS) and the role of DAF-16/FOXO in ageing

2.1.1 IIS and DAF-16/FOXO nuclear localization

One of the most intensively studied pathways linked to ageing is the Insulin/IGF-1 signaling (IIS) pathway. Originally it was described in mammalian growth and sugar metabolism and is linked to diabetes and neurodegenerative diseases (Gems and Partridge, 2013). It first appeared as a potential longevity pathway in *C. elegans* in 1988 when it was

shown that mutants of *age-1*, the nematode´s phosphoinositide 3-kinase (PI3K) homologue, are long-lived (Friedman and Johnson, 1988a; 1988b). Five years later, the temperature sensitive mutant allele of *daf-2(ts)*, which encodes the only *C. elegans* insulin receptor (insR) also led to a more than two fold lifespan extension at non-permissive temperature (Kenyon et al., 1993; Kimura, 1997). Therefore IIS not only gave the first hint that longevity can be influenced genetically but furthermore that conserved pathways, and growth hormone signaling are involved in lifespan determination. Studies in mice (Brown-Borg et al., 1996) and flies (Tatar et al., 2001) (Clancy et al., 2001) could show a conserved role of IIS in ageing in higher organisms. In *C. elegans*, ligands for DAF-2/insR are insulin like peptides/insulin (ILP/ins). These hormone peptides are components of the endocrine system. In *C. elegans* 40 ILPs are described, among them two, namely INS-1 and INS-18 that are proposed antagonists of DAF-2/insR (Pierce et al., 2001). Accordingly, transgenic lines expressing *ins-1* as well as *ins-18* overexpressor line phenocopy *daf-2(ts)* phenotypes, underlining their inhibitory effect on DAF-2. Notably *ins-1* is the closest homolog to human insulin and expression of insulin also leads to DAF-2 inhibition. Interestingly, in humans insulin has an agonistic function, though.

In general, it is difficult to assign certain function to specific ILPs, since they have potentially redundant functions (Pierce et al., 2001). Accordingly complexity and redundancy of insulin like peptides for several functions was also shown in *Drospohila* (Grönke et al., 2010). DAF-2/insR itself is a transmembrane receptor tyrosine kinase. When DAF-2/insR is bound by agonistic ILP/insulin the adaptor protein IST-1/IRS is recruited (Wolkow et al., 2002), leading to activation of the AGE-1/PI3K and resulting phosphatidylinositol-3,4,5,-triphosphate (PIP3) (Morris et al., 1996). In the presence of PIP3 and pyruvate dehydrogenase lipoamide kinase isozyme (PDK)-1 (Paradis et al., 1999) AGE-1/PI3K phosphorylates AKT-1/2 (Paradis and Ruvkun, 1998) that in turn together with the serine/threonine-protein kinase (SGK)-1 (Hertweck et al., 2004) phosphorylates the transcription factor DAF-16/FOXO. Phosphorylated DAF-16/FOXO is excluded from the nucleus and sequestrates in the cytoplasm (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), mediated by FTT-2/14-3-3 (Wang et al., 2006). Thus, mutations of *age-1* or *daf-2* counteract phosphorylation of DAF-16/FOXO and promote subsequent nuclear localization. In the nucleus, DAF-16/FOXO promotes expression profiles to enable longevity and stress resistance (Murphy et al., 2003).

2.1.2 Co-regulators of IIS and DAF-16/FOXO

Notably, there are many other factors that regulate DAF-16/FOXO. PIP3 for examples is influenced by DAF-18/PTEN, a phosphatase that converts PIP3 to phopsphatidylinositol-4,5-bisphosphate (PIP2). DAF-18/PTEN therefore indirectly dephosphorylates AGE-1/PI3K and abrogates DAF-16/FOXO nuclear translocation (Mihaylova et al., 1999; Ogg and Ruvkun, 1998). DAF-16/FOXO can also alternately be phosphorylated by c-Jun N-terminal Kinase JNK-1. The E3 ubiquitin ligase RLE-1 provides an additional level of DAF-16/FOXO regulation. It ubiquitinates DAF-16/FOXO for proteasomal degradation (Li et al., 2007). Additionally, it was shown that constitutive localization of DAF-16 to the nucleus is not sufficient to recapitulate longevity and stress resistance of IIS mutants(Lin et al., 2001). This suggests more coactivators that modulate DAF-16/FOXO-dependent expression profiles according to the cell´s demand, such as response to starvation, oxidative, or UV stress. One of these nuclear factors is SMK-1/SMEK. It is required for longevity and thermal resistance but not for UV and oxidative stress response (Wolff et al., 2006). Many factors are involved in the regulation of DAF-16/FOXO nuclear localization and the plasticity of its transcriptional outcome, emphasizing multidimensional regulation of this important transcription factor. (***Figure 1***)

In *C. elegans,* these endogenous modulators are influenced by environmental conditions. Under suitable temperature, low population density and proper food availability, the intracellular phosphorylation cascade is activated and DAF-16 is sequestered in the cytoplasm. Under unfavorable conditions DAF-2 is not activated and DAF-16 enters the nucleus to drive stress resistance and longevity expression profiles (Fielenbach and Antebi, 2008). DAF-16 nuclear localization is a response to environmental challenges, therefore among DAF-16 target genes there are many genes involved in cellular stress response like heat shock proteins (*hsp-16*), superoxide dismutases (*sod-3*), glutathione-S-transferases and cytochrome P450s (Murphy, 2006). Consequently, manipulations leading to DAF-16 nuclear localization are also associated with stress resistance (Murphy, 2006; Murphy et al., 2003) and DAF-16 null mutants like *daf-16(mgdf50)* and *daf-16(mu86)* are short lived, less stress resistant and suppress phenotypes of upstream IIS mutants like *daf-2(e1368)* and *age-1* (Kenyon et al., 1993; Larsen et al., 1995; Ogg et al., 1997). In addition, in *C. elegans* SKN-1/Nrf was identified as second target for IIS phosphorylation. SKN-1/Nrf promotes longevity DAF-16 independently (Tullet et al., 2008) and is an upstream component of the cellular xenobiotic stress response.

Another downstream component of DAF-16/FOXO is *lipl-4* a triglyceride lipase, essential for IIS longevity. Its knockout abrogates *daf-2(ts)* longevity and overexpression is sufficient to extend lifespan (Wang et al., 2008). Most notably, a second lipase *lips-7* was shown to be essential for DAF-16 dependent longevity induced by the conserved NAD(H)-dependent corepressor CTBP-1 (Chen et al., 2009). These findings connect nutrition sensing and IIS function to induced longevity and alterations in fat metabolism. Taken together, many genetic, pharmaceutical, and environmental manipulations lead to ageing phenotypes depending on IIS and in particular on specific nuclear DAF-16/FOXO activity.

2.1.3 Small molecule modulators of IIS

One of these *daf-16*-dependent ageing modulators is glucose, a component of every diet. Glucose feeding shortens lifespan in *C. elegans*, thus exhibiting the opposite phenotype of a DAF-2 mutant (Lee et al., 2009; Schlotterer et al., 2009). Glucose inhibitory effect on IIS is possibly mediated by the ILPs *ins-7* because *ins-7* mRNA levels are lower under glucose feeding. Neither the exact mechanism of how glucose lowers INS-7 nor whether this is the cause for shortevity was unambiguously shown. Nevertheless, it is clear that the nutritional molecule glucose interacts with the genetics of ageing. Further support for this comes from experiments with 2-deoxy-D-glucose, a synthetic sugar, that restricts glucose availability for worms, and leads to *daf-16* dependent lifespan extension (Schulz et al., 2007).

The same effect on IIS and lifespan was proposed for another sugar - the non-reducing disaccharide trehalose. It consists of two α-α-1-1 glucoside bound D-glucose monosaccharides. It was shown that feeding trehalose (5 mM) to *C. elegans* extends lifespan up to 30%. Moreover it delays the end of reproductive phase and delays onset of age related phenotypes like lower pumping-rate and polyglutamine accumulation, reflecting improved protein homeostasis. These positive effects on health and lifespan of trehalose were linked to IIS, because the longevity phenotype is *daf-2* and *daf-16* dependent. Moreover, trehalose-induced *daf-2* longevity partially depends on the DAF-16/FOXO target genes trehalose-6-phosphate synthase 1 and 2 (*tbs-1* and *tbs-2*) (McElwee et al., 2003; Murphy et al., 2003). Accordingly, *tbs-1* and *tbs-2* mRNA, as well as trehalose levels are increased in *daf-2(ts)* background. Unfortunately, it is not known if adding trehalose to *tbs-1* and *tbs-2* RNAi-treated *daf-2* animals restores longevity. Moreover, it was not tested, if transgenic overexpression of *tbs-1* or *tbs-2* abrogates *daf-16* dependence of trehalose-induced longevity. Nevertheless, trehalose is a natural endogenous compound acting downstream of DAF-

16/FOXO to regulate lifespan. Thus, both sugars glucose and trehalose are linked to the IIS function in lifespan. Whereas glucose probably acts indirectly on cellular functions by decreasing DAF-16 induced stress response, trehalose itself has a proteoprotective function as a chemical chaperone to stabilize protein integrity a cellular mechanism linked to youthfulness. Interestingly, it is known that trehalose can be converted to D-Glucose in mammals and nematodes by trehalase (Behm, 1997; Oesterreicher et al., 2001). This metabolic relation adds another level of crosstalk to the complexity of ageing networks. Ratio of glucose and trehalose might play a role for ageing and is in turn influenced by availability food and other sugars. Thus, it suggests that there are more sugars involved in the regulation of IIS longevity. Other endogenous compounds that were shown to affect *C. elegans* lifespan are oxaloacetate and malate. Providing these intermediates of tricarboxylic acid (TCA) cycle increased lifespan DAF-16/FOXO dependently (Edwards et al., 2013; Williams et al., 2009). Glucose is the precursor of glycolysis. The TCA cycle and glycolysis are tightly coupled via the intermediate pyruvate to regulate energy demands of the cell. Thus, these results demonstrate a link between the nutritional sensor IIS, energy homeostasis and longevity. Moreover it suggests involvement of additional intermediates of energy metabolism in the biology of ageing.

2.1.4 Connectivity of IIS within the ageing network

IIS regulates many metabolic processes, among others, by the uptake of glucose. If food availability is reduced, IIS is down-regulated, which in turn enhances cellular recycling of macromolecules by autophagy a component of cell clearance and marker of youthfulness. IIS longevity depends on autophagy. Note that autophagy (Hars et al., 2007; Meléndez, 2009; Meléndez et al., 2003)is distinguished at least into micro-, macro-, mito-, and lysophagy, but it is referred to in this thesis as a single event of cell clearance. As mentioned above, IIS is also linked to fat metabolism, as *lipl-4* is essential for *daf-2(ts)* longevity. Nevertheless the exact role of fat metabolism in longevity remains elusive, it is evident that there is no correlation of body fat and lifespan in *C. elegans*. Long-lived *daf-2(e1368)* have increased adiposity (Kenyon et al., 1993) but on the other hand long-lived mutants like *eat-2*, that are in a genetic induced CR status have decreased fat deposit (Lakowski and Hekimi, 1998) suggesting rather a role for the quality of free fatty acid than for quantity (Ackerman and Gems, 2012). In line with low nutritional intake, IIS inhibition leads to high AMP to ATP ratio (Apfeld, 2004), reflecting a status of low energy availability that activates AMP activated kinase (AMPK). AMPK is a central molecule in the regulation of energy

homeostasis and longevity in response to starvation and other stresses like heat. It was also linked to other lifespan modulators like TOR, CR, sirtuins and CRCT-1 (see following paragraphs). A widely unappreciated role of IIS in worms is that it positively regulates the protein g kinase (PGK)/EGL-4, that in turn regulates feeding behavior in response to satiety (You et al., 2008). Thus, to some extent IIS feedback regulates intake of its own small molecule modulators. Taken together, although IIS is studied intensively there are still open questions. Among others, which particular dietary molecules other than glucose regulate IIS? What is the role of fatty acids and lipase activity in IIS induced lifespan modulation?

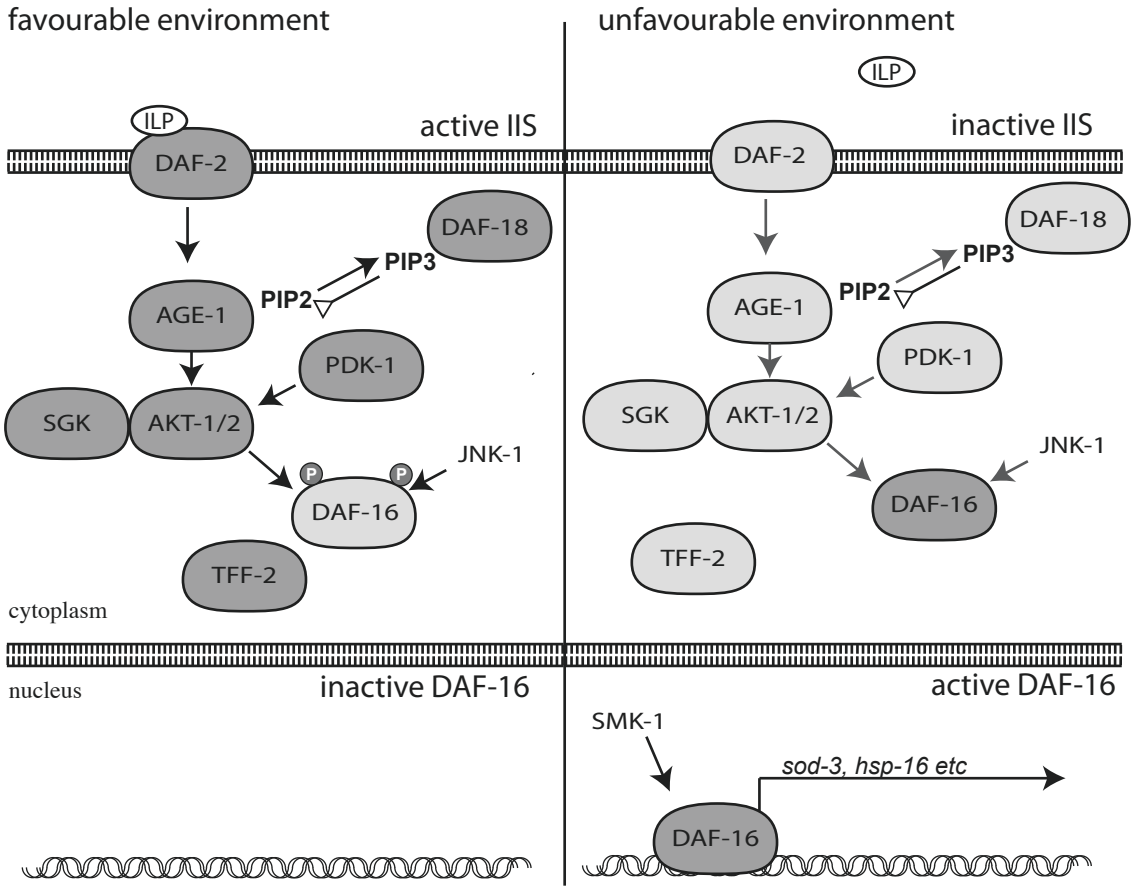


Figure 1 IIS pathway in *C . elegans*

Left panel: activated IIS. Under favourable environmental conditions, binding of agonistic ILPs to DAF-2 activates a phosphorylation cascade (black arrows), that via AGE-1, AKT-1/2, SGK finally leads in the presence of PDK-1 and PIP-3 to phosphorylation of DAF-16. Phosphorylated DAF-16 remains cytosolic and promotes reproductive development and regular life-span. Right panel inactive IIS: Under unfavorable environmental conditions, unbound (or mutated) DAF-2 receptor inhibits phosphorylation of DAF-16, that in turn enters the nucleus to promote stress resistance and longevity. DAF-16 can also be phosphorylated by JNK-1 and needs nuclear co-factors like SMK-1.

2.2 TOR signaling and the role of caloric restriction in ageing

2.2.1 TOR signaling, caloric restriction and AMPK

For TOR signaling indeed the small molecule regulating it was identified first and the pathway is named after it – **T**arget **O**f **R**apamycin. Just like IIS, TOR strongly responds to quality and quantity of nutritional intake. TOR senses amino acid and is activated by high ATP to AMP ratio. It is also target of growth hormones. In mammalian systems the core protein of TOR signaling is mTOR. It is a Serine/Tyrosine kinase of the PI3K family forming two complexes: mTOR complex I and mTOR complex II (mTORC1 and mTORC2). mTORC1 regulates several cellular function linked to ageing: it represses autophagy, promotes lipid synthesis and regulates mitochondrial function via HIF-1α. Active mTORC1, therefore reflects a cellular status of growth and in line with this promotes higher translational activity and protein syntheses. In yeast, worms, flies and mice these outcomes were linked to the translation factor S6 kinase (S6K) (Hansen et al., 2007; Pan et al., 2007; Steffen et al., 2008). Accordingly, in *C. elegans* knockout or knockdown (RNAi) of mTOR/let-363 or the mTORC1 component *daf-15/raptor* lead to longevity (Jia, 2004; Vellai et al., 2003). mTOR knockdown mediated longevity is conserved in *Drosophila melanogaster* (Kapahi et al., 2004)and *Mus Musculus* (Harrison et al., 2009).

Notably, other than mTORC1 inhibition, the only intervention extending lifespan throughout all model organisms from yeast to rodents is CR (Braeckman et al., 2001; Chapman and Partridge, 1996; Jiang et al., 2000; Lakowski and Hekimi, 1998; Weindruch et al., 1986; Yu et al., 1985). CR-longevity in *C. elegans* was shown to be induced via several different regimes: reduced pumping rate, bacterial dilution, in solid or liquid medium, feeding or no feeding (Greer et al., 2007; Honjoh et al., 2009; Kaeberlein et al., 2006; Klass, 1977; Lakowski and Hekimi, 1998). CR depends on *pha-4*. PHA-4 is a FOXA forkhead transcription factor homolog and mTORC1 has been placed downstream of it in many epistatic experiments in yeast, *C. elegans* and flies (Hansen et al., 2007; Kaeberlein et al., 2005; Zid et al., 2009)Based on these findings CR is thought to mediate longevity via mTOR inhibition. Nevertheless, the actual relation of TOR and CR seems to be more complex. In *C. elegans* it was shown that the different CR regimes lead to activation of multiple different downstream targets of TOR and interestingly, only bacterial dilution on solid medium was DAF-16 dependent (Greer and Brunet, 2009). Moreover *C. elegans* knockdown of S6K had an additive effect on CR induced longevity contradicting the suggested epistatic relation

(Hansen et al., 2007).

Another link between mTOR and CR longevity is the AMP-activated protein kinase (AMPK). AMPK senses the ratio of AMP to ATP and is activated under low energy availability such as under starvation and CR conditions. Accordingly AMPK overexpression is sufficient to extend lifespan in *C.elegans* (Apfeld, 2004). AMPK phosphorylates tuberous sclerosis protein 2 (TSC2). TSC2 in turn inhibits mTORC1(Inoki et al., 2003). Therefore AMPK couples low ATP availability e.g. during CR to TOR signaling. Nevertheless, regarding longevity phenotypes this hypothesis is discussed controversially. In *C. elegans*, which lacks TSC2, it was shown that AMPK - dependent on *daf-16* - mediates longevity via the worm homologue of CREB regulated transcriptional coactivator (CRTC-1) by rendering it cytoplasmic (Mair et al., 2011). Cytoplasmic sequestration of CRTC-1 was also shown under starvation activated AMPK (Inoki et al., 2003). Taken together, there is a broad consensus that CR is mediated partially via TOR signaling, but the exact mechanism and the role of AMPK remains largely elusive. This example outlines how nutritional availability and most certainly specific small dietary molecules can modulate energy availability and the activity of genetic pathways.

2.2.2 Small molecules modulators of TOR signaling- and AMPK

Consistent with its regulation by nutritional availability mTORC1 is also activated by amino acids (REF). Apart from these and other postulated nutritional small molecules the most prominent small molecule modulator of TOR is rapamycin. Rapamycin was extracted from the *Streptomyces hygroscopius* named after its place of discovery, RapaNui (Easter Island). It binds FK506-binding protein FKBP12, a unit of mTORC1, and thus inhibits mTORC1 activity. It is used to treat chronic inflammation, to prevent organismal rejection of organ transplants and as cancer therapy. Only mTORC1 but not mTORC2 was identified as a direct target of rapamycin, first in yeast (Cafferkey et al., 1993; Kunz et al., 1993) and later in human cells (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995; Stan et al., 1994). Although rapamycin targets solely mTORC1, mTORC2 plays an important role when rapamycin is used as a drug. It can be influenced by prolonged rapamycin treatment, since mTOR might be sequestered from mTORC2. Interestingly, mTORC2 accounts for many side effects of chronic rapamycin treatment, like glucose intolerance and altered lipoprofiles (Lamming et al., 2012) mTORC2 is not required for rapamycin-induced longevity (Lamming

et al., 2012). Rapamycin is one of the compounds confirmed as a lifespan extending drug in mammals by the NIA Interventions Testing Program (Harrison et al., 2009).

Grandison et al. showed that in flies amino acid quality plays an important role in CR regulation. CR in flies reduces fecundity and extends lifespan. Availability of methionine alone was sufficient to rescue CR-induced reduction of fecundity but not longevity. Adding back all other essential amino acids without methionine to CR restricted flies does not rescue longevity, whereas provision of all essential amino acids including methionine abrogates longevity. Additionally, methionine starvation is sufficient to extend lifespan in flies and mice. Taken together these findings suggest that rather the balance of certain amino acids play a key role in CR longevity (Grandison et al., 2009). A concept that might be true for other lifespan extending compounds as well e.g. glucose vs. trehalose. However it emphasizes the possibility of single small molecules treatments to understand ageing networks, and highlights that especially metabolites can lead to imbalance modulating longevity and potentially healthspan.

Metformin represents a different category of small molecules - it is a synthetic small molecule that was described to extends lifespan in *C. elegans*, mice and flies. It is used as anticancer drug and has approval of national drug administration (NDA) since 1994 as an antidiabetic drug against type II diabetes in humans. In mice, metformin activates AMPK to represses TOR via TSC2 and extends lifespan of otherwise short-lived cancer prone mice (female only) (Anisimov, 2010; Anisimov et al., 2010). Metformin was also reported to extend lifespan in wild type *C. elegans*. It is described to mimic CR status and in agreement does not further extend CR longevity in *C. elegans* (Onken and Driscoll, 2010). Notably concentrations used in the study by Onken and Driscoll, are rather high. Only 50 mM but not 10 mM metformin could induce longevity conditions that might suggest a partial involvement of a stress response to osmolarity. Increased osmolarity was linked before to transcriptional targets of DAF-16, a major regulator of lifespan (Lamitina, 2005). Interestingly, it was described recently, that metformin alters the folate-metabolism of *E. Coli*, the food of *C. elegans* food, and that this accounts for longevity (Cabreiro et al., 2013). This study uncouples metformin-induced longevity from AMPK signaling. In line with this, metformin was shown to inhibit TOR independent of AMPK in *Drosophila* cell culture (Kalender et al., 2010). Taken together, the metformin mechanism to extend lifespan is not completely understood and might differ in different model organisms.

Another synthetic molecule that was shown to activate AMPK is 50-aminoimidazole-4-car-boxamide-1-b-D-ribofuranosid (AICAR). It was shown to activate AMPK in muscles of young rats (Reznick et al., 2007). It was not tested if AICAR extends lifespan so far but might help to understand the role of AMPK within the ageing network. In sum, the connection of AMPK activity and TOR to regulate lifespan in *C. elegans* is not unambiguously understood and also the role of pharmaceutical AMPK activators is not clear up to date.

A third group of molecules linked to CR longevity is N-acylethanolamines (NAEs). NAEs are lipid signaling molecules and part of the endocannabinoid signaling pathways. Provided in a feeding assay they suppress CR- and TOR-induced longevity (Lucanic et al., 2011). Notably NAE are synthesized from fatty acids - connecting fat metabolism to TOR and CR. The small molecule group of lipid-derived signaling molecules has great potential to connect metabolic status to genetic modulations of ageing (see also section 4).

2.2.3 Connectivity of TOR signaling, caloric restriction and AMPK in the ageing network

Interactions between TOR and IIS were shown in many instances. First, both pathways are regulated by quality and quantity of food availability – they are nutrient sensors. But they also interact on genetic level. mTORC1 was shown to be activated by insulin, PI3K and AKT kinase signaling (Potter et al., 2002). Second, mammalian mTORC1 regulates availability of IRS via S6K (Takano et al., 2001). In mammalian cells, tumor suppressor genes tuberous sclerosis protein I (TSC1) was shown to be a target of FOXO3 a downstream transcription factor of IIS. TSC I in turn represses mTOR (Inoki et al., 2002). Despite these established genetic crosstalk - a clear epistatic relation of both pathways to regulate lifespan remains largely elusive. It is known that in *C. elegans daf-16(null)* suppresses *daf-15*/raptor longevity (Jia, 2004) placing TOR upstream of IIS or at least upstream of DAF-16/FOXO. In contradiction to this, mTOR mutant longevity and longevity induced with rapamycin are DAF-16/FOXO independent (Robida-Stubbs et al., 2012; Vellai et al., 2003). The same was shown for mutations of mTOR downstream targets S6K and eIF4E, placing mTOR and its targets either downstream or in parallel to IIS (Hansen et al., 2007; Pan et al., 2007). Common consensus is that IIS and mTOR have no clear epistatic relation but regulate overlapping targets, like *lipl-4* (Kapahi et al., 2010; Wang et al., 2008). In *C. elegans* gonadal longevity (see below), TOR regulates *lipl-4* expression. This example again underlines the potential role of fatty acid as modulators of ageing networks.

2.3 The role of sirtuins in ageing

2.3.1 Sirtuins

Sirtuins are highly conserved NAD^+ - dependent deacetylase family, class II histone deacetylases that have been originally described as silent information regulators (Rine and Herskowitz, 1987) targeting histone and non-histone proteins (Haigis and Guarente, 2006). Later, in yeast Sir2 was shown to be a genetic modulator of lifespan. Sir2 overexpression led to an extension of replicative lifespan and deletion led to shortened replicative lifespan (Kaeberlein et al., 2006).

2.3.2 Small molecule modulators of sirtuins

Resveratrol is probably the most controversially discussed molecule that might play a role in lifespan regulation. It is a polyphenolic compound first isolated from plant roots and received wide attention when a cardio protective role was first described in 1992 (Renaud and de Lorgeril, 1992). At the same time it was found in red wine - potentially solving the so called French Paradox, describing a low rate of cardiovascular disease in French population but a comparatively high fat diet (Liu et al., 2007). Subsequent studies revealed a perspective role for resveratrol in the protection against age related diseases. It was shown to extend lifespan in yeast (Howitz et al., 2003), worms (Wood et al., 2004), flies (Bass et al., 2007) and in mice that receive high fat diet (Baur et al., 2006). Due to these results resveratrol raised public attention. However, subsequent studies revealed a more complex role. First, resveratrol did not extend lifespan of mice on regular food (Pearson et al., 2008). Second, not all experiments in yeast, flies and worms, could reproduce lifespan extension of earlier studies (Timmers et al., 2012). Another question that remains elusive is if resveratrol really directly binds to sirtuins. Different model organisms have been used to address this question and at least two reports raise doubts about a direct resveratrol-sirtuin interaction. First, resveratrol fails to induce SIRT1 deacetylation of its endogenous targets (Beher et al., 2009). Second, in *C. elegans*, other than *sir-2.1* (oe) induced longevity, resveratrol longevity is DAF-16/FOXO independent (Viswanathan et al., 2005). Thus, if they have common targets at all they must be downstream of DAF-16. This strongly suggests that resveratrol does not regulate *sir-2.1*. Furthermore many of these studies found resveratrol-induced phenotypes of dietary restriction but only some were SIRT1 dependent (Kaeberlein and Powers, 2007).

2.4 Connectivity of sirtuins within the aging network

Another effect of resveratrol is increased energy expenditure and therefore in mice it protects against obesity and obesity related diseases under high fat diet (Baur et al., 2006). This high energy expenditure is probably based on a higher number of mitochondria, that is induced by Sirtuin activation of PCG-1α, the key- regulator of mitochondrial biogenesis (Lagouge et al., 2006). In other studies AMPK is identified as a target of resveratrol, which could as well explain the increased mitochondrial activity (Hwang et al., 2007). This is further supported by the finding that resveratrol activates AMPK in mouse embryonic fibroblast cell cultures of SIRT1 knockout mice uncoupling it from Sirtuin. Another report showed activation of SIRT1 by NAD^+ induced AMPK activity, placing it rather downstream of AMPK (Cantó et al., 2009). Sirtuins are therefore closely connected to TOR, CR and mitochondrial biogenesis via AMPK. As mentioned, *C. elegans sir-2.1* overexpression extends lifespan *daf-16* dependent. It was suggested that *sir-2.1* activates DAF-16/FOXO by deacetylation since mammalian SIRT1 was described to act on mammalian FOXO proteins in response to oxidative stress. Consistently, oxidative stress can likewise induce longevity (see below) in *C. elegans* and this is *sir-2.1* and *daf-16* dependent (Heidler et al., 2009). Taken together, sirtuin-activity seems to promote longevity, but the mechanism is far from being understood. Part of the problem might be that sirtuins can have many targets.

3 The role of cellular functions in ageing

Taken together we only just begun to understand how genetic networks and small molecules interact to sustain cellular functionality and youthfulness. But one important aspect was not discussed so far: What exactly do these pathways regulate? How do cells, tissues and organisms become long-lived? What are the physiological functions that need to be improved?

3.1 The role of Translational activity in ageing

One cellular function that was linked to ageing is protein translation. Down regulation of translation was shown to be sufficient to increase lifespan in yeast, worms, flies and mice (Kaeberlein et al., 2006). It is one of the effects of reduced mTOR signaling mediated via S6K. Accordingly, in *C. elegans* (Hansen et al., 2007; Pan et al., 2007) knockdown of certain ribosomal proteins and translation initiation factors also leads to longevity and in flies, overexpression of 4E-BP results in longevity (Zid et al., 2009). Moreover at least in yeast and worms, knockout mutants of S6K have less global mRNA translation and less protein synthesis (Pan et al., 2007). But what are the lifespan extending effects of reduced translation? Translation is an energy consuming process and its down-regulation might allow a shift of energy to cell maintenance processes implicated by less cell proteotoxicity. It was hypothesized that attenuated translation and reduced protein abundance lead to more efficient cleaning of damaged and misfolded proteins and thus preventing structural and functional decline of the cell. Although this might be part of the beneficial effect it might also be that longevity appears due to a certain subset of mRNAs that is differentially translated under mTORC1 inhibition. Evidence for this hypothesis comes from yeast, worms and flies, where special 5´ structures were suggested to mark a subset of mRNAs for TOR regulation. The mammalian situation might be even more complicated since S6K mutation in mice leads to longevity but no translational inhibition was observed (Selman et al., 2009). Thus, translational activity has clearly been linked to longevity, but again the exact mechanism and role of TOR signaling in it remains elusive.

3.2 The role of mitochondrial activity and ROS in ageing

3.2.1 Mitochondrial Longevity and ROS

Apart from being the powerhouse of the cell, mitochondria have multiple functions in regulation of cellular physiology. They are involved in multiple metabolic pathways, calcium signaling, apoptosis and ROS signaling. Mitochondria are the place of oxidative phosphorylation in enzymatic transport of electrons across the inner mitochondrial membrane by the electron transport chain (ETC). This results in the release of protons into the mitochondrial intermembrane space to create a potential over the inner mitochondrial membrane. This potential provides the energy to drive ATP synthesis. Downregulation of mitochondrial function by inhibiting components of ETC was found as a conserved longevity mechanism in worms, flies and mice (Copeland et al., 2009; Ewbank et al., 1997; Feng et al., 2001).

The question how inhibited ETC extends lifespan is not completely understood. Interestingly, in *C. elegans* there are also mutations of ETC inducing the opposite phenotype-shortevity (e.g. *mev-1*) (Ishii et al., 1998) and longevity (*isp-1*) (Yang and Hekimi, 2010). One explanation for longevity of these mitochondrial mutants is an alteration in reactive oxygen species (ROS). During respiration ROS are generated. ROS can start chain reactions with free radicals that oxidize and damage molecules like fatty acids, DNA and proteins. DNA and protein integrity are important markers of youthfulness (López-Otín et al., 2013). ROS damage was therefore one of the first hypotheses to explain ageing (HARMAN, 1956). The cell's way to counter oxidative stress is to initiate the oxidative stress response. This response includes expression of glutathione-S-transferases and superoxide dismutases to prevent oxidation of molecules and to clean out damaged molecules. Support for the idea that oxidative stress plays a role in lifespan arose from the finding that long-lived *age-1* worms (Friedman and Johnson, 1988a; 1988b) were significantly more resistant to oxidative stress and to the superoxide anion inducing paraquat (Vanfleteren, 1993). In general, the activation of DAF-16/FOXO stress target genes in many long-lived mutants seems to support this hypothesis. But there are other papers supporting an idea that increased ROS stress rather extends than shortens lifespan (Schulz et al., 2007). Notably also in mice a genetic up-regulation of cellular antioxidant functions could not extend lifespan (Bokov et al., 2004;

Pérez et al., 2009b). To date it is not clear if ROS modulates ageing by causing molecular damage - and the oxidative stress hypothesis of aging is questioned and under intensive investigation (Gems and Partridge, 2013; Pérez et al., 2009a). Part of the issue of addressing the role of ROS in ageing is the effect of mitohormesis. Hormesis describes the phenomenon that a small dose of toxicity in early life can have beneficial effects later in life. Mitohormesis states that minor increases in ROS themselves might contribute to longevity by inducing cellular stress defense mechanisms (Schulz et al., 2007) including particular mitochondrial unfolded protein stress response (mitoUPR). The cell's stress response is far-reaching and helps clearing out damages on every level, which in turn might lead to a more healthy state of the organism than before the stress cue.

3.2.2 Small molecule modulators of mitochondrial activity and ROS

Nevertheless, based on the idea that lowering ROS levels might extend lifespan, antioxidants were tested for life-extending effects. Antioxidants are mostly reducing compounds that inhibit the oxidation of macromolecules by free radicals. Interestingly, experiments with antioxidant compounds revealed promising but still contradictory results. So far no compound was found that could reproducibly prolong lifespan in *C. elegans*, *Drosophila* and mice. Nevertheless, some antioxidants did affect lifespan at least in one or two model organisms. In drosophila, N-acetylcystein (NAC) was found to extend maximum and median lifespan by 27% (Brack et al., 1997). NAC is a precursor of the antioxidant glutathione, helping to refold proteins. Also in worms the synthetic antioxidants (EUK-134&EUK-8) extended lifespan (Melov et al., 2000). Notably another group could not repeat the results with EUK-8 (Keaney et al., 2004). Gamma-tocopherol (vitamin E) slightly increased lifespan in *C. elegans* but not in drosophila whereas α tocopherol (Vitamin E) had no effect on both model organisms (ZOU et al., 2007). α-lipoic acid extends lifespan in worms (Benedetti et al., 2008; BROWN et al., 2006) and female flies (Bauer et al., 2004) but not in mice (Lee et al., 2004). On the other hand tocotrienol, an even stronger antioxidant, prevents protein carbonylation and extends lifespan of *C. elegans*. Coenzyme Q10 (ubiquinone) was shown to extend lifespan in *C. elegans* (Ishii et al., 2004), but a Q10 free diet also had a lifespan extending effect (Larsen and Clarke, 2002). A high variance in results between model organisms makes an underlying biological principle questionable. Antioxidants are small molecules and as such they might have additional effects on other metabolic processes. Furthermore, considering hormetic effects, feeding antioxidants must be

timed precisely because they might have a narrow time and dose window to act. Experiments in different organisms are therefore hardly comparable.

3.2.3 Connectivity of mitochondrial activity and ROS within the aging network

There are direct links between mitochondrial activity and the longevity pathways IIS and TOR. The highly conserved transcription factor HIF1-α is a downstream factor of TOR signaling and its translation and stability is promoted by active mTORC1(Johnson et al., 2013). Under normoxic conditions it is targeted by the cullin ubiquitin E3 ligase VHL-1 for proteasomal degradation (Mehta et al., 2009; Müller et al., 2009). But under low oxygen conditions (hypoxia) it is activated and promotes a metabolic shift from oxidative phosphorylation towards glycolysis to produce ATP. ATP in turn is reflecting energy availability and might influence AMPK activity. In contrast, long-term hypoxia can lead to mTORC1 downregluation (Zhang et al., 2009). In *C. elegans,* lifespan extending effects of *hif-1* null mutants were shown at 20°, 25° but not at 15°C, adding a temperature dependence to the system. Moreover, stabilization of HIF-1 VHL-1 knockout also leads to longevity IIS and CR independent (Mehta et al., 2009). Wheras another group found the mechanism to be daf-16/FOXO dependent (Zhang et al., 2009). In flies mRNAs coding for components of the respiratory chain are widely immune to TOR induced down-regulation of translation.

3.3 The role of cell clearance in ageing

3.3.1 Proteostasis and autophagy

As mentioned above, one mechanism to improve cellular functions broadly is to assure protein integrity (proteostasis). Many longevity mutations have been shown to actually improve protein homeostasis (He et al., 2013). Proteins fulfill tasks in every essential cellular function: in transcription, in translation, in enzymatic processes and as structural components. Moreover they fulfill communicational task either by forming direct cell-cell contacts such as integrins or connexins or in the endocrine system as peptide-hormones, such as insulin or growth hormone. It is understood that proteostasis is declining with age (Douglas and Dillin, 2010). Thus, many neurodegenerative diseases caused by proteotoxicity are age-related. Corea Huntington, Alzheimer and Parkinson disease are caused by misregulation of certain proteins namely huntingtin, α-synuclein and β-amyloid, respectively. These proteins all form amyloid-like inclusions (Carrell and Lomas, 1997). Amyloids are fibrillar aggregates,

harboring β-sheets that form hydrogen bonds with other β-sheet-rich proteins leading to amyloid depositions in neuronal cells (Selkoe, 2003). Recently a paradigm shift is supported by the finding that accumulation of amyloids can have beneficial function in yeast (Shorter and Lindquist, 2005; True et al., 2004)and humans (Fowler et al., 2006). Currently it is believed that a soluble toxic intermediate during amyloid formation might rather be the cause for deteriorating effects, and the formation of insoluble amyloid structure is part of the cell's response to defend against proteotoxicity. In line with this idea are recent findings in Alzheimer mouse models (Chui et al., 1999; Hsia et al., 1999; Kumar-Singh et al., 2000; Tomiyama et al., 2010). In *C. elegans*, tissue culture, and mice it was shown that promoting the formation of insoluble aggregates from the presumably more toxic soluble oligomer A-β structures ameliorates deteriorating effects (Cheng et al., 2007; Cohen et al., 2006).

The integrity of protein homeostasis under the influence of intrinsic and external stressors is a major challenge for all cells. In principle there are two major sites for cells to assure proper homeostasis under stress conditions. One approach is to prevent proteotoxicity by increasing mechanisms involved in proper protein glycosylation and folding. Chaperones help in this context to protect spontaneously folding of nascent polypeptide chains that are release from the ribosome. The other approach is to defend proteotoxicity by increasing clearance mechanisms. Such mechanisms are mitochondrial unfolded protein responses (mt)UPR, endoplasmic reticulum (ER)UPR and heat shock response- all of the above also include increased chaperone activity. Moreover two highly conserved mechanisms autophagy and proteasomal degradation are involved in clearance of misfolded and dysfunctional proteins. Proteasomes degrade ubiquituinated proteins. It is a highly specific process and involves different E3 ligases that attach the right upbiquitine chain structure (Komander and Rape, 2012). Autophagy is a process in which cell-organelles and macromolecules are delivered to the lysozyme for degradation (Cuervo, 2008). Especially under starvation, autophagy is used to recycle amino acids and other molecular building blocks such as fatty acids (Mizushima et al., 2008). Evidence suggested that its performance declines with age and, moreover, gonadal longevity (see below) and IIS longevity are autophagy-dependent. Interestingly it was shown that in some cases ubiquitin can also function to mark proteins for autophagic vesicles. Autophagy therefore is a good candidate as an ageing modulator. Indeed in a recent paper it was even shown that up-regulation of autophagy alone is sufficient to extend lifespan of mice (Pyo et al., 2013).

3.3.2 Small molecule modulators of cell clearance mechanisms

In particular, its established role in neurodegenerative diseases makes proteotoxicity a promising target for drug development against age-related diseases. In *C. elegans* aggregation-models, many pro-longevity compounds ameliorate formation of aggregates (Dillin and Cohen, 2011). Interestingly most of them act on one of the upstream pathways. Recently a compound that seems to directly affect proteostasis has been identified. N-acetylglucosamine is an upstream compound of N-linked glycan oligosaccharides that are synthesized in the hexosamine pathway and attached to proteins in the ER for proper function. Feeding of N-acetylglucosamine as well as genetic activation of the hexosamine pathway could extend *C. elegans* lifespan. This longevity is depending on ER-associated protein degradation, proteasomal activity, and autophagy, three mechanisms that are linked to proteostasis (Denzel et al., 2014). Nevertheless, it remains elusive if the improved N-glycosylation of only one protein or of a certain set of proteins mediates longevity or if the overall improved N-glycosylation leads to lifespan extension. Furthermore, as mentioned above trehalose was suggested to extend lifespan as a chemical chaperone involved in protein protection (Honda et al., 2010).

A third group of small molecules that extend lifespan by improving cell clearance mechanism, namely autophagy, are ω-6 polyunsaturated fatty acids (PUFAs). It was shown that ω-6 PUFAs are increased upon *lipl-4* activation and that they in turn increase autophagy (O'Rourke et al., 2013). Furthermore autophagy performance is improved by another small molecule the endogenous polyamine spermidine. It was found to extend lifespan in yeast, worms and flies. Lifespan extension depends on up-regulated autophagy (Eisenberg et al., 2009).

Regarding the complexity of protein homeostasis it is likely that there are more small molecules that might directly affect protein integrity. Among those might be sugars involved in glycosylation, amino acids as protein building bocks, chemical chaperones and signaling molecules reflecting starvation and activate cell clearance mechanism like autophagy.

3.3.3 Connectivity of cell clearance within the aging network

IIS-, TOR- and sirtuin- longevity was shown to be modulators of proteostasis and depend on autophagy. CR can delay onset of conformational pathogenic proteins in an amyloid model of *C. elegans* (Steinkraus et al., 2008). This beneficial effect of CR might be conserved, since

in mice (Duan and Mattson, 1999; Wang et al., 2005) and human (Hendrie et al., 2001; Luchsinger et al., 2002) deleterious effects of neurodegenerative related symptoms have been found to be ameliorated by direct or indirect effects of CR. It can be assumed that proteostasis is one of the most important cellular functions to assure organisms health and youthfulness and that it is a downstream target of most if not all longevity pathways. Identification of small molecule modulators to improve the clearance of toxic proteins might therefore be the most promising target to develop drugs against age-related diseases.

4 Endocrine signaling and the role of the reproductive system in ageing

4.1 Gonadal longevity in *C. elegans*

So far, I have discussed how genetic pathways regulate cellular function to influence ageing. The next paragraph is focused on how tissues communicate to coordinate and orchestrate lifespan in the whole organism. Usually tissues communicate by endocrine signaling. Hormones are secreted from one tissue to bind to a receptor on cells of another tissue to induce transcriptional changes. This hormonal control traditionally was investigated in growth, development and physiology. But an open question that remains is: which hormones might be involved in the regulation of longevity?

Interesting first insights to this came from the reproductive system of *C. elegans*. Here it was shown that microsurgery to ablate reproductive germline precursor cells Z2, Z3 of L1 larvae abolishes germline stem cells, while maintaining the somatic germline, and extends lifespan by 60% (Hsin and Kenyon, 1999). Genetic removal of the germline by *glp-1*/notch receptor knockout also extends *C. elegans* lifespan (Arantes-Oliveira et al., 2002). This so-called gonadal longevity was found in flies (Flatt et al., 2008) and mice (N. Kagawa et al., 2010). Interestingly, it is not due to a simple trade-off of energy from germline to somatic tissue as additional removing of the somatic gonad abrogates longevity (Hsin and Kenyon, 1999). This suggests that there are opposing endocrine signals produced by germline and somatic gonad to influence whole organism ageing. Removal of life shortening signals of the germline induces longevity but additional removal of life lengthening signals from somatic gonad that abrogates longevity. The identity of both these signals are not unambiguously understood yet, but a steroid hormone and micro RNAs play an important role and will be subject for further investigations.

4.2 Connectivity of gonadal longevity within the ageing networks

Endocrine signaling adds a third dimension to the ageing network; it demonstrates that ageing is not only about the epistatic and temporal order but also about spatial organization of cellular processes. Gonadal longevity is strictly dependent on a number of IIS associated factors, DAF-16/FOXO (Hsin and Kenyon, 1999), DAF-18/PTEN (Berman and Kenyon,

2006), HSF-1 (Hansen et al., 2005) and SMK-1/SMEK-1 (Wolff et al., 2006). But whereas in reproductive animals IIS inhibition induces DAF-16/FOXO translocation to the nucleus in intestinal cells and neuronal cells, germline ablation only induces DAF-16/FOXO nuclear localization in the intestine, pointing to the importance of the intestine to mediate gonadal longevity (Yamawaki et al., 2010). It seems evident that an endocrine signal from somatic gonad induces longevity, and that this signal needs to target IIS signaling in a specific tissue, namely the intestine to modulate lifespan. Notably, mediation of gonadal longevity is more complex than just via IIS, since there are more essential factors specific for gonadal longevity like the anykrin-repeating transcription factor kri-1/KRIT-1, which is essential for DAF-16/FOXO nuclear localization specifically in gonadal but not in IIS longevity (Berman and Kenyon, 2006).

Another factor is the transcription elongation/splicing homologue TCER-1 (Ghazi et al., 2009), outlining that it is not only *daf-2*/InR that is directly targeted by the somatic germline longevity signal. Another target of the gonadal longevity pathway is fat metabolism. First, as mentioned above, gonadal longevity depends on the activity of the lipase *lipl-4*. Second, it is dependent on PHA-4/FOXA to induce autophagy (Lapierre et al., 2011). Thus, it seems essential to gonadal longevity that autophagy and lipases together modulate fat metabolism. Third, it was shown that stearoyl-Co-A-Δ9-desaturase FAT-6 is essential for gonadal longevity. It desaturates stearic acid to oleic acid. *Fat-6* expression as well as oleic acid levels are up-regulated in germline-less animals. Knockdown of *fat-6* abrogates gonadal longevity and it can be restored by nutritional oleic acid. Interestingly, *fat-6* is regulated by NHR-80/HNF4α-like (Goudeau et al., 2011). NHR-80 is an orphan nuclear hormone receptor suggesting the unknown gonadal longevity signal and its mediation involves steroid signaling.

Strongest evidence that the signal from somatic gonad actually is a steroid hormone, comes from a recent papers. It was shown that gonadal longevity depends on the dafachronic acid (DA) pathway (Gerisch et al., 2007; Shen et al., 2012; Yamawaki et al., 2010). The DA pathway is a lipophilic hormone synthesis pathway first discovered as a key regulator of the dauer pathway. To discuss the role of DA pathway in lifespan determination of germline ablated and also reproductive *C. elegans* it is advisable to first understand metabolic and endocrine properties of the dafachronic pathway.

4.3 The role of steroid endocrine signaling for dauer formation and lifespan in *C. elegans*

Steroid hormones are another group of regulatory hormones. They are small lipophilic molecules that bind to nuclear hormone receptors (NHRs) to regulate transcription of many target genes. NHRs are usually comprised of a ligand binding domain and a DNA binding domain. The number of NHRs strongly varies among species. In drosophila, there are 18 NHRs, in human 48, and in *C. elegans* 284, most of which are orphan receptors, meaning that the ligand is not known. The best-studied NHR in *C. elegans* is DAF-12. It has homology to human vitamin D receptor, farnesoid-X and liver X receptor (VDR, FXR,LXR) and it was found to act in dauer formation, developmental timing and longevity. Moreover it is the major target of the dafachronic acid pathway (Antebi, 2013).

Dafachronic acid is a cholesterol-derived steroid. Steroids are C17 molecules creating three cyclohexane rings (called A, B, C) and one pentane ring (D). Cholestens are special form of steroids they consist of 10 C atoms. Cholestens carrying an OH moiety group in position 3 are called sterols (3- hydroxyl-cholestens). Cholesterol is the simplest sterol and regulates membrane liquidity. But most notably, in mammals it is the precursor for steroid hormones, bile acids and oxysterols. Steroid hormones are important signal molecules in developmental decisions like puberty and metabolic processes like fat metabolism (Wollam and Antebi, 2011). Two, primary bile acids are produced in the liver and released to small intestine where host bacteria produce two other, so called secondary bile acids. Bile acids render micelle formation possible in small intestine. Moreover they are formed to drain spare cholesterol to prevent toxicity (Chiang, 2013). A large group of steroids in mammals are the oxysterols. They are oxidized forms that posses either one or more hydroxyl or keto-groups. They function as signaling molecules and bind to oxysterol binding proteins to regulate cellular processes like lipid metabolism, bile acid production, cholesterol and their own synthesis. In *C. elegans* the DA pathway is the only in detail described steroid pathway with cholesterol as an upstream substrate and is involved in dauer formation and longevity.

4.3.1 Dauer formation and its connection with ageing

As discussed before, environmental conditions and certain small molecules can have a significant impact on genetic networks modulating lifespan of *C. elegans*. During development, *C. elegans* uses similar signals to make another decision - reproductive growth or larval arrest?

Under harsh environmental conditions such as high temperature, crowding or food scarcity, *C. elegans* can arrest development at several stages to enter so called diapauses. Best studied is the L3 larvae alternate dauer arrest that is entered after L2 molting to endure until more favorable conditions occur. Executing the appropriate developmental program must have been evolutionary optimized to integrate a large number of environmental and genetic signals into a binary decision: Dauer Formation - Yes or No?

Accordingly, up to date several pathways have been identified that contribute to this important decision. Originally, many genes were identified in dauer screens (Albert and Riddle, 1988; Morris et al., 1996; Pierce et al., 2001; Riddle et al., 1981; Wolkow et al., 2002) and named according to their phenotype either **da**uer **f**ormation **c**onstitutive (Daf-c) or **d**auer **f**ormation **d**efective (Daf-d). Many of these genes have been placed in pathways and epistatic interactions are described. Notably among them are also ageing pathways like transforming growth factor β (TGF-β), IIS and TOR. All of them impinge on the lipophilic dafachronic acid pathway ultimately regulating the dauer decision (Hu, 2007). Although not all pathways and interconnections are understood completely within the network regulating dauer formation and ageing, the idea immediately suggests that the big overlap results from functional orthology: a vast variety of endogenous and environmental signals needs to be processed and translated into an orchestrated and organism-wide response (Schaedel et al., 2012). For both decisions signals like food availability, temperature and population density are taken into account. From the evolutionary point of view it is likely that a network established to process these signals is used for distinct decisions regarding biological timing, such as dauer entry or survival at different time points during an animal's life history. The concept of using the same molecular switches to guide different decisions in life history was recently illustrated by Horn et al. (Dev. Cell in press). The interaction of the F-BOX protein DRE-1 with its target BLMP-1 regulates dauer formation, epidermal maturation and lifespan (Horn et al., 2014).

4.3.2 Features of dauer larvae

Dauer larvae themselves are long-lived. Compared to reproductively growing worms, dauer larvae feature increased resistance to many different kinds of stresses, such as heat stress, hypoxia, anoxia, UV radiation and food scarcity. They are longer and thinner than L3 larvae due to a radial constriction (Cassada and Russell, 1975). They appear darker under dissecting microscope due to higher fat content. Moreover they form a specific cuticle that is

robust and is even resistance to 1% SDS, dauer larvae can be identified under a microscope by a surface structure, the dauer alae. Further, dauer larvae do not feed, their oral orifices are closed by an internal plug and therefore they have no pharyngeal pumping (Cassada and Russell, 1975; Riddle et al., 1981). They utilize the glyoxylate cycle instead of aerobic respiration and TCA to generate carbohydrates (Wadsworth and Riddle, 1989). This allows them to survive for several months. Upon improved conditions, dauer larvae resume reproductive growth and develop to fully mature fertile adult hermaphrodites.

4.3.3 The genetics of dauer decision

C. elegans mainly sense nutrition with processes of sensory head neurons directly exposed to the outside. Information is then processed first in neurons, involving cGMP and serotonergic signaling (Birnby et al., 2000; Thomas et al., 1993). Under favorable conditions IIS and TGF-β signaling are activated (Murakami et al., 2001). Released hormones of these two pathways in turn lead to the activation of the dafachronic acid pathway that stepwise converts cholesterol to bile acid-like steroids called dafachronic acids (DA) (***Figure 2***). How these hormones actually activate DA synthesis remains mainly unknown. However, DA then binds to the nuclear hormone receptor DAF-12. DA-bound DAF-12 promotes reproductive programs in L2 larvae (Antebi et al., 2000; Motola et al., 2006). In addition to this function prior to sexual maturation, later in life the DAF-12 DA interaction ascertains a normal lifespan. On the other hand unbound DAF-12 recruits the co-repressor DIN-1 to repress reproductive development, promote dauer formation and extended lifespan (Ludewig et al., 2004). Accordingly, the *daf-12* null mutant *rh61rh411* is dauer defective and short-lived (Antebi et al., 2000). In this context, DA acts on DAF-12 to determine its function as an activator or as a repressor. Thus, DA binding and the regulated activity on the NHR DAF-12 connects *C. elegans* developmental decision and lifespan determination. Consequently, upstream pathways of DA synthesis like IIS and TOR have dauer and ageing phenotypes. Nevertheless, not all of these lifespan phenotypes are strictly DAF-12 dependent indicating a more complex network underlying determination of lifespan.

4.3.4 Biochemistry of the dafachronic acid pathway

C. elegans is cholesterol auxotroph and relies on diet as the only source. Thus, cholesterol can become a limiting factor for DA synthesis. Accordingly, knockouts of the two known genes involved in sterol transport, the Nieman-Pick Type C-1 (NPC) homologues *ncr-*

2 and *ncr-2* are Daf-c (Li, 2004). The DA pathway is separated in at least two distinct branches that both convert cholesterol to dafachronic acid (DA), 3-keto-4-cholestenoic-acid (Δ^4-DA) and 3-keto-7-cholestenoic-acid (Δ^7-DA), respectively. In the first step of the Δ^7-DA branch the DAF-36/Rieske Oxygenase reduces cholesterol to 7-dehydrocholesterol (Wollam et al., 2011; Yoshiyama-Yanagawa et al., 2011), introducing a 2nd double bound in position seven of steroid backbone (B ring). In a second step, a so far elusive enzyme reduces the steroid B ring of 7-dehydrocholesterol in position four to synthesize lathosterol (Motola et al., 2006). Lathosterol in turn is oxidized to lathosterone by 3-hydrosteroid dehydrogenase DHS-16 by introducing a keto-group to C3 of steroid A ring (Wollam et al., 2012). The last step is carried out by DAF-9/CYP27A1 acting on the side chain of lathosterone to convert it to a carboxylic acid similar to human bile acids (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006). DAF-9/CYP27A1 also has a postulated role in the second branch of DA synthesis it supposedly oxidizes the side chain of 4-cholesten-3-β-one to generate Δ^4-DA. Biochemical evidence supporting this role of HSD-1 *in vivo* remains elusive, up to date (Dumas et al., 2010; Patel et al., 2008; Wollam et al., 2012).

In a recent study using comparative metabolomics more endogenous ligands of DAF-12 were identified 3-oxo-1,7-cholestadie-noic acid ($\Delta^{1,7}$-DA), 3α-hydroxy-7-cholestenoic-acid (3a-OH- Δ^7-DA). $\Delta^{1,7}$-DA was also shown to have a physiological function, restore gonadal longevity in *glp-1;daf-36*. Interestingly, with 3a-OH- Δ^7-DA a DA that carries up an OH- moiety in in position 3 was identified, supporting earlier findings (Held et al., 2006), that the keto-group of other DAs in this position is not essential to bind to DAF-12 (Mahanti et al., 2014). Although Δ^4-DA itself was not detected in worms, a physiological function to activate DAF-9 and a subsequent rescue of DA depletion-induced phenotypes was shown repeatedly (Gerisch et al., 2007; Shen et al., 2012; Yamawaki et al., 2010).

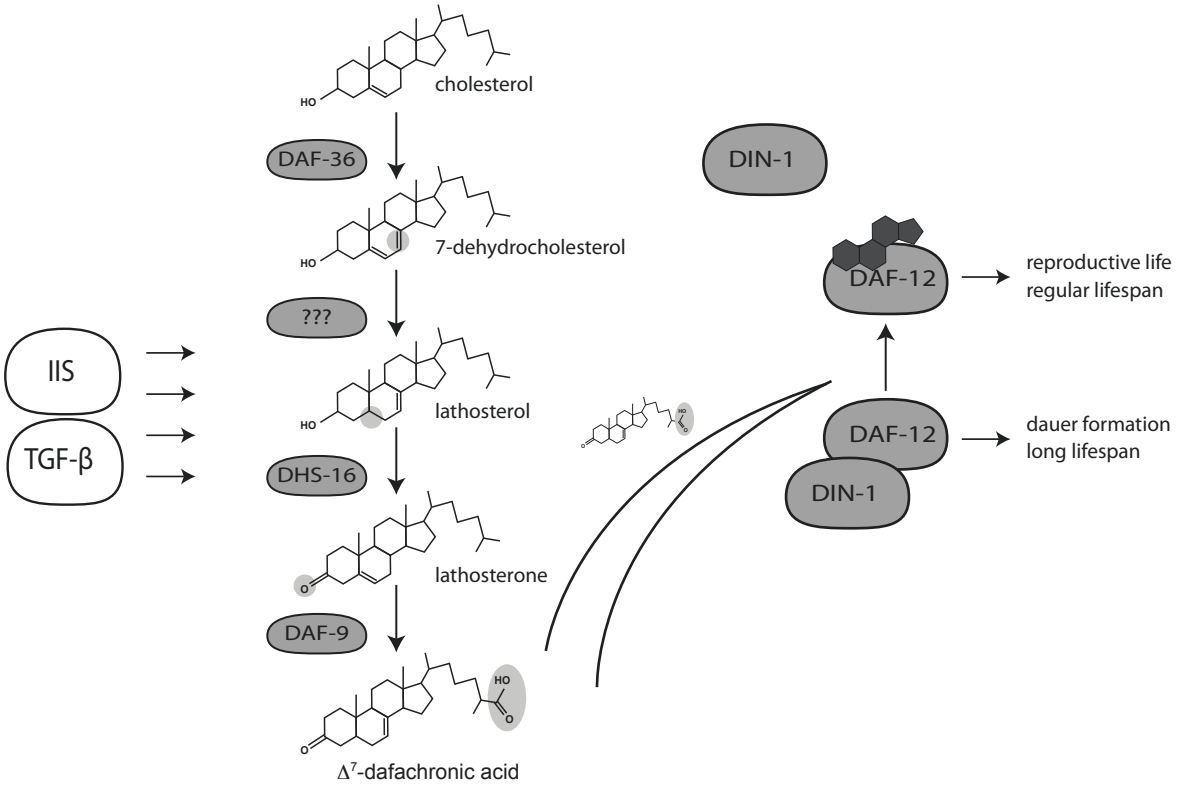


Figure 2 IIS and TGF-β induce dafachronic acid pathway

Delta 7 dafachronic acid synthesis: Under favourable conditions IIS and TGF-β signals activate DA pathway. In the DA pathway cholesterol is first converted to 7-dehydrocholesterol by DAF-36, that in turn is oxidzed to lathosterol by an so far elusive enzyme, next DHS-16 converts lathosterol to lathosterone and finally lathosterone is oxidized to Δ^7-DA -dafachronic acid by DAF-9. Dafachronic acid binds to DAF-12 to promote reproductive growth and regular lifespan. Under unfavourable condition the pathway synthesizes less DA and unbound DAF-12 recruits DIN-1 promotes dauer and longevity

4.4 Endocrine character of the dafachronic pathway

Interestingly, expression profiles of the involved enzymes are distributed in different tissues. DAF-12 is expressed in all tissues but the final step of DA synthesis is restricted to a few neuronal cells, because *daf-9*/Cyp27A1 is expressed in a distinct set of neuroendocrine cells the XXX cell, the hypodermis and spermatheca (Gerisch et al., 2001; Jia et al., 2002). Notably, DAF-36 is expressed mainly in gut and DHS-16 is found in neurons, pharynx, and hypodermis (Rottiers et al., 2006; Wollam et al., 2012). The only enzyme of the postulated second branch, HSD-1, is expressed in XXX- cells (Patel et al., 2008). Thus, the intermediates of the DA pathway must be transported cell non-autonomously between tissues. It remains elusive how and at which developmental time-point DA is transported to a tissue where it binds to DAF-12. One reasonable speculation is that the spatial distribution separates the

multiple functions of DA/DAF-12 interaction (Wollam and Antebi, 2011). This distribution of the DA pathway further supports its endocrine character.

4.4.1 The role of DA pathway in longevity

In regular reproductively growing animals, DA signaling seems to have rather mild effects on lifespan. Nevertheless, strong DAF-9 mutations that result in low DA levels are long-lived at 15 °C and 20 °C, and this longevity is *daf-12* and *din-1* dependent(Gerisch et al., 2001; Ludewig et al., 2004). Moreover it was found, that a synthetic inhibitor of DAF-9 (dafadine) also induces dauer formation and extends lifespan (Luciani et al., 2011), underlining the essential role of DAF-9 to ascertain a regular lifespan. The putative DAF-12 null allele *(rh61rh411)* that carries nonsense mutations in the DBD *(rh411)* and in the LBD *(rh61)* is slightly short lived, suggesting a lifespan-extending role for unliganded DAF-12. Another LBD mutant*(rh273)* was reported to be long-lived (Antebi et al., 2000; Fisher and Lithgow, 2006). It seems likely that DAF-12 can work in both directions to either extend or shorten lifespan (Rottiers et al., 2006). This ambivalent role is reflected in its relation to IIS longevity. Longevity of strong *daf-2* alleles is further extended whereas longevity of weak alleles is abrogated (Gems et al., 1998; Larsen et al., 1995).

Recently it was found that the DAF-12/DA interaction is also essential to gonadal longevity. It depends on three components of the dafachronic acid pathway: DAF-36/Rieske like oxygenase, DHS-16, DAF-9 and DAF-12. Knockouts of DAF-36 and DAF-9 have lowered levels of Δ^7-DA (Wollam et al., 2012). Supplementation of DA to germlineless animals in *daf-9* and *daf-36* background restores gonadal longevity (Gerisch et al., 2007). This suggests that the longevity signal from the somatic gonad is dafachronic acid. It is supported by the finding that gonadal longevity of animals without reproductive system is restored by feeding Δ^4-DA (Gerisch et al., 2007; Yamawaki et al., 2010). Accordingly, in germline-less animals DAF-36 expression is increased and Δ^7-DA levels are higher. DA activates DAF-12 target genes *mir-84* and *mir-241* (Bethke et al., 2009). These genes are also up-regulated in germline-less animals and in line, gonadal longevity is abrogated in *mir-84*; *mir-241* mutants (Shen et al., 2012). Therefore, dafachronic acid and the *let-7* micro RNAs *mir-84* and *mir-241* play an essential role in gonadal longevity and are involved in the mediation of longevity signals coming from somatic gonad. Steroid signaling therefore is a promising target to look for other components that can extend *C. elegans* lifespan.

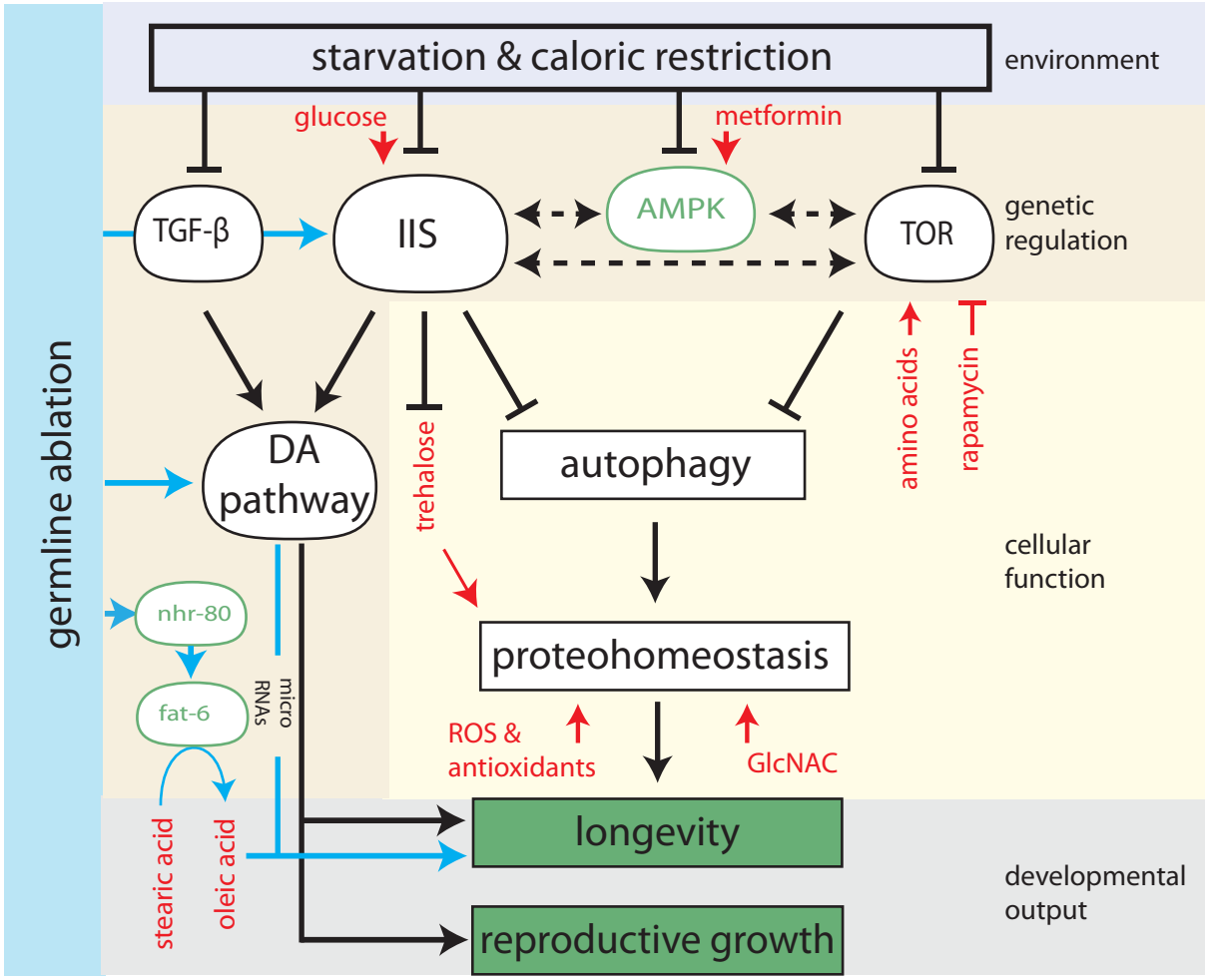


Figure 3 The role of small molecules in modulating longevity pathways

Direction of arrows in this figure are based on proposed genetic epistasis and do not indicate a direct biochemical interaction. Dotted lines represent interactions of which direction is not unambiguously understood. Environmental conditions are signaling via small molecules (red) to inhibit or activate genetic networks - IIS and TOR pathway. Pharmaceutical manipulation is possible by feeding TOR inhibitors like rapamycin or changing availability of sugars and amino acids. This genetic level of life span determination (beige background) includes strong involvement of the energy sensor AMPK, that was also identified as a small molecule target for ageing-manipulation (metformin). IIS, TOR and AMPK together regulate cellular functions like mitochodrial and translational activity (not shown). A common downstream target of this network seems to be autophagy, an important mechanism to uphold proteohomeostasis. These two cellular functions (yellow background) emerged as major regulators of lifespan determination. Accordingly many small molecules (antioxidants, GlcNAC and trehalose) found to improve proteohomeostasis and also extend lifespan. In *C. elegnas*, gonadal ablation leads to longevity that is depending, among others, on the IIS transcripton factor DAF-16, components of the DA pathway and activity of FAT-6 that converts stearic to oleic acid. During development IIS together with TGF-β impinge on DA pathway to regulate dauer decision and lonegvity in reproductive animals. But also TOR was shown to be involved in regulation of dauer entrance.

Aim of the study

5 Aim of the study

As discussed, many longevity pathways such as TOR and IIS are targets of metabolic small molecules such as amino acids and glucose. But also cellular functions that are regulated in lifespan determination such as autophagy and protein homeostasis are targets of such endogenous molecules like trehalose or spermidine. Moreover synthetic small molecule modulators of these longevity pathways such as metformin have been identified. In brief, endogenous and synthetic small molecule modulators of longevity pathways emerged as an important part helping to solve the puzzle of ageing. Regarding the fact that some of these molecules are components of our daily diet little is understood about how they signal to modulate the complex network that regulates lifespan.

What is role of endogenous small molecule metabolites in ageing? How can such molecules of our daily diet like sugars, amino acids, fatty acids and sterols modulate lifespan? What are the signaling pathways that they might work through? How does the quality of diet influence ageing? How can small molecules be used to alter the onset of age-related diseases and potentially help to develop drugs to treat them?

Notably, most of the longevity pathways and their regulation by small molecules are conserved in *C. elegans*. Interestingly, similar pathways regulate dauer formation of *C. elegans*. Thus, I use dauer formation as a primary readout to screen sugars, amino acids, fatty acids and sterols regarding their potential to alter dauer formation. Candidate molecules might also influence lifespan. By unraveling the effects of such small molecule modulators on lifespan in *C. elegans*, we hope to learn about conserved mechanisms that help to understand human- ageing and age-related diseases.

Results

6 Screening small molecules

6.1 Establishment of screening conditions

We intended to create a highly sensitive assay to identify new compounds that play a role in dauer formation and lifespan determination. Therefore we decided on screening in animals carrying a mutation in DAF-2. DAF-2 is the only known homologue of human insulin receptors (InR). In general, alterations of the insulin signaling were described as a conserved mechanism to modulate lifespan from nematodes to human and in particular it was shown as one of the major players in dauer decision in *C. elegans* (Hu, 2007). DAF-2 promotes reproductive growth and regular lifespan. Therefore *daf-2* thermo sensitive mutations loss of function alleles such as *daf-2(e1368)* and *daf-2(e1370)* promote dauer formation and longevity (Gems et al., 1998; Hsin and Kenyon, 1999). By using such a sensitive background we hoped to also identify compounds with rather moderate effects on dauer formation. Moreover we designed the assay to be capable to monitor increase as well as a decrease in dauer formation. We tried several genes of the two described *daf-2* allele classes to establish an assay that under control conditions will lead to around 50% dauer formation at moderate increase in temperature. We finally decided to perform the screen in class two allele *daf-2(e1368)* background at 22.5 °C and to use 10 mM D-glucose as control for rescue.

6.2 Screening for dauer modulators in *daf-2(e1368)*

As key components of the diet and messengers of metabolic balance, we focused our small-scale-screen on basic building blocks of metabolism, including sugars, amino acids, fatty acids, and sterols. To assess secondary effects of bacterial metabolism, we performed dauer assays on living and on UV-killed bacteria. Moreover we initially tested two different concentrations (10 mM and 100 mM).

6.2.1 Sugars

Sugars are an important carbon and energy source of the diet and it was shown that they play a role in lifespan determination and dauer formation. Glucose was shown to decrease lifespan and decrease dauer formation via IIS (Lee et al., 2009; Schulz et al., 2007).

We decided to test other sugars for a potential modulation of dauer formation. Glucose was established as a control to rescue dauer formation. The assay was adopted from the Kenyon Lab (Lee et al., 2009). Interestingly, 10 mM and 100 mM D-glucose, D-galactose and the sugar alcohol glycerol partially rescued dauer formation of *daf-2(1368)* on living (black) and UV inactivated (light grey) bacteria. D-galactose had the strongest effect and reduced dauer down to 5 % on living bacteria and down to 10 % on UV treated bacteria. Interestingly, there was no difference between 10 mM and 100 mM treatment.

100 mM trehalose, but not 10 mM trehalose significantly rescued dauer formation on living and UV-inactivated bacteria. And D-fructose slightly reduced dauer formation under all conditions but only rescue with 100 mM on living bacteria was significant (***Figure 4***). The only provided sugar that had no effect on dauer formation was sucrose. These data suggest that various carbohydrates influence dauer formation assumable via IIS.

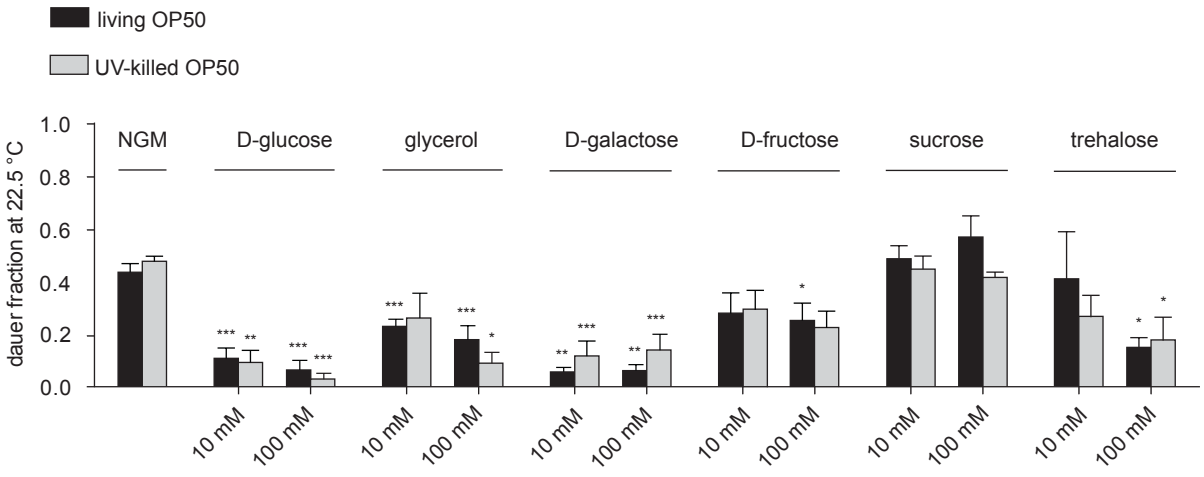


Figure 4 Multiple sugars decreased dauer formation

***Daf-2(e1368)* was grown from egg on at 22.5 °C on NGM plates containing one of multiple sugars either at 10 mM or 100 mM. NGM plates were either seeded with living OP50 (black bars) or UV-inactivated OP50 (light grey). Populations were scored after 60 hours for the fraction that formed dauer larvae. Water and glucose were provided as controls. The only sugar showing no effect on dauer formation as compared to ddH20 control was sucrose. All other tested sugars decreased dauer formation at both concentrations.*p<0.05, **p<0.005, ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each)**

6.2.2 Amino acids

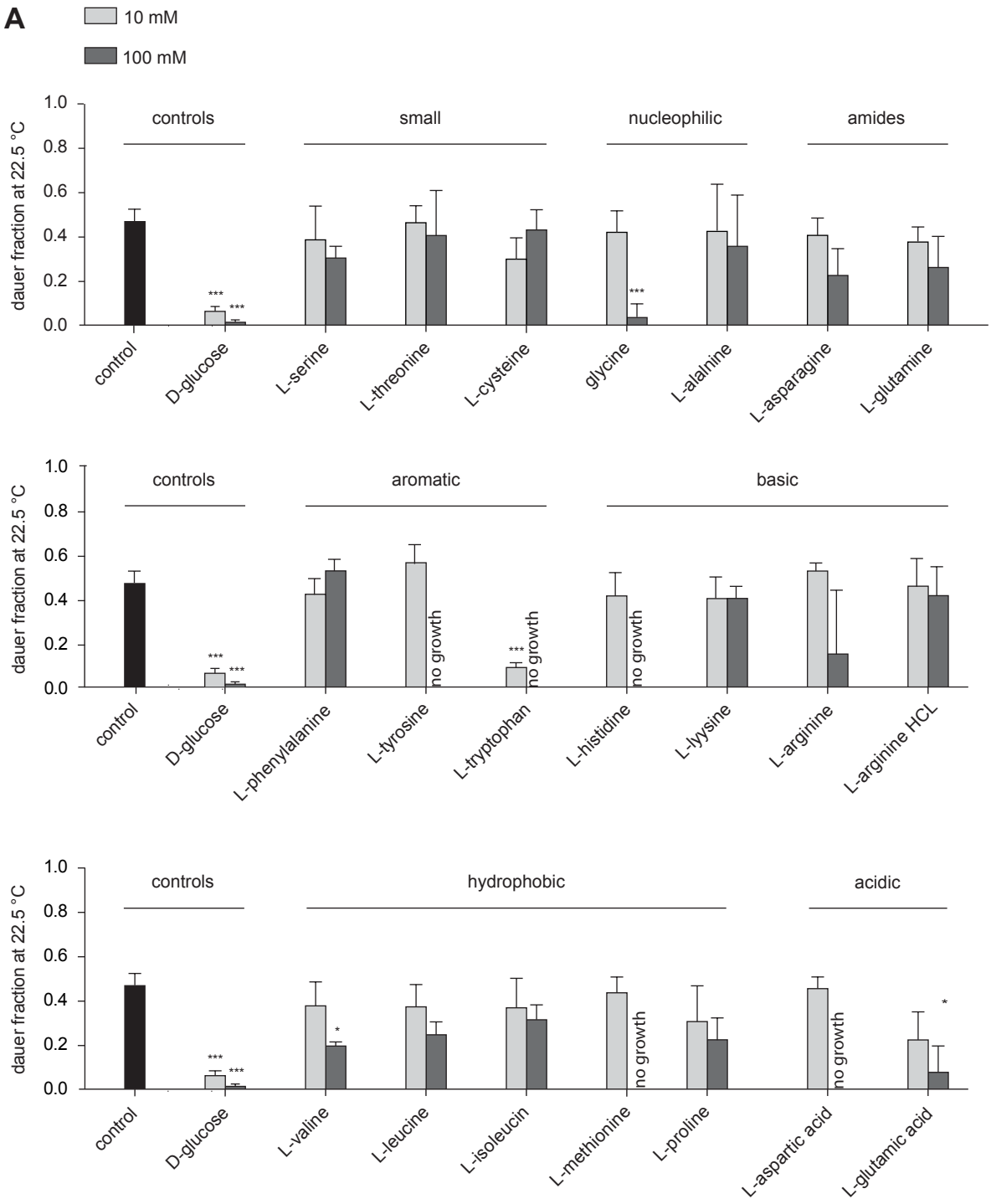
Amino acids are important building blocks for growth and reproduction. In flies it was shown that their limitation influences dietary restriction mediated longevity and reproductive output (Grandison et al., 2009). One way they may impact metabolism is through the nutrient sensor TOR, a major pathway modulating ageing and CR. We therefore decided to test all proteinogenic amino acids regarding their potential to modulate dauer formation and used D-

glucose and ddH_2O as controls. Again we used two concentrations (10 mM and 100 mM) and tested dauer formation, first on living (***Figure 5 A***) and then on UV inactivated bacteria (***Figure 5 B***).

On living bacteria in the group of small, nucleophilic and amide amino acids no effect on dauer formation was observed for L-serine, L-threonine, L-cysteine, L-alanine, L-asparagine and L-glutamine neither at 10 mM nor at 100 mM. The only effect was with 100 mM glycine. A tendency of decreased dauer formation that did not reach significance was observed with 100 mM serine, asparagine and glutamine. In the groups of aromatic, basic, hydrophobic and acidic amino acid tryptophan (10 mM) and valine at 100 mM significantly decreased dauer down to 9 % and 20 %, respectively. At 100 mM tyrosine, tryptophan, histidine, methionine and aspartic acid were toxic and inhibited either growth of bacteria or hatching of larvae. Arginine at 100 mM had a high variability in the single experiments. To exclude a potential effect of basic conditions we provided arginine-HCl and observed no effect on dauer formation. Moreover with 100 mM we observed decreased dauer fraction that did not reach significance for leucine and proline (***Figure 5 A***).

With UV treated bacteria we observed no effect of serine, threonine alanine, asparagine glutamine phenylalanine lysine, isoleucine, proline aspartic acid and glutamine. Glycine at 10 mM decreased dauer formation and 100 mM abolished it. On UV inactivated bacteria more amino acids had toxic effects. Cysteine, tyrosine, tryptophan inhibited growth at both concentrations, leucine and methionine at 100 mM and valine at 10 mM. We assume that living bacteria have an ameliorating effect on amino acid toxicity (***Figure 5 B***).

In addition, we observed a shift towards reproductive growth, when amino acids were provided on living bacteria at 100 mM. This apparent rescue may potentially be a consequence of increased osmolarity, since increased osmolarity was linked before to transcriptional targets of DAF-16. DAF-16 is the key-regulator of IIS and upstream of dauer pathway. Alterations in its transcriptional output, thus might lead to dauer rescue. This tendency towards reproductive growth seems to be ameliorated in UV treated bacteria, suggesting that there is an osmolaric effect on living bacteria that leads to differential dauer formation of *C. elegans*. Additionally we did not see an osmolaric effect when sugars were provided. Anyhow since effects of osmolarity on lifespan is not scope of this study we decided to prevent them and only use compounds at 10 mM or lower for further screening. The only amino acid that significantly decreased dauer formation under all conditions was glycine. It might be an interesting candidate to follow up in the future.


A
10 mM
100 mM
dauer fraction at 22.5 °C
1.0
0.8
0.6
0.4
0.2
0.0
controls
small
nucleophilic
amides
***
***
***
control
D-glucose
L-serine
L-threonine
L-cysteine
glycine
L-alalnine
L-asparagine
L-glutamine
controls
aromatic
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L-tryptophan
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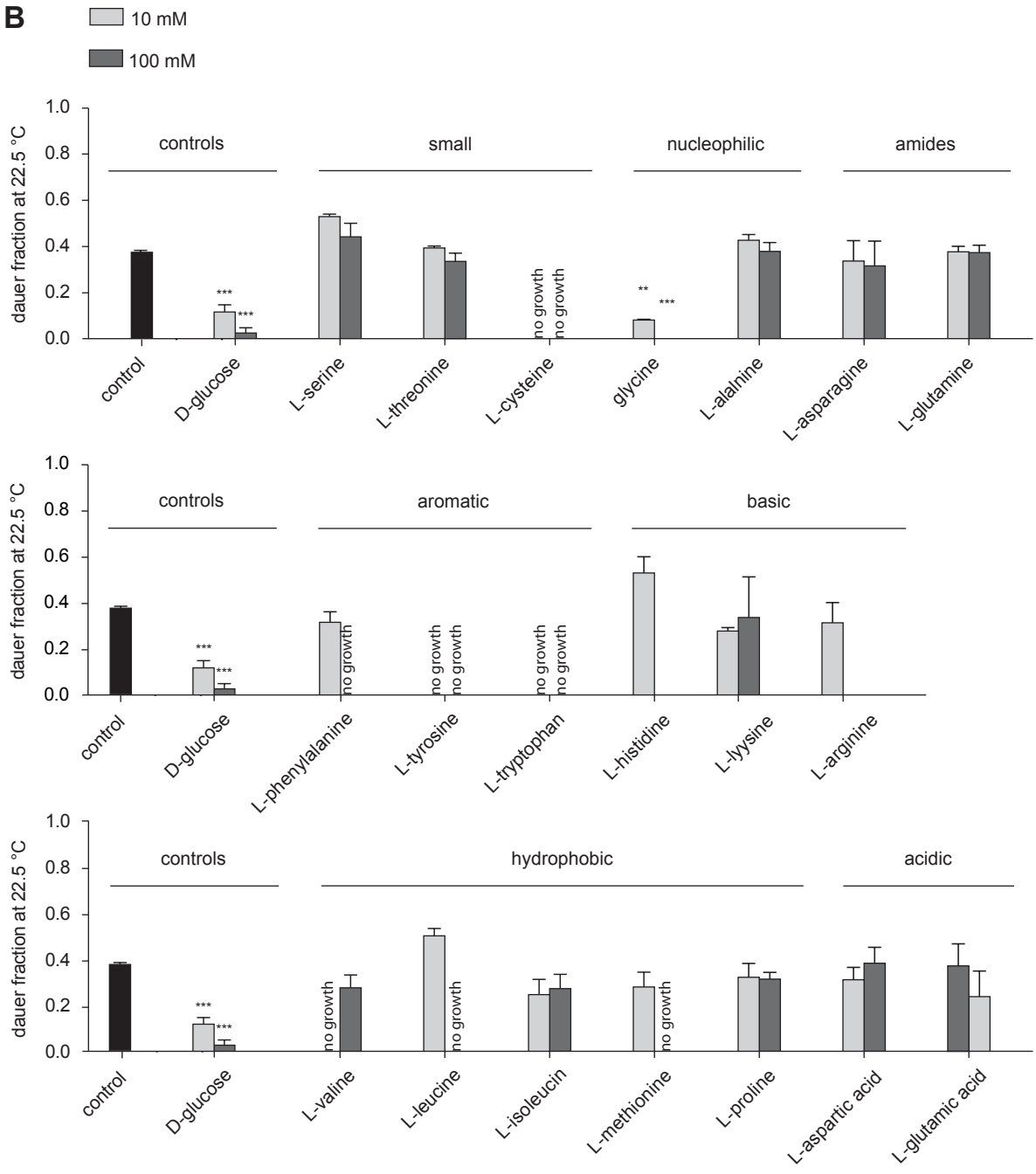

Figure 5 Only few amino acid decreased dauer formation of *daf-2(e1368)*

***Daf-2(e1368)* was grown from egg on at 22.5 °C on NGM plates containing one of the 20 proteinogenic amino acids either at 10 mM or 100 mM. NGM plates were either seeded with (A) living OP50 or (B) UV-inactivated. Populations were scored after 60 hours for the fraction that formed dauer larvae. Water and glucose were provided as controls. Some amino acids inhibited bacterial growth or prevented worms from hatching (no growth). *p<0.05 **p<0.005, ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each)**

6.2.3 Fatty acids

Fatty acids are a rich source of energy, provide building blocks for membranes and organelles and serve as signaling molecules in many pathways. Several lipids such as N-ethanolamides, oleic acid, and DGLA have been implicated in *C. elegans* ageing (Goudeau et al., 2011; O'Rourke et al., 2013; Watts and Browse, 2006). Moreover, IIS and TOR are nutrient sensing pathways partly regulated by fatty acids. Under starvation conditions, stored fatty acids are released through lipolysis, in some cases closely coupled to autophagy. Because fatty acid are important signals in nutrient sensing we decided to test major classes of fatty acids found in *C. elegans* including representative saturated, monounsaturated and polyunsaturated fatty acids. Fatty acids were provided in the presence of NP40 to facilitate their solubilization and uptake into *C. elegans* (Nomura et al., 2009). Under these conditions, only arachidonic acid partially rescued dauer formation of *daf-2(e1368)* ***(Figure 6)***.

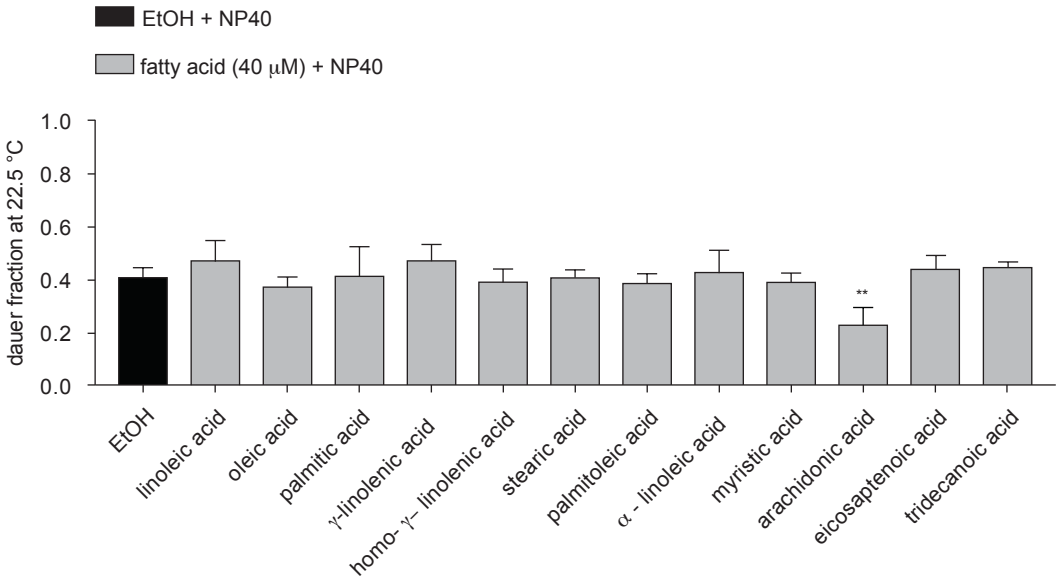

Figure 6 Arachidonic acid decreased dauer formation of *daf-2(e1368)*

***Daf-2(e1368)* was grown from egg on at 22.5 °C on NGM plates with NP-40 containing one of 12 fatty acids previously described as part of *C. elegans* fat metabolism. Corresponding volume of vehicle (EtOH) was used as control. Populations were scored after 60 hours for the fraction that formed dauer larvae. Arachidonic acid was the only fatty acid that showed a significant affect (decrease) of dauer formation. **p<0.005 (ANOVA). Mean+SEM (n≥3, >50 worms each)**

6.2.4 Steroids

C. elegans cannot synthesize cholesterol and must obtain it from the diet (Hieb and Rothstein, 1968). The bile acid-like steroids, the dafachronic acids, are derived from dietary cholesterol, and are thus far the only identified steroid pathway in the worm. They play an important role in preventing dauer formation and regulating longevity.

Given the role of sterols and steroids in physiological regulation, we thus asked if various steroids affect *daf-2* dauer formation. In particular we examined precursors of mammalian steroid hormone synthesis, mammalian bile acids, intermediates of the DA pathway, several other plant mammalian and insect steroids, two retinoic acids, C. elegans molting hormone lophenol and a group of oxysterols known to target liver x receptor (LXR) in humans (Wollam and Antebi, 2011)(***Figure 7***). Most of the steroids were as well tested under low cholesterol conditions to down-regulated synthesis of cholesterol-derived sterols.

We observed no significant effect on dauer formation of steroids in the group related to human steroid synthesis and in the group of human bile acids (***Figure 7, upper panel***). In the group of mixed steroids, lophenol increased dauer formation significantly under regular and low cholesterol conditions (***Figure 7, medium panel***). All steroids in the group of DA synthesis intermediates reduced dauer formation in *daf-2(e1368)* but under regular and low cholesterol conditions, whereas 7-deyhdrocholesterol, lathosterol and 4-cholesten-3-one had a rather mild effect. Lathosterone reduced dauer formation down to 10 % and only Δ^7- DA completely abrogated dauer formation. In the group of oxysterols, we found that 22(S)-hydroxycholesterol, 22(R)- hydroxycholesterol and 5-cholesten-3β-ol-7-one increased dauer formation. All other compounds had no significant effect (***Figure 7, lower panel***).

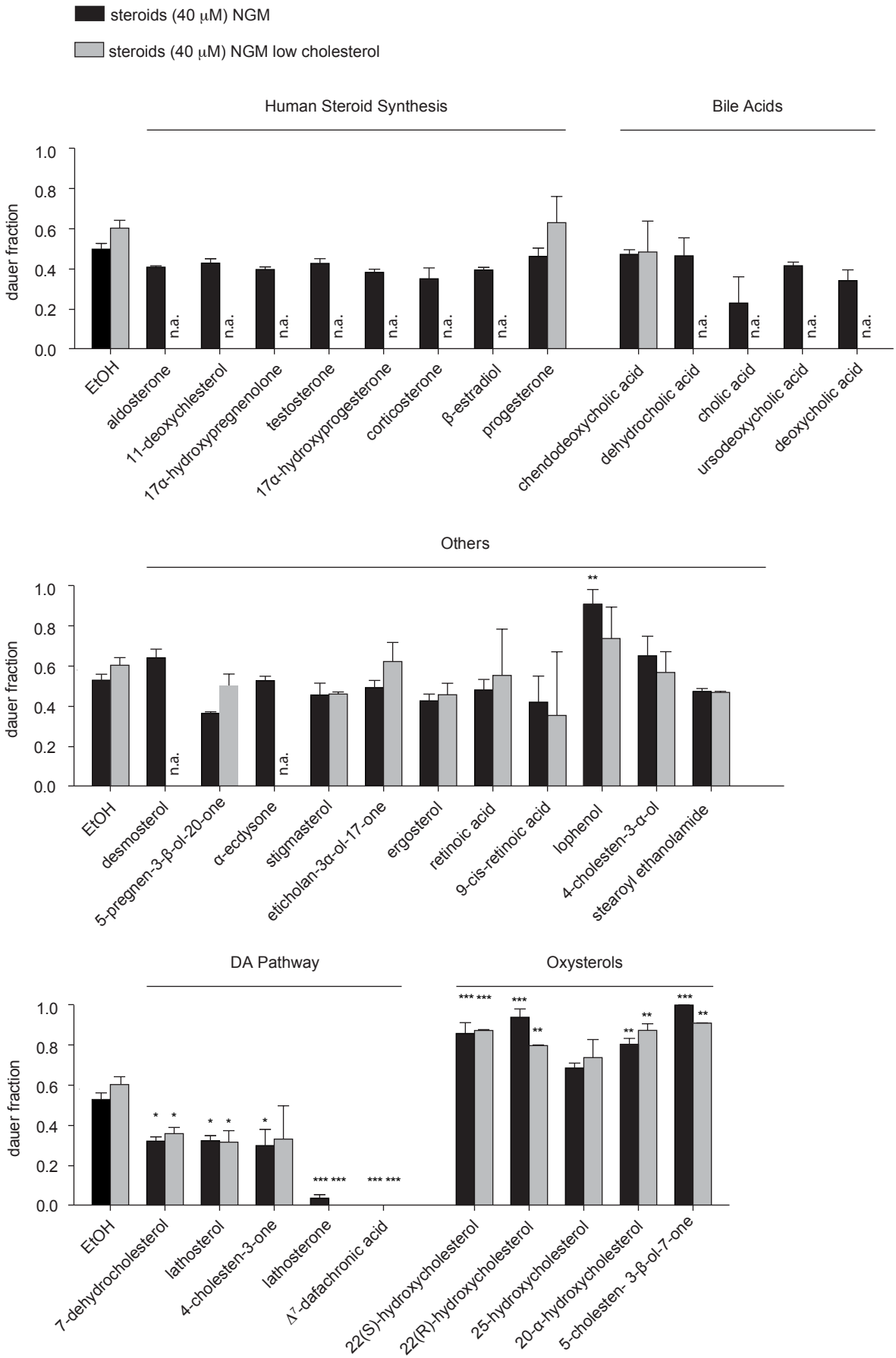

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Figure 7 DA intermediates and oxysterols altered dauer formation in daf-2(e1368)

***Daf-2(e1368)* was grown from egg on at 22.5 °C on regular NGM (black bars) or NGM plates without additional cholesterol (grey bars). Steroids, provided at 40 µM belonging to either (A) the group of human steroid hormone synthesis or human bile acids (B) steroids of the DA pathway, (C) oxysterols or (D) to no of the above. Corresponding volume of vehicle (EtOH) was used as control. Populations were scored after 60 hours for the fraction that formed dauer larvae. Some steroids of the DA pathway rescued dauer formation and some oxysterol increased dauer formation independent of nutritional cholesterol. **p<0.01, ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each)**

7 7-Ketocholesterol induced phenotypes

7.1 7-ketocholesterol enhanced dauer formation in *daf-2(e1368)* at low concentrations

Because 22(R)-hydroxycholesterol, 20-α-hydroxycholesterol and 25-hydroxycholesterol were identified as among the few molecules to enhance dauer formation of *daf-2(e1368)*, we followed up on their effects by examining their dose response to 40 μM, 4 μM and 0.4 μM. We only observed reproducibility of significant increased dauer formation for lophenol and 22-(R)-hydroxycholesterol with 40 μM (***Figure 8 A***). We further tested, 5-cholesten-3β-ol-7-one that had the strongest effect on dauer formation in the screen (97%). We found that it induced dauer phenotypes even at lower concentrations. Indeed 5-cholesten-3β-ol-7-one reliably increased dauer formation of *daf-2(e1368)* with 40 μM, 4 μM and 0.4 μM, but did not increase dauer formation at 40 nM (***Figure 8 B***). 400 nM lies within a reasonable range for a specific ligand-receptor interaction. Thus, we decided to focus further analyses on 5-cholesten-3β-ol-7-one. 5-cholesten-3β-ol-7-one is a sterol that differs from cholesterol only in position 7 at the B ring, where it contains a keto-group and is therefore colloquially called 7-ketocholesterol (7-KC) (***Figure 8 C***).

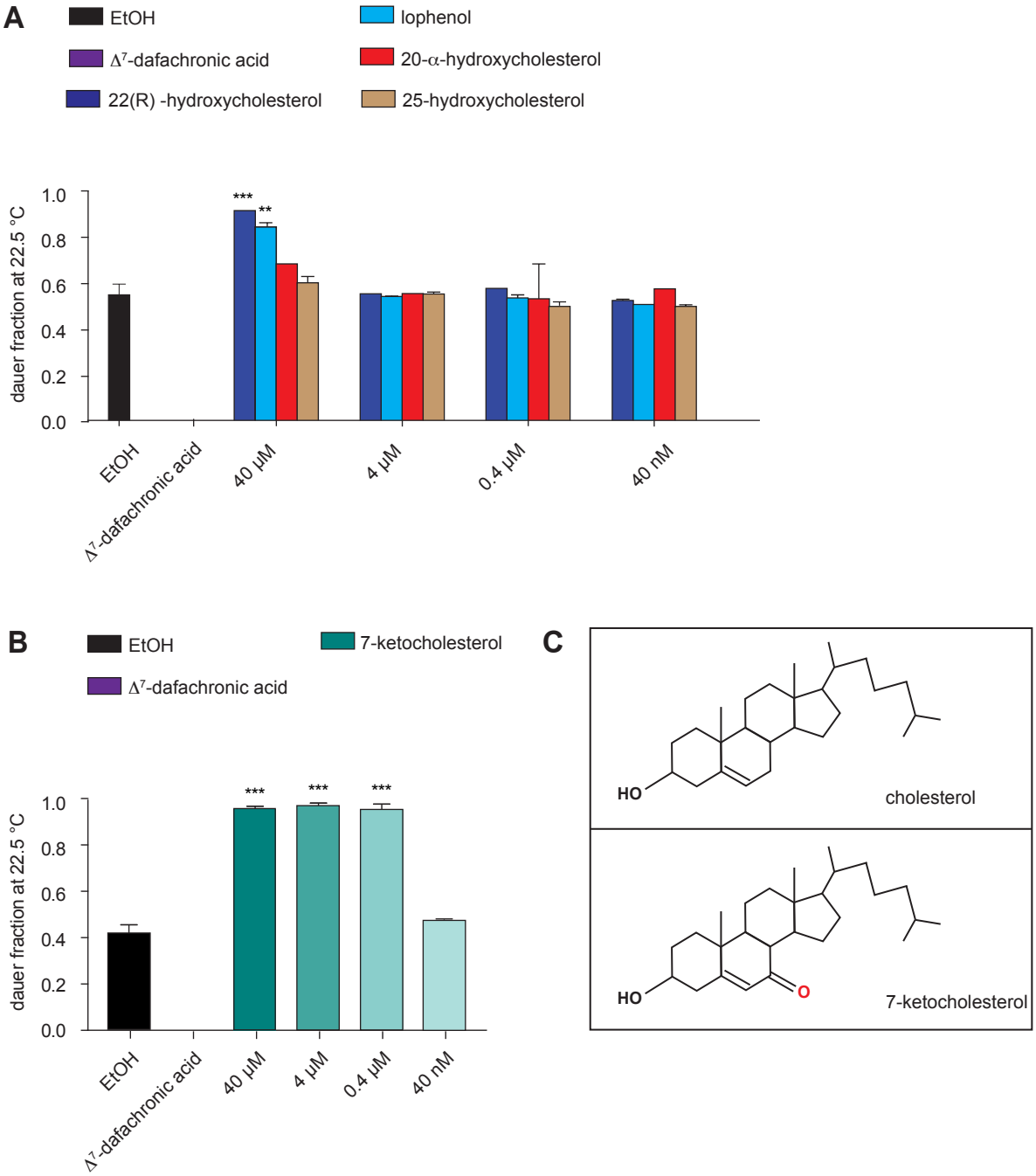

Figure 8 400 nM 7-ketocholesterol increased dauer formation in *daf-2(e1368)*

(A-B) *Daf-2(e1368)* was grown from egg on at 22.5 °C on NGM plates containing 7-ketochoesterol 40 µM, 4 µM, 400 nM or 40 nM, respectively. Δ^7-dafachronic acid and corresponding volume of vehicle (EtOH) were used as controls. Populations were scored after 60 hours for the fraction that formed dauer larvae. (B) All 7-KC concentrations ≥ 400 nM significantly increased dauer formation. (C) Structure of cholesterol and its oxidized form 7-ketocholesterol, carrying a keto-group at C7. ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each).

7.2 7-KC did not affect dauer formation of N2

As a first step in characterizing 7-KC, we asked if it also affects dauer formation in wild type N2 or if it is restricted to *daf-2(e1368)*. We fed 40 μM 7-KC or the vehicle EtOH to N2 worms throughout development. N2 animals on regular NGM plates with vehicle control at 20 °C, 22.5 °C and 25 °C form no dauer larvae. At 27 °C they formed dauer larvae at a fraction of around 10 %. Surprisingly, animals grown on 7-KC supplemented plates also formed no dauer at 20 °C, 22.5 °C and 25 °C and the fraction dauer larvae formed at 27 °C was not increased by 7-KC compared to EtOH (***Figure 9 A***). Thus, we conclude that the effect of 7-KC on N2 is not strong enough to induce dauer formation in otherwise reproductively growing animals.

We decided for a non-genetic way to create a more dauer-prone environment, namely cholesterol deprivation. *C. elegans* is auxotroph for cholesterol (Hieb and Rothstein, 1968), and cholesterol is the precursor of DA. Accordingly, the F1 generation on low cholesterol is more prone to enter dauer (Gerisch et al., 2001). Thus, we decided to measure dauer formation on NGM plates without additional cholesterol supplementation (NGM low) and monitor dauer formation of F1 generation. Under this conditions no dauer larvae were formed at 20°C, 22.5°C and surprisingly neither at 25 °C with EtOH, which would be expected according to literature. However, as expected in populations grown at 27 °C, significantly more animals entered dauer compared to regular NGM plates. Provision of 7-KC did not further enhance the amount of dauer larvae. (***Figure 9 B***).

DA deficiency cannot only lead to dauer, but also to gonadal cell migration defects (Mig) in which migratory distal tip cells fail to reflex. This phenotype is seen for example with mutations in hormone biosynthetic genes *daf-36*/Rieske null mutants (Rottiers et al., 2006), and daf-9/CYP27A1 hypomorphs(Gerisch et al., 2001)). However, 7-KC did not induce gonadal Mig phenotypes in the wild type background. Therefore we conclude that either effect of 7-KC is specific with *daf-2(e1368)* or is too mild to induce dauer in N2.

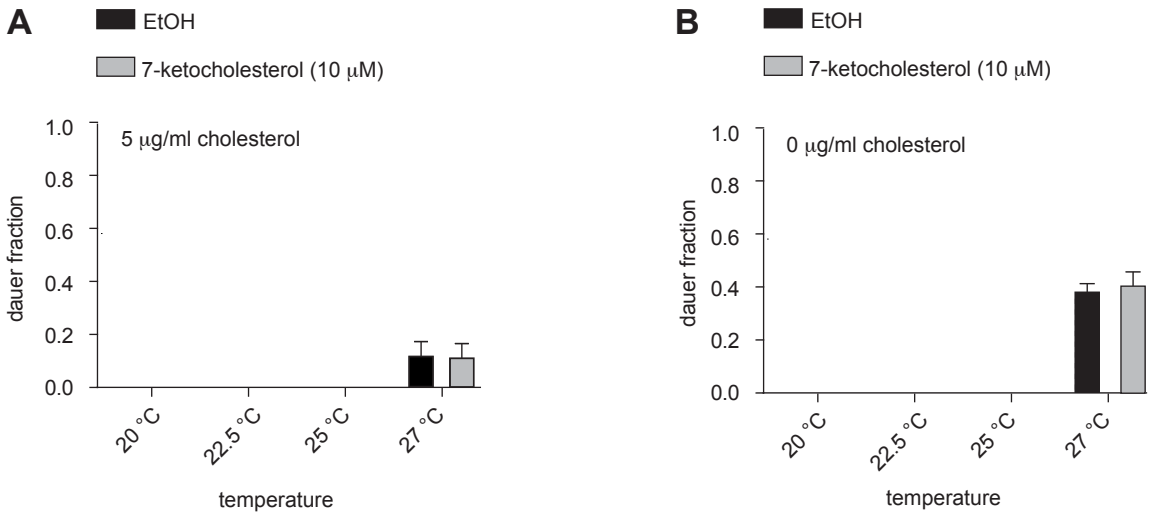

Figure 9 7-ketocholesterol does not increases dauer formation of N2

N2 was grown from egg on at 20 °C, 22.5 °C, 25 °C and 27 °C on NGM plates containing 7-ketochoesterol (40 µM) containing (A) regular cholesterol (5mg/ml) or (B) no additional cholesterol. Populations were scored after 48 h (20 °C, 25 °C, 27 °C) or 60 h (22.5 °C) for the fraction that formed dauer larvae. 7-ketocholesterol did not increase dauer formation of N2 (ANOVA). Mean+SEM (n≥3, >50 worms each)

7.3 7-KC induced hypodermal *daf-9* expression in N2

To elucidate which of the above is the case, we decided to examine a third phenotype induced by mild DA depletion- namely hypodermal DAF-9 expression (*daf-9 hyp*). Under favorable conditions DAF-9 as the key-enzyme in DA synthesis is expressed from embryo until adulthood in the XXX neuroendocrine cells, leading to a sufficient level of DA to activate DAF-12 driven reproductive growth programs. Phenotypes, Mig and *daf-9(hyp)* are associated with rather mild DA depletion. Under such mild depleting conditions low DA feedback regulates DAF-9 expression in hypodermal cells from late L2 stage on through adulthood. Switching DAF-9 expression profile from the XXX cells to all hypodermal cells is thought to result in more DA synthesis and the induction of reproductive growth (Gerisch et al., 2001; Wollam et al., 2011).

We compared fluorescence of the hypodermis of L3 larvae carrying a *daf-9::gfp* reporter in N2 (Gerisch et al., 2001)fed with 10 μM 7-KC or vehicle EtOH. If DA pathway is down-regulated by 7-KC supplementation, but not far enough to induce dauer programs in N2, we expect to find hypodermal expression of DAF-9. As a positive control we used the same GFP marker in *daf-36(k114)* background (Wollam et al., 2011).

We first checked with microscopy for induction of hypodermal GFP expression in the positive control background *daf-36(k114)* and fluorescence was increased significantly. We

next checked GFP of 7-KC fed N2 and found a similar induction of hypodermal DAF-9 expression (***Figure 10 A***). With this approach a basal level of fluorescence in all samples was detected due to *daf-9::gfp* expression in the XXX cells. Moreover, 7-KC significantly up-regulated hypodermal GFP of L3 larvae compared to EtOH fed animals (***Figure 10 B***). We thus conclude that 7-KC inhibits the DA pathway as well in wild type. This hypothesis is further in support with the finding that Δ^7-DA suppressed 7-KC induced *daf-9(hyp)*.

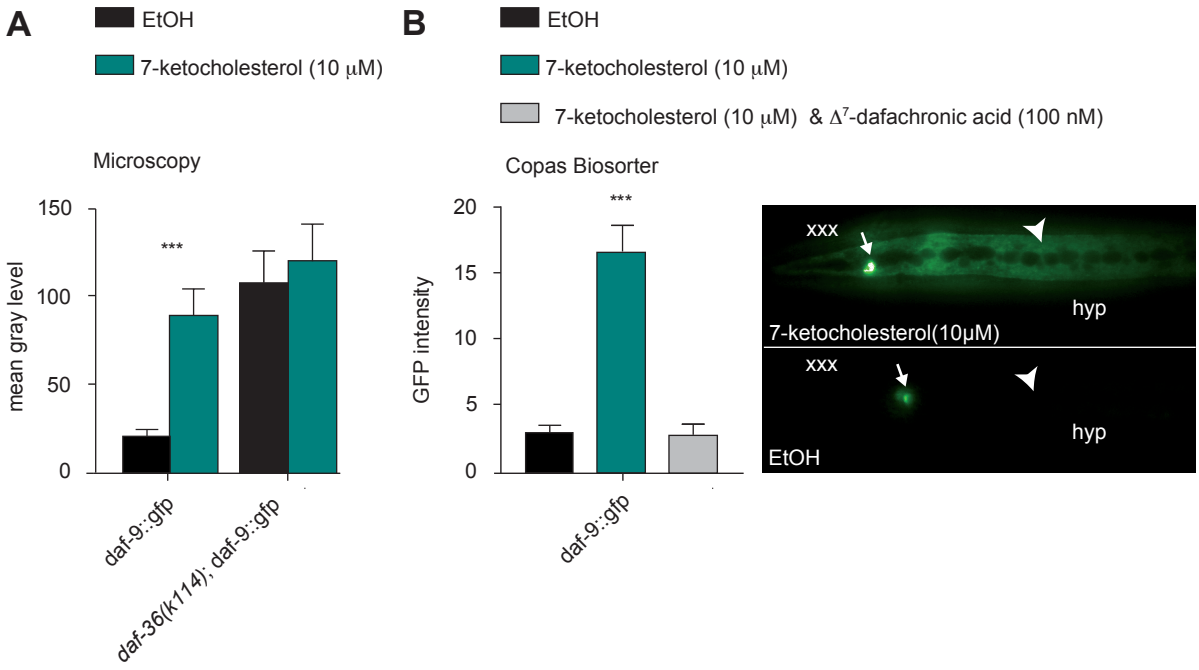

Figure 10 7-ketocholesterol induce daf-9::gfp hypodermal expression

***daf-9::gfp* and *daf-36(k114);daf-9::gfp* was grown from egg on at 20 °C on NGM plates provided with 7-ketochoesterol (10 µM) or corresponding volume of vehicle control (EtOH). Hypodermal GFP intensity of L3 larave was analyzed after 40 h by (A) microscopy or GFP intensity was analyzed with (B) Copas Biosorter. 7-ketocholesterol and daf-36(k114) induce hypodermal daf-9..GFP expression and the latter is rescued by additional Δ^7 - dafachronic acid (100 nm). Right Panel: Representative picture of L3 larvae fed with either vehicle control or 7-ketocholesterol (10 µM) xxx=XXX cells (arrows), hyp= hypodermis (arrow heads). ***p<0.001 (ANOVA, for (A) Mean+SEM n≥3, >12 worms each., for (B) Mean+SEM n≥3, >2000 worms each.**

7.4 7-KC extended lifespan on living and dead bacteria

The initial screen was based on the hypothesis that dauer modulators are also potential lifespan modulators in *C. elegans*. 7-KC increased dauer formation - thus we tested it for its effect on lifespan. Different concentrations of 7-KC were fed to N2 animals from L4 stage on (40 μM, 10 μM, 1 μM, 0.1 μM and 0.01 μM). Compared to EtOH fed animals 0.01 μM and 0.1 μM had no significant effect on lifespan. 1 μM and 10 μM significantly extended median lifespan by 5 % and 20%, respectively, but over all experiments not maximum lifespan

(***Figure 11 A, Table S1, Appendix table 1***). No extension was observed at and at 40 μM. Notably for observed median lifespan extension, it made no difference whether 10 μM 7-KC, was provided from egg on or from L4 stage on, uncoupling effects on lifespan from development. Notably feeding of 40 μM, led to no longevity when fed from egg on but to slightly increased lifespan when fed from L4 (***Figure 11 B&C, Table S1 Appendix table 1***). We conclude that 7-KC acts in a rather narrow range to extend *C. elegans* median but not maximum lifespan. Moreover we suggest that accumulation of 7-KC over time causes toxicity, counteracting and ultimately abrogating longevity. We decided on a concentration of 10 μM for further experiments.

Feeding assays on living bacteria always entail the risk of secondary effects of altered bacterial metabolism. Thus, we decided to perform control lifespan assay on UV inactivated bacteria. Feeding 7-KC (10 μM) induced the same lifespan extension (around 20 %) as on living bacteria. (***Figure 11 D, Appendix table 1***), suggesting that 7-KC directly affects worms metabolism to extend lifespan (hereafter called 7-KC longevity).

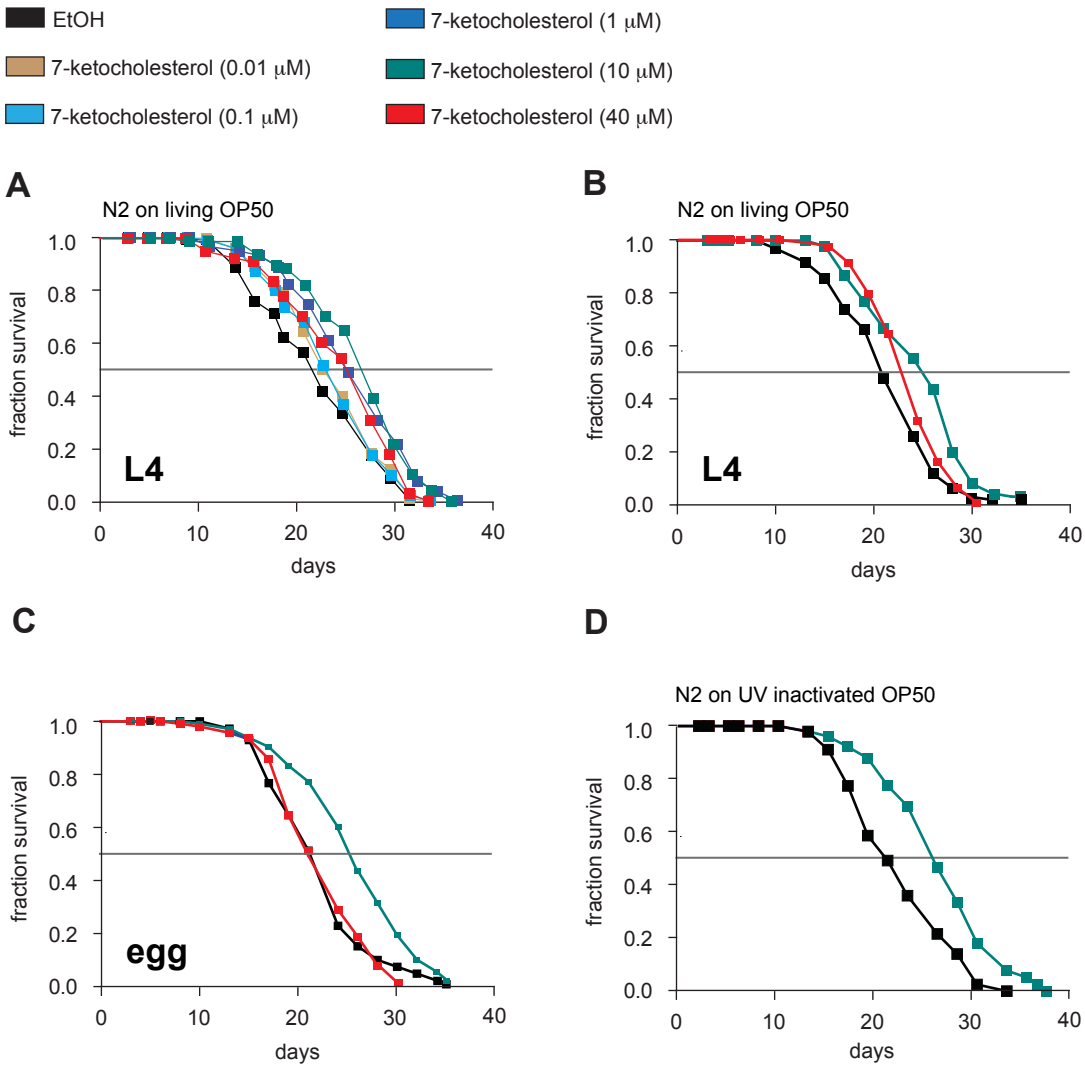

Figure 11 7-KC extends lifespan of N2 on living and dead bacteria

N2 was grown at 20 °C on NGM plates and fed with 7-ketocholesterol either (A, B and D) from L4 on or (C) from egg on at indicated concentrations or with corresponding volume of vehicle control (EtOH). N2 was either grown on living OP50 (A,B,C) or on UV-inactivated OP50 (D). 10 µM 7-ketocholesterol extends lifespan on living and UV treated OP50 (20 %) provided from egg or L4.

7.5 7-KC effects on egg lay and pumping rate

To further characterize the effects of 7-KC on N2 developmental and physiology, fecundity and pumping rate were monitored. A potential mechanism for lifespan extension is the energy tradeoff from reproduction to somatic maintenance. Therefore, egg-laying behavior of 7-KC fed animals was analyzed and compared to EtOH fed animals. We found that 7-KC fed animals laid slightly but significantly less eggs (***Figure 12 A, upper panel***). However, over the course of the reproductive period there was no delay in egg laying ***(Figure 12A, lower panel)***. Thus, we conclude that this minor change in total fecundity does not account for 7-KC longevity.

Another mechanism to delay ageing is CR. Therefore we asked if investigated if 7-KC (10 μM) fed animals take up less food. Pumping rate of the posterior pharyngeal bulb was used as a proxy measure for food uptake. 7-KC slightly decreased pumping rate in fed animals on day one but not later in life (day six and day ten) (***Figure 12 B***). We presume that this effect is negligible and not sufficient to induce CR longevity.

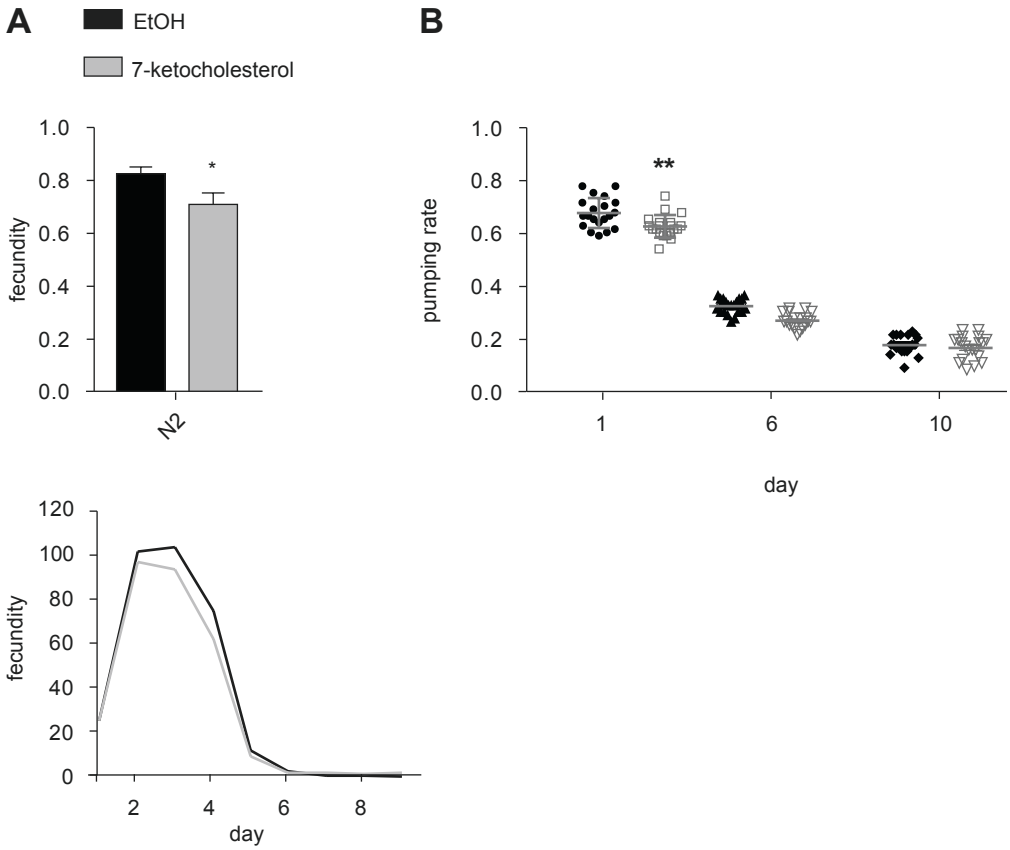

Figure 12 7-KC effects on egg lay and pumping rate

N2 grown from egg on at 20 °C on NGM plates containing 10 µM 7-ketocholesterol or corresponding volume of vehicle control (EtOH). Fecundity that hatched was scored over 10 days of adulthood starting from L4+1 day (72 h). (A, upper panel) Bars represent total number of progeny. t-test *p<0.05 Mean+SD (n ≥36).(B, lower panel) Time course of total number of living progeny of N2 treated and untreated. Mean (n ≥18) (C) N2 was grown from egg on at 20 °C on NGM plates containing 10 µM 7-ketocholesterol or corresponding volume of vehicle control (EtOH). Pumpingrate of pharyngeal bulb was scored at L4 + 1 day, +6 days and +10 days respectively. Mean+SD (n ≥20).

8 Mechanism of 7-ketocholesterol induced dauer formation

8.1 DA pathway intermediates suppressed 7-KC induced dauer formation and *daf-9(hyp)*

Next, we asked what mechanism underlies 7-KC induced dauer formation and *daf-9(hyp)*. The presented data provide several lines of evidence 7-KC acts in proximity to DA pathway. First, dauer formation and *daf-9(hyp)* are phenotypes linked to interference of DA synthesis. Second, 7-KC induced *daf-9(hyp)* is suppressed by DA. Third, the DA pathway shows characteristics of longevity pathways and could also account for 7-KC longevity. Thus, we decided to more intensively study the effects of 7-KC on the DA pathway.

7-KC structure is related to all steroids of the DA pathway. We therefore hypothesize, that it might thwart one or more compounds from being processed by according enzymes. Thus, the epistatic relation of 7-KC and DA pathway was investigated by feeding each of the intermediates in addition to 7-KC. We expected to see rescue of 7-KC induced dauer formation and *daf-9(hyp)* with those compounds that are downstream of the inhibited DA synthesis step. Same assays as above were used but this time in addition to 7-KC all known intermediates of DA pathway were provided.

7-KC induced dauer formation in *daf-2(e1368)* was rescued with 7-dehydrocholesterol and lathosterol back to levels similar d*af-2(e1368)* to EtOH. We observed high variability between biological replicates but never saw complete rescue of *daf-2(e1368)* induced dauer formation. With lathosterone dauer formation was rescued almost completely (and with Δ^7-DA no dauer larvae were formed (***Figure 13 A***). We observed before, that *daf-2(e1368)* dauer formation was not suppressed by 7-dehydrocholesterol and lathosterol (***Figure 7***). We conclude that all provided intermediates rescue at least the 7-KC induced portion of dauer formation.

7-KC induced *daf-9(hyp)* was rescued back to wild type level with 7-dehydrocholesterol, lathosterol, lathosterone and Δ^7-DA. Additionally upstream substrate cholesterol rescued *daf-9(hyp)* (***Figure 13 B***). These data suggest that intermediates upstream of 7-KC actually might enhance DA pathway activity and superimpose mild effect of 7-KC. However we decided to first follow the initial hypothesis that only downstream components rescue 7-KC induced phenotypes and conclude that 7-KC acts upstream or at the level of cholesterol.

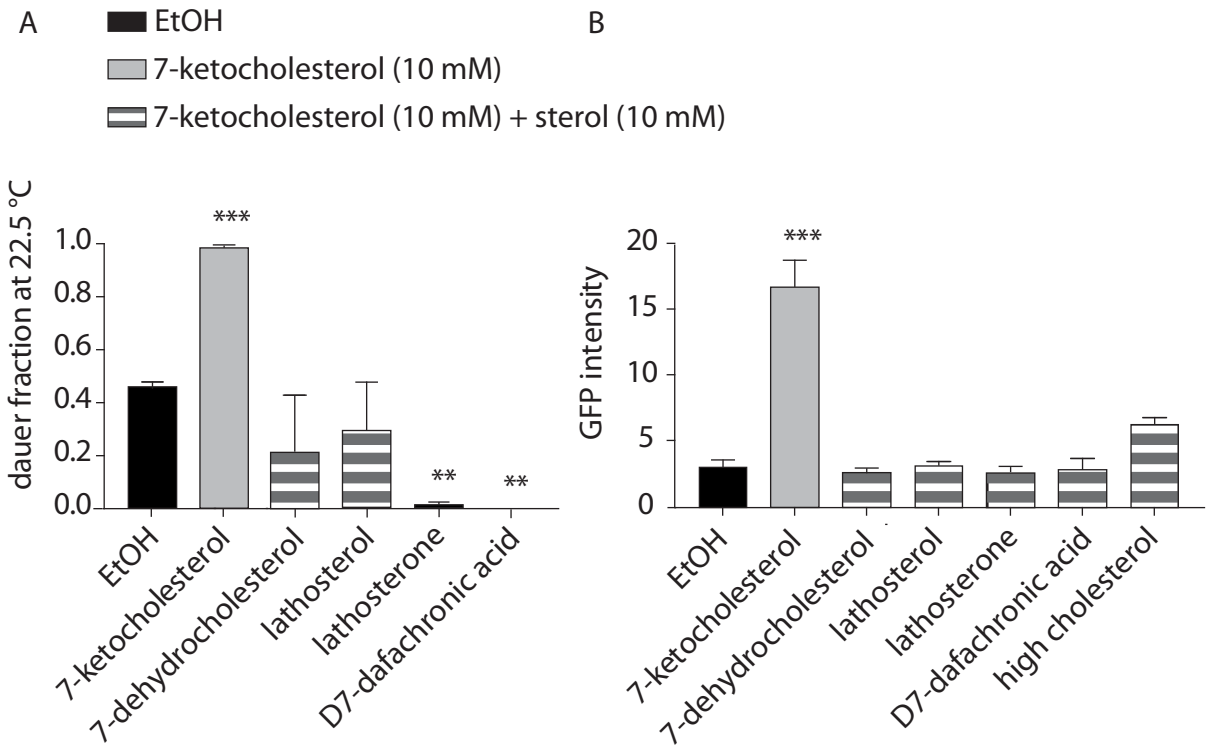

Figure 13 7-KC dauer formation and hypodermal daf-9 expression is linked to DA pathway

(A) *Daf-2(e1368)* was grown from egg on at 22.5 °C on NGM plates containing provided with 7-ketochoesterol (10 µM) alone, 7-ketocholesterol (10 µM) and DA intermediate, namely 7-dehydrocholesterol (10 µM), lathosterol (10 µM), lathosterone (10 µM), D7-dafachronic acid (100 nM). Corresponding volume of vehicle (EtOH was) used as control. Dauer fraction was scored after 60 h. ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each) (B) *daf-9::gfp* was grown from egg on at 20 °C on NGM plates provided with 7-ketochoesterol (10 µM) alone, 7-ketocholesterol (10 µM) plus 7-dehydrocholesterol (10 µM), lathosterol (10 µM), lathosterone (10 µM), Δ^7 -dafachronic acid (100 nM). Corresponding volume of vehicle (EtOH) was used as control. GFP intensity of L3 larave was analyzed after 40 h with Copas Biosorter. ***p<0.001 (ANOVA). Mean+SEM (n≥3, >2000 worms each)

8.2 7-KC induced dauer formation is *ncr-1;ncr-2* independent

C. elegans is cholesterol auxotroph and thus the sole source of cholesterol uptake in the diet. Thus, if 7-KC inhibits cholesterol availability, it must interfere cholesterol-uptake or distribution. In *C. elegans* not much is known about steroid transport in general and about cholesterol transport in particular. Nevertheless it is understood that homologues of human Niemann-Pick Type C-1 (NPC) *ncr-1* and *ncr-2* are essential for proper steroid transport and functional DA syntheses (Li, 2004). Consequently the double mutant *ncr-1(tm2022); ncr-2(tm2023)* is Daf-c. If 7-KC competes with cholesterol for uptake by NCR1 and/or NCR-2, in a double null mutant we expect no phenotype. Thus, we assayed dauer formation of *ncr-1(tm2022); ncr-2(tm2023)* at 20 °C. As shown before at 20 °C, N2 formed no dauer neither on EtOH nor on 7-KC (10 μM). Whereas 18 % of *ncr-1(tm2022); ncr-2(tm2023)* entered

dauer on EtOH. Interestingly, this fraction was increased significantly up to 80 % with 7-KC (10 µM) (***Figure 14***). Thus, we conclude that *ncr-1* and *ncr-2* are dispensable for 7-KC induced dauer formation and that 7-KC does not inhibit cholesterol uptake via *ncr-1* and *ncr-2*. However, we cannot exclude that there is another unknown steroid transport system that is inhibited by 7-KC and decided to investigate the actual levels of cholesterol after 7-KC feeding.

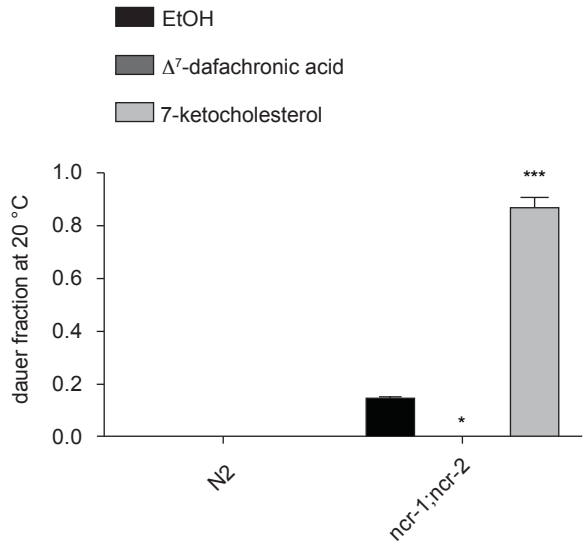

Figure 14 7-ketocholesterol enhanced dauer formation *ncr-1;ncr-2* independent

N2 and *ncr-(nr2022); ncr-2(2023)* were grown from egg on at 22.5 ° C on NGM plates containing 7-ketochoesterol (10 µ M). Corresponding volume of vehicle (EtOH was) used as control populations dauer larvae were scored after 60 hours. DA rescued and 7-ketocholesterol increased dauer formation of *ncr-1;ncr-2*. *p<0.05, ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each)

8.3 7-KC increased cholesterol and decreased 7-dehydrocholesterol levels

If cholesterol uptake is blocked by 7-KC then after 7-KC feeding whole body cholesterol levels should be decreased. We therefore measured levels of cholesterol in whole worm lipid extracts with GC/MS/MS analyses. Analyses were performed on N2 L3 larvae, a stage right after dauer decision and therefore the established stage to reflect metabolic state of enacted dauer decision. 7-KC (10 µM) significantly increased cholesterol levels (three fold) compared to L3 larvae grown on EtOH (***Figure 15 A***). Thus, we assumed that whole body limited cholesterol availability is not the reason for 7-KC induced dauer formation. However, we cannot exclude that cholesterol distribution in the worm is altered. Regarding the

endocrine character of the DA pathway proper distribution might actually play an important role (4.4). Unfortunately this problem cannot be addressed with GC/MS/MS technology.

Most notably we also found that 7-KC (10 μM) significantly decreased whole body 7-dehydrocholesterol levels (three fold) (***Figure 15 A***). 7-dehydrocholesterol is synthesized from cholesterol by DAF-36. (Wollam et al., 2012; Yamawaki et al., 2010). Taken together these results suggest that 7-KC inhibits DAF-36 activity. These findings lead to two conclusions: First we cannot argue convincingly that higher cholesterol levels are resulting from uptake, because they might occur due to decreased processing rate and accumulation over time. Second, 7-dehydrocholesterol depletion might be the actual cause of 7-KC induced dauer formation.

To clarify if this is the case, the levels of downstream intermediates, lathosterone and lathosterol in 7-KC fed animals were monitored. If DA synthesis is impaired at the level of DAF-36 and this inhibition is responsible for dauer formation we expect lower levels of all downstream components. Thus, we determined levels of lathosterol, lathosterone and Δ^7-DA. Interestingly, 7-KC (10 μM) did not significant change their levels (***Figure 15 B&C***). We conclude that enough 7-deyhydrocholesterol is processed to assure wild type levels of lathosterone and lathosterol and to promote reproductive growth in 7-KC fed worms. These findings suggest that 7-dehydrocholesterol-depletion is uncoupled from the dauer formation phenotype.

Consistent with the idea that DA synthesis in N2 is not inhibited by 7-KC strongly enough to induce dauer formation, 7-KC (10 μM) slightly increased Δ^7-DA levels (***Figure 15 C***). It seems counterintuitive at first glance that increased Δ^7-DA in N2 L3 larvae match the DA depletion phenotype of dauer formation. This supports the idea of moderate DA pathway inhibition. As mentioned above slight inhibition leads to expression of DAF-9 in the hypodermis and enhanced DA synthesis and we showed *daf-9(hyp)* phenotype in 7-KC fed N2 larvae (***Figure 10***). Taken together we conclude that neither inhibition of cholesterol uptake nor 7-dehydrocholsterol depletion is the cause of 7-KC induced dauer formation.

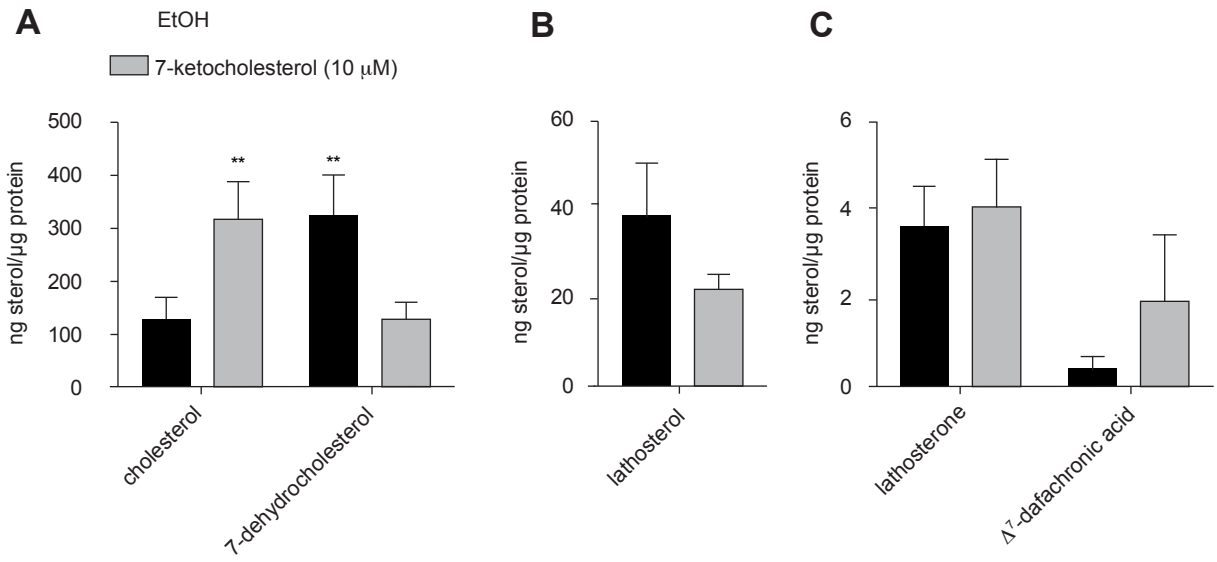

Figure 15 GC/MS/MS 7-ketocholesterol effects steroid profile of N2

N2 was grown from egg on at 20 °C on NGM plates provided with 7-ketochoesterol (10 μM) or corresponding volume of vehicle control (EtOH). L3 larvae were harvested and lipids were extracted with chloroform-methanol. Whole lipid extracts were derivatized and analyzed with GC/MS/MS relative to an added internal standard (5-β-cholanic acid for Δ^7-dafachronic acid and cholesterol-d7 for all other steroids). Cholesterol was increased and 7-dehydrocholesterol was decreased upon 7-ketocholesterol treatment. **p<0.001 (ANOVA). Mean+SEM (n≥3).

8.4 7-KC induced dauer formation is *daf-36* independent

To further test the hypothesis that DAF-36 inhibition and resulting 7-dehydrocholesterol depletion does not induce the dauer phenotypes we used a *daf-36* null mutant - *daf-36(k114)*. If our hypothesis is right, *daf-36(k114)* would not abrogate 7-KC induced dauer formation. Thus, we assayed *daf-36(k114)* animals at 25 °C fed with 7-KC (10 μM). On EtOH control plates no dauer larvae were formed but 85 % dauer larvae were formed on 7-KC conditions. Thus, DAF-36 can be excluded as the sole target of 7-KC and as the mediator of 7-KC induced dauer formation (***Figure 16***). This leaves the question open, what the actual target of 7-KC is.

8.5 7-KC modulated dauer formation downstream of IIS and TGF-β

To identify the target that mediates 7-KC induced dauer formation we utilized not only null mutants of the DA pathway but also null mutants of its upstream regulators IIS and TGF-β. TGF-β and IIS pathways converge on downstream transcription factors. If the hypothesis that 7 –KC acts on the DA pathway is right, it is expected that dauer induction is independent on these upstream pathways.

The role of TGF-β and IIS in 7-KC induced dauer formation was investigated. The smad homologs DAF-3 and DAF-5 are the downstream targets of TGF-β signaling and DAF-16/FOXO is the homolog of mammalian FOXO transcription factor and the downstream target of IIS and there activation supports reproductive growth. The single mutants *daf-3(mgDf90)*, *daf-5(e1386)* and *daf-16(mgDf50)* are thus Daf-d at 25 °C (Hu, 2007). Considering that 7-KC induced dauer only in dauer prone backgrounds like *daf-2(e1368)* and *daf-36(k114)* but not in wild type animals, dauer induction is neither expected for these single Daf-d mutants. Therefore in addition null mutants in *daf-36(k114)* background were analyzed. Moreover the Daf-c mutants *daf-7(e1372)* and *daf-2(e1368)* encoding the upstream receptors of TGF-β signaling and IIS, respectively, were analyzed. If 7-KC is acting downstream of IIS and TGF-β we expect 7-ketocholeterol induced dauer formation at least in *daf-36(k114)* background.

N2 formed no dauers at 25 °C, neither with EtOH control nor with 7-KC (10 μM). As expected *daf-7(e1372)* formed 89 % dauers and the fraction was increased upon 7-KC provision (100 %). Neither the single mutant *daf-5(e1386)* nor the double mutant *daf-5(e1386); daf-36(k114)* formed dauer larvae on EtOH control. 7-KC (10 μM) did not induce dauer larvae formation in the single mutant *daf-5(e1386)* but around 33 % in the double mutant *daf-5(e1386); daf-36(k114)*. The single mutant *daf-3(mgDf90)* formed no dauers on EtOH but 35% with 7-KC (10 μM) treatment. 5 % of the double mutant *daf-3(mgDf90); daf-36(k114)* entered dauer on EtOH and that fraction was increased to 70% with 7-KC (10 μM) (***Figure 16***).

The unexpected dauer larvae formed in single *daf-3(mgDf90)* with 7-KC and double *daf-3(mgDf90); daf-36(k114)* with EtOH might be explained with a more complex role of DAF-3. It was reported before that Daf-d phenotype of DAF-3 is temperature dependent and is switched to Daf-c at higher temperatures (27°C) (Ailion and Thomas, 2000; Thomas et al., 1993). It is therefore possible that it also loses Daf-d abilities in more dauer prone conditions induced by 7-KC feeding or *daf-36* background. We concluded that there might be a partial dependence on TGF-β since dauer formation is only partial restored. On the other hand this could be explained by the strong dependence of 7-KC dauer induction on Daf-c backgrounds. Interactions and relations of IIS, TGF-β and DA pathway are not completely understood and null mutants might induce feedback loops to regulate other parts of the network- resulting in less dauer prone background. However, based on these data we presume that 7-KC acts downstream of TGF-β.

Next, we investigated 7-KC dauer dependence on IIS. At 25 °C *daf-2(e1368)* formed 100 % dauer so 7-KC cannot further increase this fraction. *Daf-16(mgDf50)* did not form dauers with 7-KC but neither abrogated 7-KC induced dauer formation of *daf-36(k114)* (***Figure 16***). Thus, we conclude that 7-KC acts downstream of IIS and *daf-16*. Taken together this supports the hypothesis that 7-KC acts on DA pathway.

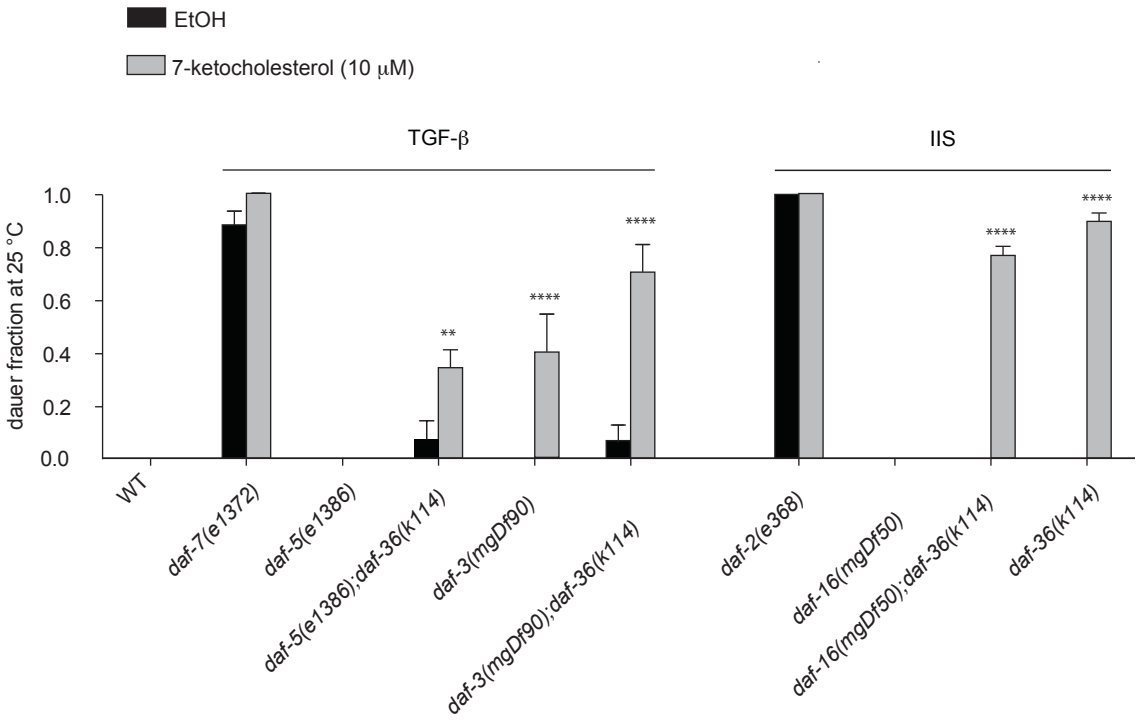

Figure 16 7-KC increased dauer independent of IIS and TGF-β signaling

Epistasis of 7-ketocholesterol to TGF-β and IIS: Indicated genotypes were grown from egg on at 25 °C on NGM plates containing 7-ketochoesterol (10 μM) or corresponding volume of vehicle (EtOH) as control. Dauer fraction was scored after 48 h. **p<0.05, ****p<0.0001 (ANOVA). Mean+SEM (n≥3, >50 worms each).

8.6 7-KC modulated dauer formation downstream of DA synthesis

So far it was shown that 7-KC acts downstream of IIS and TGF-β and independent of *daf-36* to induce dauer. Thus, we decided to investigate if 7-KC dauer induction is depending on other enzymes of the DA synthesis pathway. We used the Daf-c null mutations *dhs-16(tm1890)* and *daf-40(hd100)*. In addition we used the Daf-c *daf-9(k182),* since the null mutation *daf-9(dh6*) forms constitutively dauer at all temperatures.

dhs-16(tm1890) and *daf-9(k182)* did not form dauers on EtOH and with 7-KC (10 μM) dauer in both few dauer larvae were observed but with high variability between biological replicates and significance. *daf-40(hd100)* formed no dauer larvae on EtOH but 7-KC

significantly induced dauer larvae formation (90 %) (***Figure 17 A***). To further clarify if there is an effect of 7-KC in *dhs-16* we scored Mig at 26 °C. Notably, 7-KC increased fraction of Mig but again significance was not reached. For *daf-9(k182)*, we combined scored for DA related phenotypes at 26 °C (Mig and dauer) and found a significant increase with 7-KC (***Figure 17 B***).

Taken together 7-KC is placed downstream of TGF-β, IIS and DA synthesis, so we next investigated whether downstream targets of DA are essential for 7-KC induced dauer formation. As expected the Daf-d null mutant *daf-12(rh6hrh411)* does not form dauer larvae upon 7-KC feeding. As for the DA synthesis pathway mutants we thus crossed it into a Daf-c background. It was suggested that DAF-12 activity might have direct feedback loops on *daf-36*. Therefore we decided to use an upstream regulator of the DA pathway namely the IIS mutant *daf-2(e1368)* because. Other than TGF-β and IIS mutants in Daf-c background, 7-KC induced dauer formation is abolished in *daf-12(rh61rh411);daf-2(e1368)*. We observed the same dependence on *din-1*. Dauer formation thus is *daf-12* and *din-1* dependent (***Figure 17 C***).

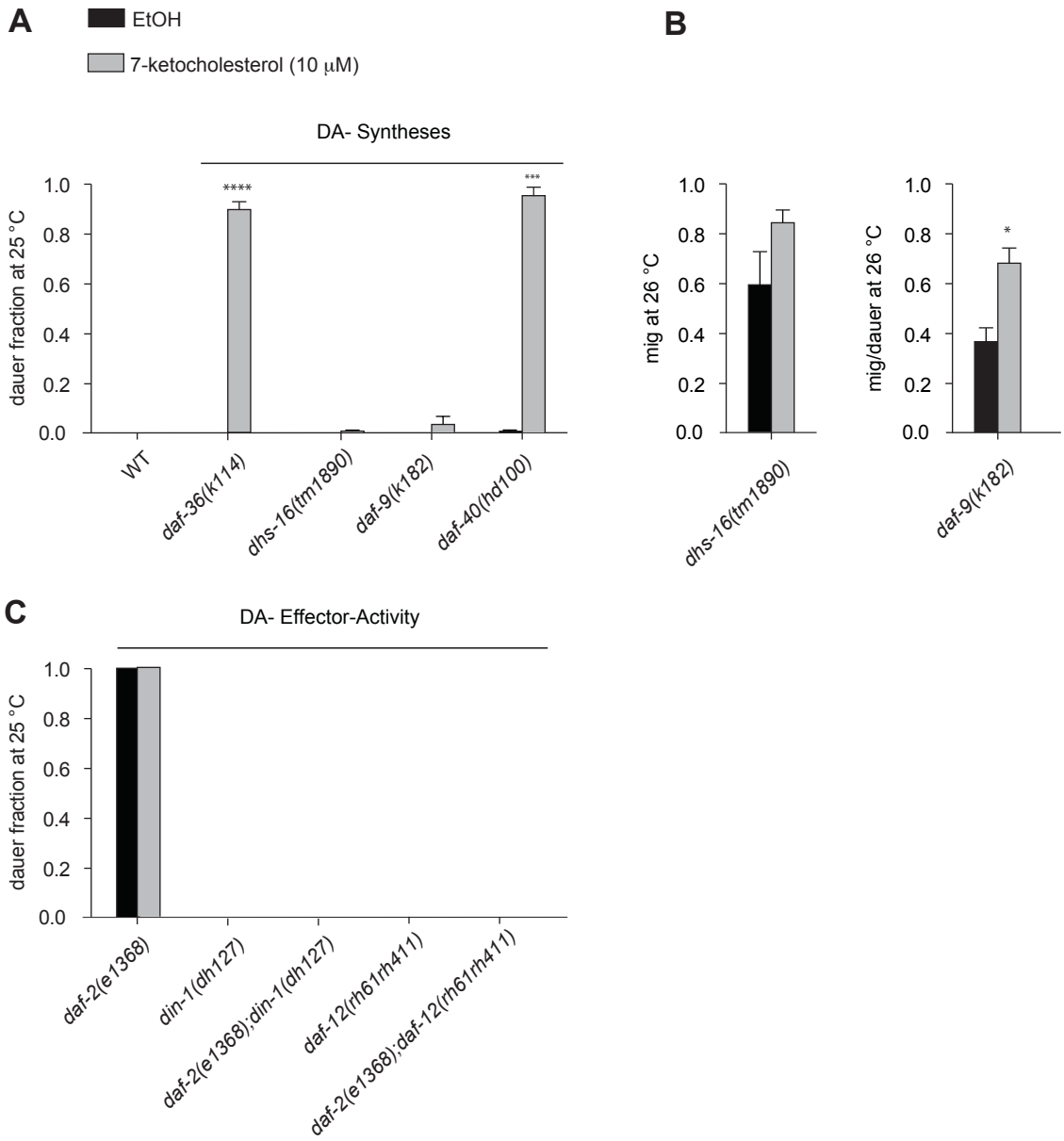

Figure 17 7-KC suppressed DA- effector- activity but not DA- synthesis

Indicated genotypes were grown from egg on at 22.5 °C on NGM plates containing provided with 7-ketochoesterol (10 μM) or corresponding volume of vehicle (EtOH) as control. Dauer fraction was scored after 60 h. (A) Epistasis of 7-ketocholesterol to DA- pathway (B) *mig* fraction of *dhs-16(tm1890)* was scored and dauer fraction an mig fraction of hypomorph *daf-9(k182)* was scored, both after 60 h. (C) Epistasis of 7-ketocholesterol to DA- Effector-activity *p<0.05, ***p<0.001 (ANOVA). Mean+SEM (n≥3, >50 worms each).

9 Mechanism of 7-ketocholesterol induced longevity

9.1 7-KC longevity was *daf-12* dependent

It is known that mutations in the DA pathway such as *daf-12(rh411rh611)* show lifespan phenotypes. Additionally DA is the longevity signal of germline longevity (Gerisch et al., 2007; Yamawaki et al., 2010). The DA pathway can thus be considered as an ageing pathway in *C. elegans*. To investigate if we can place the 7-KC induced longevity with in the DA pathway we performed lifespan assays using mutants in DA and upstream IIS pathway.

First, lifespan of the IIS mutants *daf-2(e1368)* and *daf-16(mgDf50)* was monitored on 7-KC supplemented plates (10 µM). We hypothesize that 7-KC effects the same epistatic position for longevity as for dauer induction. If this is the case longevity is expected to be independent of *daf-2* and *daf-16*. In at least three biological independent repeats 7-KC extended median lifespan of IIS mutants *daf-2(e1368)* and *daf-16(mgDf50)* (***Figure 18 A,*** *Appendix table 1*). Next, DA pathway mutants *daf-36(k114)* and *daf-12(rh61rh411)* were tested. We found that 7-KC induced longevity in *daf-36(k114)* but not in *daf-12(rh61rh411)* (***Figure 18 B,*** *Appendix table 1*). We therefore conclude, that DAF-12 is essential to 7-KC longevity. Taken together our data suggest that 7-KC acts on DAF-12 to promote dauer formation and longevity.

9.2 7-KC longevity depended on *daf-12(LBD)*

Our hypothesis suggests that 7-KC suppresses DA binding to DAF-12. We therefore asked if it might bind to the same postulated ligand-binding domain (LBD) as Δ^7-DA. We used a *daf-12(rh273)* a DAF-12 mutant carrying a mutation in the postulated DA/ligand binding domain (LBD) and we observed that this mutation is sufficient to abolish 7-KC induced longevity (***Figure 18 C,*** *Appendix table 1*). A functional LBD is therefore essential for the interaction of 7-KC on DAF-12.

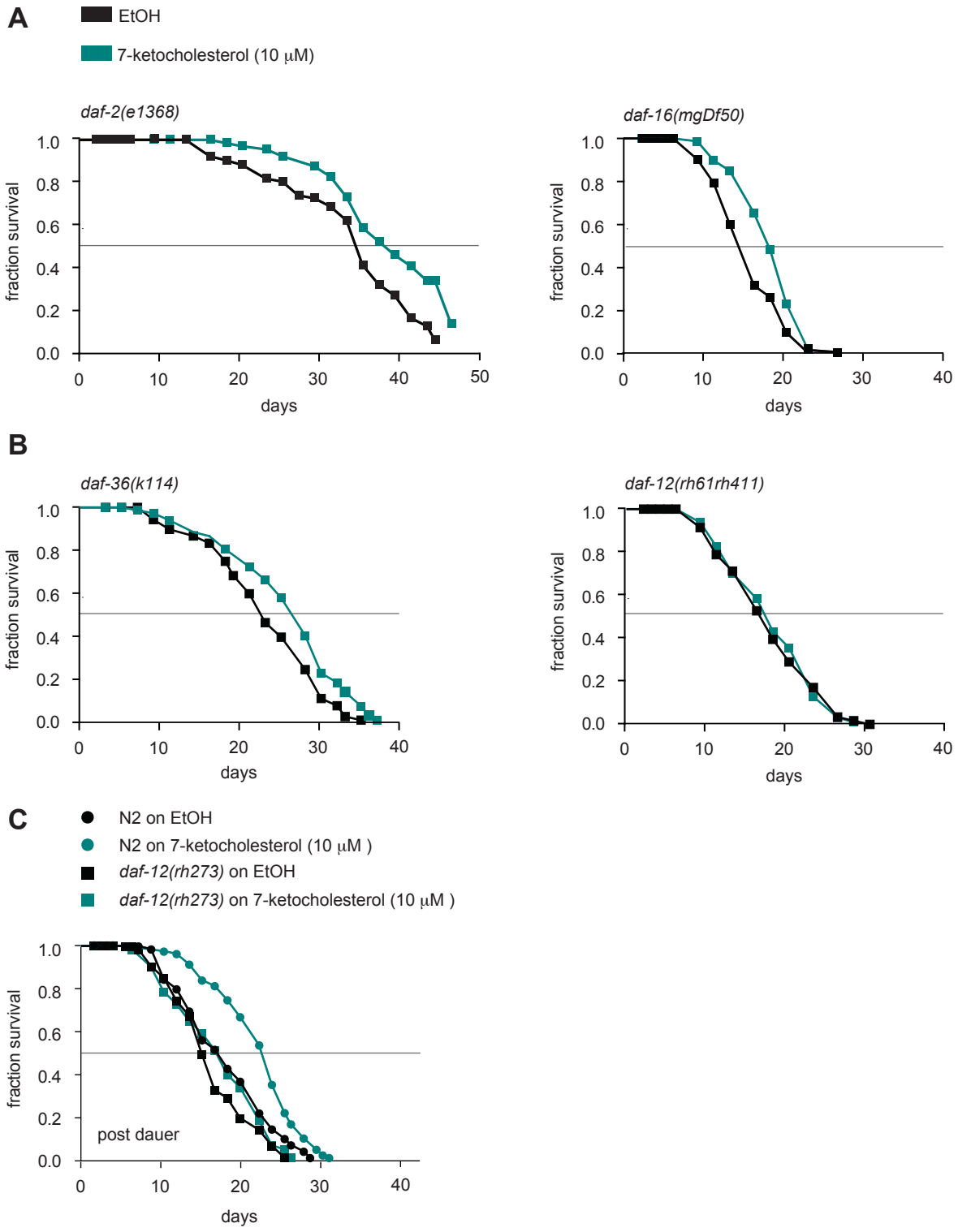

Figure 18 7-ketocholesterol extends N2 lifespan on living and dead OP50

Indicated genotypes were grown from egg on at 20 °C on NGM plates containing 10 µM or corresponding volume of vehicle control (EtOH). (A) Genes of the IIS pathway *daf-2(e1368)*and *daf-16(mgDf50)* (B) Genes of the DA pathway *daf-36(k114)* and *daf-12(rh61rh411)*. (C) *daf-12(rh273)* is a mutation that abolishes functionality of DAF-12 LBD. 7-KC longevity is IIS, independent and it is also *daf-36* independent. But it is dependent on *daf-12*, more precisely on *daf-12(LBD)*.

10 7-Ketocholesterol acts on DAF-12

10.1 7-KC down-regulated DAF-12 target genes

Our data suggest that 7-KC inhibits DAF-12 activator activity by outcompeting DA binding. To test our hypothesis we first examined levels of *let-7* family members *mir-84*, *mir-48* and *mir-273*. These microRNAs are known targets of DA bound DAF-12 (Bethke et al., 2009; Shen et al., 2012). If 7-KC inhibits that binding we also expected down-regulation of the driven target genes. To address this question we performed qPCR on N2 L3 larvae fed with either vehicle EtOH or 7-KC. Moreover we analyzed levels in *daf-12(rh61rh411)*, if down regulation of 7-KC is mediated via DAF-12 we expected to see no further down regula tion of the let-7s in this background.

In 7-KC fed N2 L3 larvae the tested microRNAs, *mir-48, mir-84 and mir-241* are all mildly but significantly down regulated (0.77, 0.70 and 0.65) compared to vehicle fed larvae. In *daf-12(rh61rh411)* vehicle fed animals all three micro RNAs are down-regulated significantly compared to wild type, as reported before. Notably there is no further down-regulation when 7-KC is fed (***Figure 19***). These data show that 7-KC down-regulates tested DAF-12 target genes and, moreover, support the idea that this effect is mediated via DAF-12 inhibition.

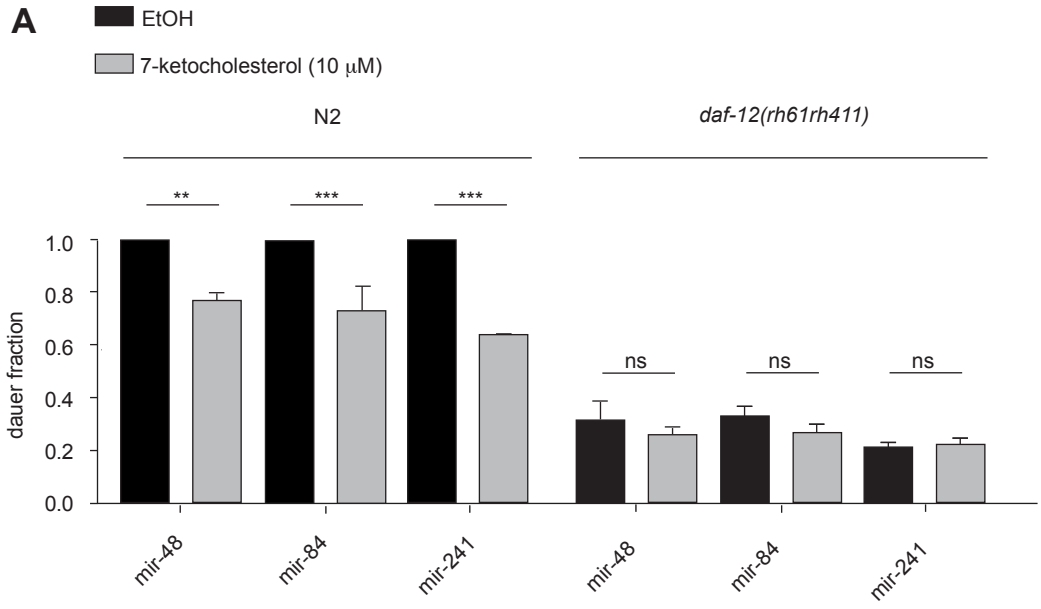

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Figure 19 7-ketocholesterol down-regulates *mir-48, mir-84* and *mir 241*

N2 and *daf-12(rh61rh411)* was grown from egg on at 20 °C on NGM plates and provided from L4 on with 7-ketochoesterol (10 µM) or vehicle control. 24 h after 7-ketocholesterol provision, RNA was isolated from 200 adult animals and levels of the micro RNAs *mir-48, mir-84* and *mir-241* were analyzed using qPCR. Shown are the relatives values to EtOH control, each sample was normalized to internal control (*U-18*). *** p<0.001 (ANOVA). Mean+SEM (n≥3).

10.2 7-KC inhibited DA binding to DAF-12 in cell culture

So far our hypothesis that 7-KC can outcompete DA is based on genetic evidence. To further test it biochemically, we performed a cell culture based competition assay. We choose an endogenous approach using a plasmid harboring DAF-12 and another plasmid carrying *mir-84* promoter driven luciferase (*pmir84::luciferase*) to co-transfect HEK293T cells(Bethke et al., 2009). We only expect activated DAF-12 to bind to *pmir84::luciferase*. We thus provided DA to activate DAF-12 and induce resulting luciferase activity (***Figure 20 A***). To control that luciferase activity is due to DA activated DAF-12 and not a general effect of DA in our system, we provided DA to cells co-transfected with the *pmir-84::luciferase* vector and the backbone of the DAF-12 plasmid expressing FLAGG. If we in addition add 7-KC and it really represses DAF-12 activity by outcompeting DA, we expect to see a down-regulation of luciferase activity (***Figure 20 A***).

In cell transfected with FLAG and *pmir84* baseline luciferase activity was not increased with DA neither with additional vehicle (***Figure 20 B, black curve with dots***) nor with 7-KC (***green curve with dots***). If DAF-12 was co-transfected with *pmir84::luciferase*, luciferase activity increased in a DA dose responsive manner from 3 nM up to 100 nM (***black curve with boxes***). No further increase was observed with 300 nM, indicating saturation of the reaction. If 30 μM 7-KC was added, in addition to each DA concentration the curve shifted to the right (***green curve with boxes***) suggesting that DA is less efficient in activating DAF-12. We therefore conclude that 7-KC thwarts DA from DAF-12 binding.

We have shown before that DAF-12 LBD is essential to 7-KC longevity. Next, we tried to answer the question if the DNA- binding domain (DBD) might play a role for 7-KC binding as well. To address this question we used a slightly different approach. We co-transfected with a plasmid carrying a DAF-12(LBD)::GAL4(DBD) fusion and a plasmid carrying luciferase driven by 4xGAL promoter. Again FLAG control showed no altered luciferase activity, with and without 7-KC (***Figure 20 C, black curve and green curve with dots***). Cells with the DAF-12 construct in contrast responded to DA in a similar manner as in the first assay (***black curve with boxes***). Interestingly, in this system 7-KC showed no

inhibitory effect (***green curve with boxes***), indicating a potential role for the DBD in mediating 7-KC inhibition of DAF-12 activity, at least in cell culture (***Figure 20 B***).

Next, we addressed the question if the observed 7-KC inhibition of DAF-12 occurs within a physiologically relevant range using the endogenous assay. We therefore fixed DA concentration to 100 nM and titrated in 7-KC in a range from 0.1 to 30 μM. We found 300 nM 7-KC to be the lowest tested concentration that decreased DA induced luciferase activity (15%) (***Figure 20 C***). We suspect that 300 nM is a reasonable concentration for a physiological interaction.

To furthermore exclude the possibility that 7-KC induces a general stress response to steroid toxicity in turn that leads to reduced DAF-12 activity we tried to induce the postulated steroid toxicity with another steroid. We decided to use 7-β-hydroxycholesterol (7-β-OH) although it harbors another stereo center and the hydroxy moiety turns it into a less planar molecule compared to 7-KC, we still think due to its bipolarity and overall structure it is reasonable close to 7-KC. Moreover, both steroids 7-KC and 7-β-OH were shown before to play a role in apoptosis induction in cell culture and were shown to bind to the human CYP7A1. If 7-KC affect happens due to a general steroid toxicity we would expect a similar down-regulation in particular with 7-β-OH (30 μM). Notably 7-β-OH (30 μM) did not reduce DA induced DAF-12 activity by adding 7- (***Figure 20 D***). Thus, we think that the observed phenotype is not a cell response to steroid toxicity. Taken together our cell culture data suggest that 7-KC thwarts the interaction of DA and DAF-12 and thereby inhibits DAF-12 activity.

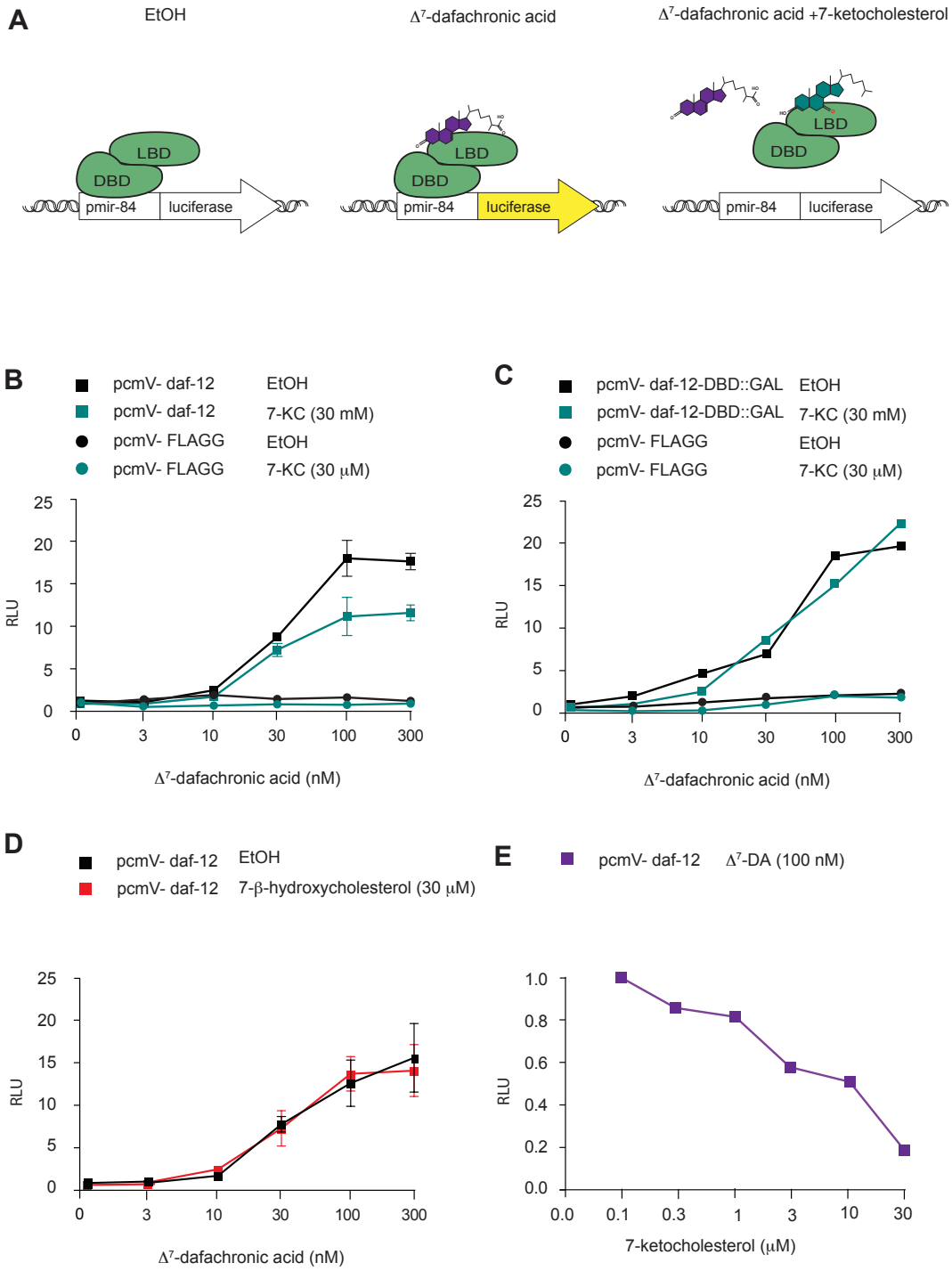


Figure 20 7-ketocholesterol outcompetes DA binding to DAF-12 in cell culture

HEK293T cells were co-transfected with DAF-12 and luciferase driven by the DAF-12 target mir-84 promoter. (A) Model of our hypothesis. (Left panel) DAF-12 in Hek293T without additional DA does not activate *mir-84* promoter driven luciferase, (middle panel) after DA binding luciferase is activated.(right panel) 7-ketocholesterol blocks DA binding to DAF-12 and inhibits luciferase activity. (B) DA activates luciferase activity in dose dependent manner and 7-ketocholesterol (30 µM) attenuates it (representative graph of 3 repeats). (C) DAF-12::LBD fused to Gal::DBD activates 4xGal promoter driven luciferase after DA binding in a dose dependent manner, additional 7-ketocholesterol (30 µM) does not inhibits luciferase activity. (D) Another sterol 7-β-hydroxycholesterol (30 µM) did not inhibit DA-DAF-12 interaction. (E) Same assay as (B) DA is fixed to 100 nM and 7-KC is titrated. 7-KC ≥ 300 nM inhibit DA-DAF-12 interaction.

10.3 7-KC longevity was *daf-9* and *din-1* dependent

So far it still remains elusive if 7-KC itself or a downstream product binds to DAF-12. It is known that at least two DAF-12 ligands, namely Δ^7-DA and Δ^4-DA can be synthesized by DAF-9, in worms from 4-cholesten-3-one and lathosterone, respectively. We therefore postulated that DAF-9 might act on 7-KC to synthesize 7-KC –DA (***Figure 21 A***). To follow up this idea we used the postulated null allele *daf-9(dh6)* to examine the role of DAF-9 and DA in 7-KC induced longevity. We used double mutants with *din-1(dh127)* null mutations to rescue developmental growth in *daf-9(dh6)* without adding DA. Assuming that 7-KC is converted to an active form by DAF-9 we expected the longevity phenotype to be rescued in a DAF-9 null background. We observed 7-KC longevity in *din-1(dh127)* single mutants but not in the *din-1(dh127);daf-9(dh6)* double (***Figure 21 B***). Interestingly, *daf-12(rh61rh411);daf-9(dh6)* decreases lifespan with 7-KC (***Table 1***). These data point at a potential function of DAF-9 in 7-KC longevity and 7-KC-DA as the active form promoting dauer formation and longevity.

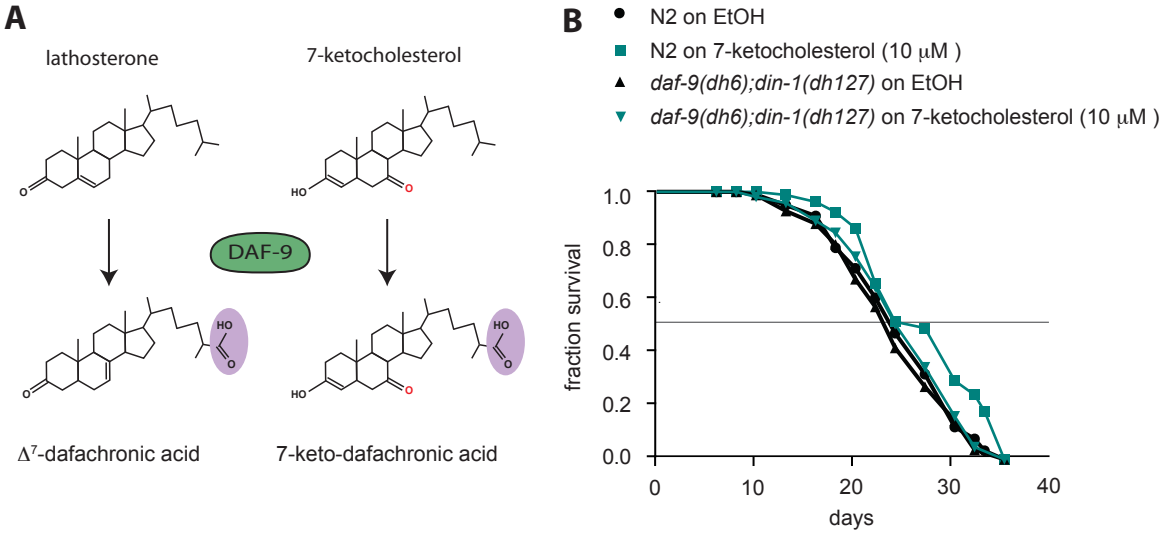


Figure 21 7-ketocholesterol longevity is *daf-9* dependent

(A) *daf-9(dh6);din-1(dh127)* was used to investigate DA free background. It was grown from egg on at 20 °C on NGM and transferred from L4 on to plates containing 7-ketcochlesterol (10 µM) or corresponding volume of vehicle control (EtOH). In two repeats 7-KC longevity depended on *daf-9*, leading to the following hypothesis: (B) DAF-9 acts on C26 of 7-ketocholesterol in a similar manner as on lathosterone to oxidize it to a dafachronic acid – 7-keto-DA.

11 7-Ketocholesterol induces transcriptional changes

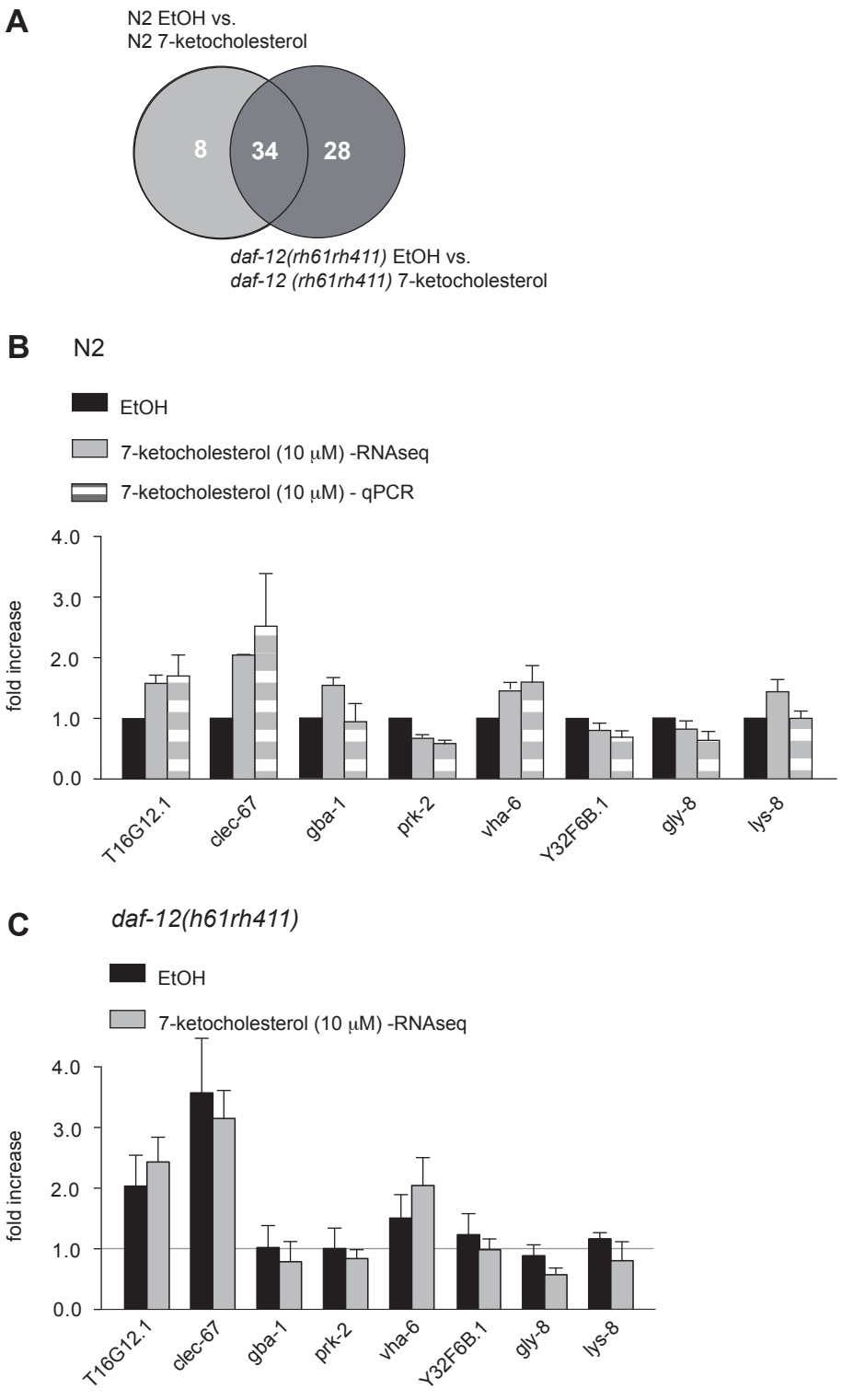
Taken together, we could show that 7-KC dauer formation and longevity is mediated via DAF-12. 7-KC might outcompete Δ^7-DA from binding to DAF-12 (***Figure 20***). But, the identification of DAF-12 as mediator of 7-KC induced phenotypes is only a first step to understand global role of 7-KC in *C. elegans*. To get a better understanding of potential other targets of 7-KC and to further unravel downstream targets of DAF-12- 7-KC interaction we decided to perform an unbiased approach using RNA sequencing (RNAseq). We performed RNAseq of N2 and *daf-12(rh61rh411)* young adults either provided with EtOH vehicle control or with 7-KC (10μM) from L4 on. Using this experimental setting we can approach a broad spectrum of questions. (All RNAseq results can be found in ***Appendix table 3 to 6***)

11.1 42 genes were regulated by 7-KC and 8 of them are *daf-12* dependent

First we checked for genes that were significantly changed (p adjusted ≤0.05) in 7-KC fed animals compared to EtOH fed animals, hereafter called 7-KC regulated genes. We identified 42 7-KC regulated genes in N2 (***Figure 22 A,*** *dark gray circle*). In *daf-12(rh61rh411)* background we identified 62 7-KC regulated genes (***Figure 22 A,*** *light gray circle*). 7-KC regulated genes in N2 and in *daf-12(rh61rh411)* were largely overlapping (34 genes ***Figure 22 A,*** *overlap*). Interestingly, moreover we identified eight 7-KC regulated genes, which were exclusively regulated in N2. This suggests that their regulation is *daf-12* dependent. They are of particular interest, because they might mediate the 7-KC induced, *daf-12* dependent phenotypes dauer formation and longevity. Notably, we cannot exclude that the set of overlapping genes plays a role in longevity as well, and only their effect on lifespan is abolished by one of the 28 7-KC regulated genes exclusively in *daf-12(rh61rh411)* (***Figure 22 A*** *light bubble*).

Before we further investigated the role of the 8 candidates we used them to validate quality of the RNAseq results with qPCR (***Figure 22 B***). We confirmed regulation of *T16G12.1*, *clec-67*, *prk-2*, *vha-6*, *Y32F6B.1* and *gly-8* in the same direction as measured with RNAseq. *gba-1* and *lys-8* were not regulated according to qPCR analyses. Next qPCR was performed on *daf-12(rh61rh411)* samples. It was confirmed that 7-KC did not regulate tested genes in *daf-12(rh61rh411)* (***Figure 22C*** black bars vs. gray bars). *T16G12.A*, *clec-67* and *vha-6* in *daf-12(rh61rh411)* with EtOH were upregulated and *gly-8* was down-regulated compared to N2 on EtOH (black bars vs. grey line). According to RNAseq results only *vha-6*

is up-regualted and *lys-8* is down-regulated (***Figure 24 B***). Thus we could only partially confirm RNAseq data with qPCR. More biological repeats will help to determine the actual regulation.



(*For figure legend see next page)*

Figure 22 Analyzes of RNA seq results

RNAseq analyzes were performed on N2 and *daf-12(rh61rh411)* animals. They were grown from egg on at 20 °C on NGM and transferred from L4 on to plates containing 7-ketcochlesterol (10 µM) or corresponding volume of vehicle control (EtOH). mRNA was isolated 24 h after shifting to experimental conditions. (A) 42 genes were regulated by 7-ketocholesterol in N2 (dark gray) 68 genes were regulated by 7-ketocholesterol in *daf-12(rh61rh411)* (light gray background. (B) Verification of RNAseq results (gray bars) using qPCR analyzes in N2 (striped bars). (C) Verification of RNAseq results (gray bars) using qPCR analyzes in *daf-12(rh61rh411).*

11.2 The gene ontology term "ageing" was DAF-12 dependent

We decided to analyze gene ontology (GO) terms of the RNAseq data. We used the online tool DAVIS to assign GO terms and to determine significance of enrichment in each GO term based on p-values. With this information at hand a cluster was created using REVIGO online tool for visualization. We used DAVIS to find GO terms for all 42 and 62 genes 7-KC regulated in N2 and *daf-12(rh61rh411)*, respectively. Regardless the low number of 7-KC regulated genes in N2, they were significantly enriched in the GO terms - cell death, death, regulation of growth, proteolysis, ageing and multicellular ageing. Notably the only GO terms not identified in 7-KC regulated genes of *daf-12(rh61rh411)* were "ageing" and "multicellular organism ageing" (***Figure 23***). A closer look revealed that 3 genes enriched in the ageing associated GO terms. These data strongly support our findings, that 7-KC longevity is *daf-12* dependent.

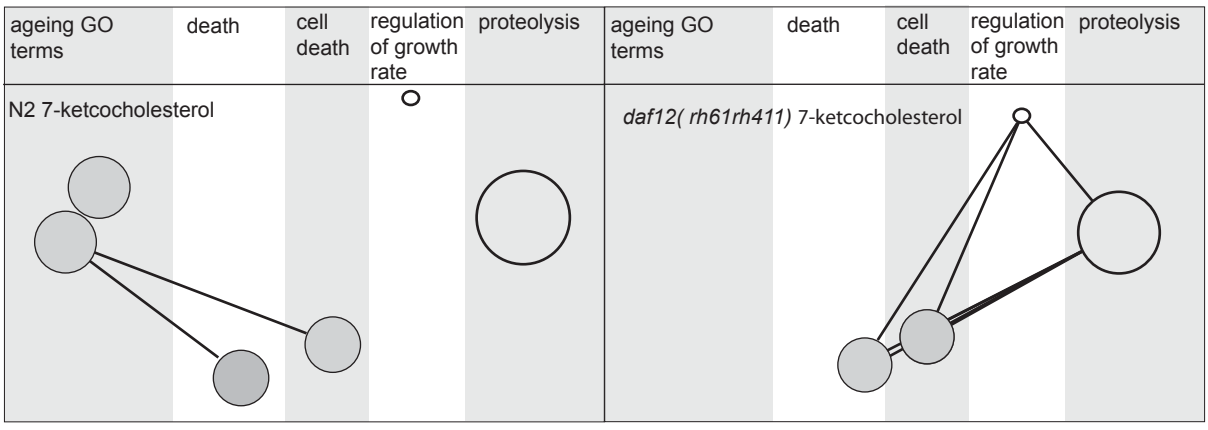


Figure 23 7-KC induced ageing is *daf-12* dependent

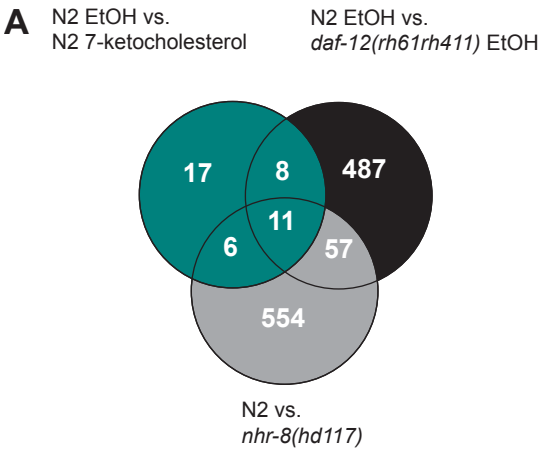
REVIGO visualization of GO terms significantly enriched ($p<0.05$) in sets of genes regulated by 7-KC in N2 (left panel) and *daf-12(rh61rh411)*(right panel), respectively. In N2 and *daf-12(rh61rh411)* backgrounds cell death, death, regulation of growth rate and proteolysis. Only the ageing related GO terms are not found in *daf-12(rh61rh411).*

11.3 7-KC regulated genes largely overlaped with DAF-12 regulated genes

Next, we asked if the RNAseq data support our hypothesis that 7-KC mediates its phenotypes via DAF-12. To address this question we compared 7-KC regulated genes with *daf-12* regulated genes. Daf-12 regulated genes are defined as all genes that were significantly changed in *daf-12(rh61rh411)* compared to N2. We found an overlap of 19 genes (***Figure 24A***). In other words, 45 % of all 7-KC regulated genes are also *daf-12* regulated. This high number of common targets supports the idea, that 7-KC mediates its effects via DAF-12.

We then further characterized the *daf-12* dependent 7-KC regulated genes. Our premise was that all *daf-12* regulated genes reflect DAF-12 activator function show up in our data set when we compare N2 on EtOH with *daf-12(rh41rh611)* on EtOH. Genes that are down regulated in this set are up-regulated by DAF-12 activity. If we further assume that 7-KC regulate genes only via the inhibition of DAF-12 activator activity, we would expect that these genes are regulated in the same direction as in a *daf-12* mutant. Interestingly, we found that only for 2 candidate genes (*lys-8, clec-67*), the other six genes (*prk-2*, Y32F6B.2, *gly-8*, *vha-6*, *gba-1*, T16G12.2) were not regulated by DAF-12 (***Figure 24 B***) according to RNAseq results. Thus, we conclude that these 6 genes might be a subset of genes that is exclusively regulated by 7-KC DAF-12 interaction. Taken together we narrowed down the number of candidate genes to 6. These 6 genes are *daf-12* dependent and might exclusively be regulated by 7-KC DAF-12 interaction. It needs to be considered that results leading to this conclusion are not entirely confirmed by qPCR.

A



B

Gene Name	Fold Regulation	
	N2 EtOH vs. 7-KC	N2 EtOh vs *rh61rh411* EtOH
prk-2	*0,61*	—
Y32F6B.2	*0,68*	—
gly-8	*0,71*	—
lys-8	*1,29*	**1,29**
vha-6	*1,32*	—
gba-1	*1,39*	—
T16G12.2	*1,42*	—
clec-67	*2,27*	**3,23**

Figure 24 A gene with potential to be exclusively regulated by 7-KC DAF-12 interaction

Venn diagram, comparing 7-KC regulated genes in N2 with genes regulated in *daf-12(rh61rh411)*, and genes regulated in *nhr-8(hd117)*. 7-KC regulated genes are largely overlapping genes regulated by DAF-12 and NHR-8. (B) Comparison of genes daf-12 dependent regulated by 7-KC with their regulation in *daf-12(rh61rh411)* background.

11.4 7-KC regulated genes largely overlapped with NHR-8 regulated genes

Subsequently we asked whether it is also possible to identify other 7-KC targets in our RNAseq data. To do so, we decided to search our data for other factors that have a large overlap in genes they regulate with 7-KC regulated genes. We thus choose one interesting candidate (*nhr-8*) from the pool of 7-KC regulated genes in *daf-12(rh61rh411)* and N2. *Nhr-8*, seemed of particular interest, since it was characterized as regulator of cholesterol, DA synthesis and fat metabolism (Magner et al., 2013)and is regulated by DAF-12 and 7-KC itsself. Mutants displayed phenotypes that are partially overlapping with our findings with 7-KC such as altered dauer formation and altered lifespan. We thus compared available microarray data of an *nhr-8* null mutant with 7-KC regulated genes in N2. Interestingly, we found a striking overlap - 40 % of 7-KC regulated genes are also regulated by NHR-8. This suggests that 7-KC mediates phenotypes also via *nhr-8*. To test the idea that this overlap is specific and not due to a general inhibition of NHR activity by 7-KC we also compared 7-KC regulated genes in N2 to *nhr-25* regulated genes identified in an available microarray assay. We found that only one gene was overlapping (4,2 %). This supports the hypothesis that 7-KC mediates phenotypes specifically via DAF-12 and NHR-8.

Thus lifespan of *nhr-8(hd117)* was measured with and without 7-KC (10 µM) (***Figure 25 A***). We found that *nhr-8(hd117)* is short-lived as described previously (Magner et al., 2013). This shortevity was rescued when 7-KC was added. Interestingly it was shown previously that cholesterol can also rescue *nhr-8* shortevity. Thus we cannot exclude that the rescue is due to cholesterol contamination in provided 7-KC we used. This hypothesis is supported by the finding that also reduced fecundity in *nhr-8(hd117)* is partially rescued by 7-KC. It was also shown that it is rescued by cholesterol.Thus it is difficult to address the question if 7-KC phenotypes are transmitted via NHR-8, since there is always additional cholesterol provision along with 7-KC.

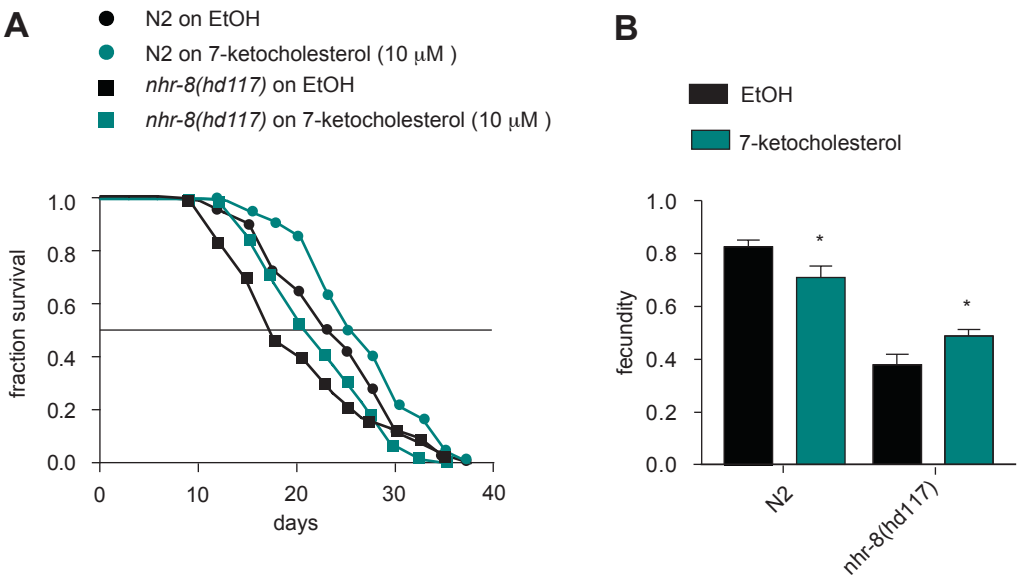


Figure 25 NHR-8 phenotypes with 7-ketocholesterol

(A) Lifespan assays: N2 and *nhr-8(hd177)* were grown from egg on at 20 °C on NGM and transferred from L4 on to plates containing 7-ketcochlesterol (10 µM) or corresponding volume of vehicle control (EtOH). In two repeats we found that 7-KC rescued *nhr-8(hd117)* shortevity (B) N2 and *nhr-8(hd117)* were grown from egg on at 20 °C on NGM plates containing 10 µM 7-ketocholesterol or corresponding volume of vehicle control (EtOH). Fecundity that hatched was scored over 10 days of adulthood starting from L4+1 day (72 h). Bars represent total number of progeny. t-test *p<0.05 Mean+SD (n ≥36) *Nhr-8(hd117*

Discussion and Future Perspectives

12 Discussion

As discussed multiple small molecule modulators regulate longevity pathways. Many of these modulators reflect nutrient and endogenous status of an organism to modulate a developmental response. *C. elegans* has been successfully used to identify small molecule modulators of which many are conserved in mammalian systems and might help to develop drugs for age-related diseases. Nevertheless, the complete metabolomic state of longevity with all its signaling molecules and all connecting points is far from being understood. We therefore asked what other molecules can modulate lifespan in *C. elegans*. Given the close connectivity of pathways regulating longevity and dauer formation we screened for novel lifespan modulating small molecules by using altered dauer formation as a first read-out. We discovered sugars (glucose, galactose, trehalose), amino acids (tryptophan, glycine) and fatty acids (arachidonic acid) novel regulator of dauer formation. Moreover 7-KC was discovered as a modulator of dauer formation and lifespan in *C. elegans* and have thus validated our screening concept.

Multiple lines of evidence suggest that 7-KC is a competitive inhibitor of DA induced DAF-12 activity. First, with epistatic experiments we placed 7-KC dauer induction downstream of IIS, TGF-β and DAF-36, which catalyzes the first step of DA synthesis. More precisely, we linked the dauer phenotype to DA-effector activity by showing *daf-12* dependence. Moreover 7-KC dauer formation was *din-1* dependent. DIN-1 is the co-repressor that is essential for DA unbound DAF-12 promotion of dauer formation, suggesting that 7-KC also recruits the co-repressor to repress DAF-12 activity. In line with these findings, 7-KC longevity phenotype was also placed downstream of IIS and downstream of DAF-36 and also 7-KC longevity is dependent on *daf-12*. We could show dependence on the LBD of DAF-12, suggesting that 7-KC might bind in the same binding pocket as DAs. Interestingly, other than dauer formation the longevity phenotype is *din-1* independent.

Second, in qPCR analyses we identified three targets of DA activated DAF-12 (*mir-48, mir-84, mir-241*) to be down-regulated by 7-KC and this down-regulation is DAF-12 dependent, again supporting inhibition of DA induced DAF-12 activity by 7-KC. Furthermore, in cell culture, 7-KC inhibited Δ^7-DA induced DAF-12 activation of a *mir-84* promoter driving luciferase, suggesting that 7-KC is capable to function as competitive

inhibitor of Δ^7-DA - DAF-12 interaction. Finally, bioinformatic analyses of RNAseq data from 7-KC fed animals revealed that 7-KC regulated genes largely overlap (41 %) with those regulated by DAF-12 (and NHR-8). In line with the idea of an inhibitory function, the overlapping genes are mainly regulated in the same direction by 7-KC supplementation and in *daf-12* null mutant. Consistent with the hypothesis of 7-KC being a competitive inhibitor of DAF-12, we found that 7-KC longevity might be *daf-9* dependent. DAF-9 is the last step of Δ^7-DA and Δ^4-DA synthesis, suggesting that DAF-9 might act on 7-KC as well to synthesize 7-keto-DA and that 7-keto-DA is the actual biological active molecule. Moreover we observed increased whole body cholesterol and decreased whole body 7-dehydrocholesterol in L3 larvae that was uncoupled from the other phenotypes.

Taken together, we suggest the following model: Supplemented 7-KC is converted to 7-keto-DA by DAF-9. 7-keto-DA then outcompetes DA from binding to DAF-12 and inhibits its activator functions. 7-keto-DA bound DAF-12 during development recruits DIN-1 and promotes DAF-12 repressor activity, which leads to increased dauer formation. In adult animals 7-keto-DA bound DAF-12 promotes longevity programs probably independent of *din-1* suggesting that the DAF-12 co-repressor is not part of the lifespan-extending complex. In addition, we suggest that 7-KC might inhibit the activity of DAF-36. Nevertheless, this inhibition can somewhat be uncoupled from dauer induction and longevity, because both phenotypes are *daf-36* independent. Moreover 7-KC inhibits NHR-8. The mechanism and epistatic of this inhibition is unknown. Also it remains elusive which of the NHR-8 regulated process in steroid metabolism of *C. elegans* (Magner et al., 2013) are overlapping with 7-KC phenotypes.

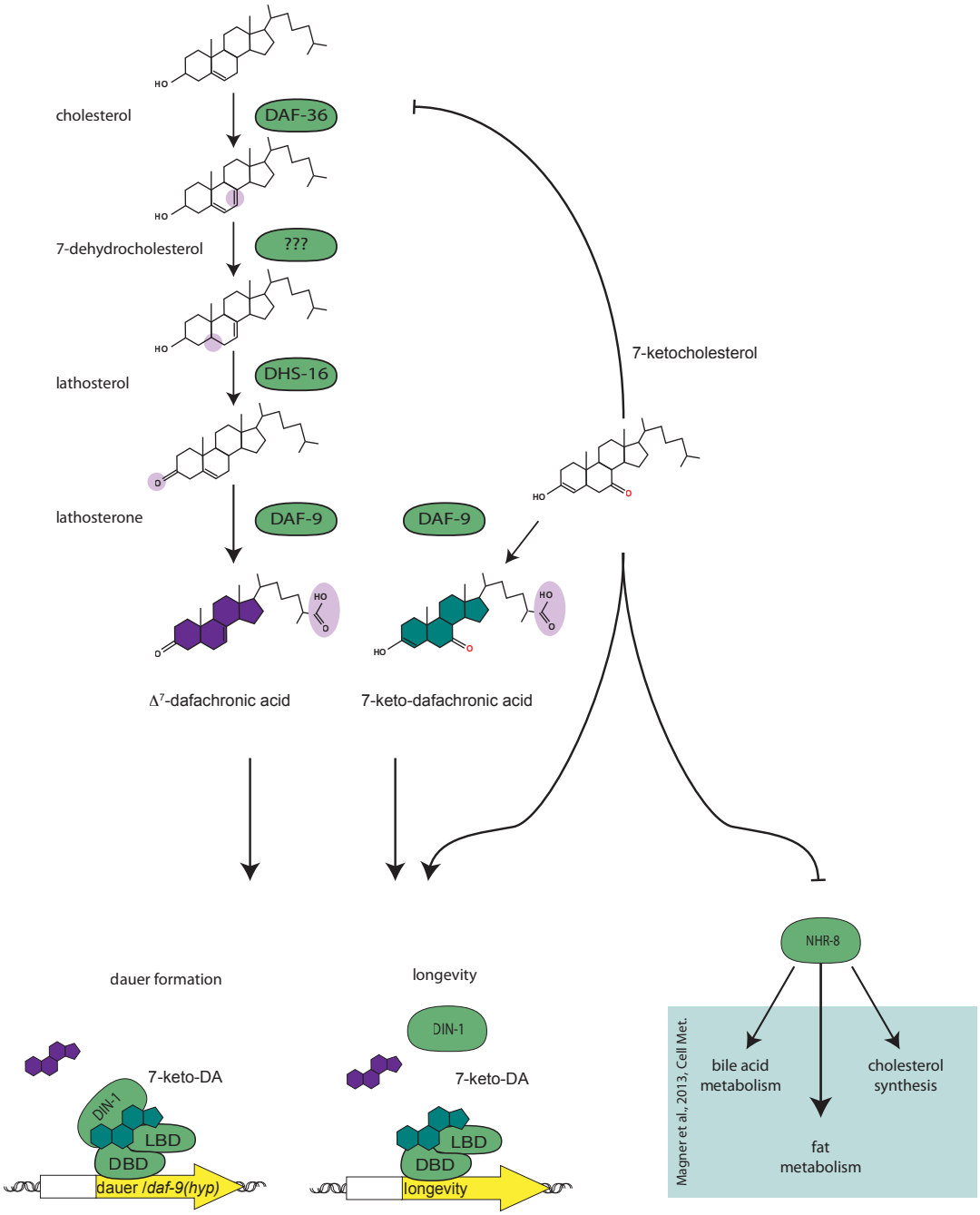


Figure 26 7-ketocholesterol activity in *C. elegans*

7-KC is converted to 7-keto-DA (green molecule) by DAF-9. 7-keto-DA competitive inhibit DA binding (purple molecule) to DAF-12. In L2 larvae 7-keto-DA bound DAF-12 recruits DIN-1 to promote DAF-12 repressor activity, leading to dauer formation in Daf-c or *daf-9(hyp)* in N2. In adult animals 7-keto-DA bound DAF-12 promotes longevity independent of *din-1* suggesting. In addition 7-KC inhibits the activity of DAF-36. Moreover 7-KC inhibits NHR-8 via a so far unknown mechanism. Also it remains elusive to what extend 7-KC regulates NHR-8 targeted processes in steroid metabolism of *C. elegans* (Magner et al. 2013).

12.1 7-Ketocholesterol feeding in *C. elegans*

It is not unambiguously clear whether 7-KC or 7-keto-DA is the biological active molecule. For this part of the discussion we assume that it is 7-keto-DA. Our data suggest that DIN-1 is recruited by 7-keto-DA bound DAF-12 for dauer formation during development but not later in life for longevity. This suggests an involvement of other coactivators or corepressor promoting 7-keto-DA DAF-12 longevity. These co-factors must fulfill the requirement to repress DAF-12 activator activity for dauer formation but not its activity for longevity. A reasonable speculation is that potential interaction partners are expressed tissue specific or dependent on developmental stage.

Up to date, the role of DAs on longevity in reproductively growing *C. elegans* is not understood unambiguously. Neither supplementation with DA intermediates nor additional surplus of cholesterol had a reliable effect on *C. elegans* lifespan. We propose 7-keto-DA as a novel DA and as the first to extend lifespan. Interestingly a clear role for DAs as longevity promoting factors was described in germline-less animals. Shen et al. showed that both, the presence of DA-DAF-12 interaction and the subsequent transcription of the target micro RNAs *mir-241* and *mir-84* are essential to gonadal longevity. Following the idea that 7-KC feeding opposes function of other DAs, and the observed down-regulation of the microRNAs, we might also expect that 7-KC feeding at least partially suppresses gonadal longevity. Nevertheless, regarding the highly orchestrated spatial and temporal organization of this pathway – down-regulation by 7-KC feeding might be as well uncoupled from gonadal longevity. Moreover we do not know whether microRNAs are down-regulated upon 7-KC feeding in longevity promoting tissues.

7-KC feeding might also help to explain the role of DAs in longevity of reproductive animals. We propose, that DAs might promote longevity in animals with an intact reproductive system as well, but it was overlooked so far due to the global character of the used methods. Usually the role of DAs and its intermediates is investigated by feeding assays. Such assays are always global approaches and, moreover, compounds are provided during development or during whole adult lifespan or both. Whereas, regarding the hormonal character of DAs, indeed it might be rather a precisely timed pulse in a certain target tissue that is required for lifespan extension. The longevity promoting ability of DAs was shown in germline-less animals.

If DAs indeed can promote longevity in reproductive animals by the activation of DAF-12 in a certain tissue, an alternative hypothesis is conceivable to explain 7-KC induced *daf-9(hyp)* and longevity phenotypes. In brief, 7-keto-DA inhibits Δ^7-DA binding to DAF-12 leading to the activation of *daf-9(hyp)* accompanied by globally increased Δ^7-DA levels in N2 (suggested in our data). It might thus be that it is a certain concentration that promotes DA longevity (***Figure 27***).

So far we assumed that 7-keto-DA is present in every tissue to inhibit Δ^7-DA binding to DAF-12 and somehow leads to a balance of Δ^7-DA-DAF-12 induction and 7-keto-DA-DAF-12 inhibition. This balance is based on the assumption that 7-KC enters all DA targeted tissue. But 7-KC might be excluded from certain longevity promoting tissues accordingly net DA-DAF-12 activity is higher in such tissues. Thus 7-KC would induce a DAF-12 activity pattern up-regulation in some and down-regulation in other tissues that might promote lifespan.

If this is the case, 7-KC longevity might actually be a tissue specific DA-longevity. A regulation in a tissue hormesis manner– a small inhibitory effect in one tissue leads to beneficial effects in another tissue (***Figure 27***). Moreover this model would explain why DIN-1 is not required for 7-KC longevity. A potential target tissue for DA- longevity is the intestine, since it was shown to be the target tissue of gonadal longevity. Doubtlessly, this very speculative hypothesis needs appropriate experiments to be validated or falsified.

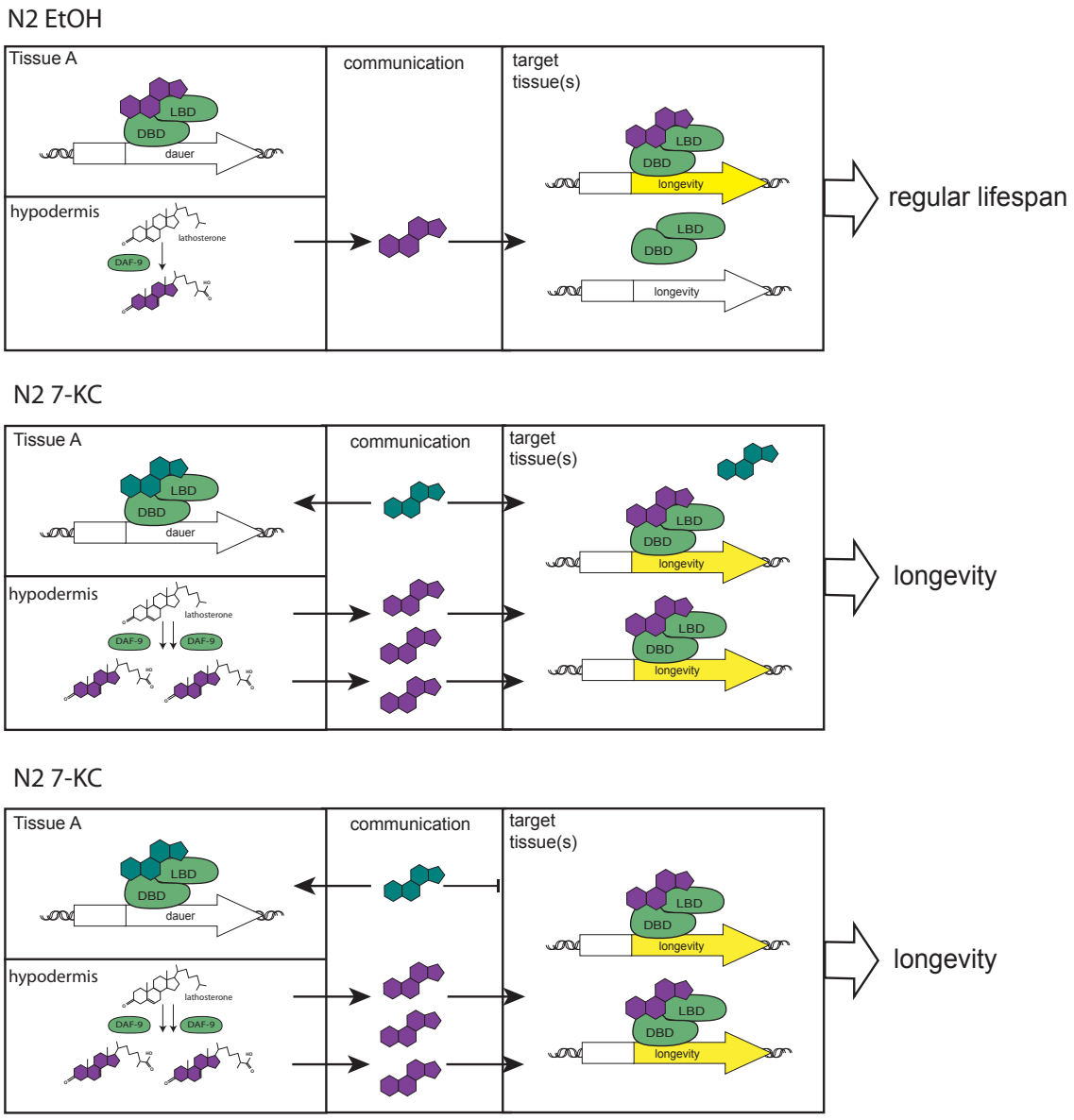


Figure 27 Tissue hormesis model of DA-longevity

Upper panel: In N2 background without 7-KC provision, tissue A synthesizes enough DA (green molecule) and targets DAF-12 in a lifespan regulating tissue (target tissue). Levels of DA- DAF-12 interaction are sufficient to promote normal lifespan. Middle panel: With 7-KC provision 7-KC inhibits binding of DA to DAF-12 in tissue A that leads to more DA synthesis in the hypodermis. The increased DA levels in lifespan regulating target tissue then leads to more DA-DAF-12 interaction sufficient to outcompete 7-KC from binding and to promote longevity. Lower panel: This effect might be enhanced if 7-KC is excluded from the lifespan determining tissue.

Especially since *in vivo* situation might actually be even more complex, because in addition to spatial and temporal regulations of DA synthesis and requirement, there is another level of DAF-12 regulation. It is not only Δ^7-DA that activates DAF-12. Up to date two other endogenous DAs were found ($\Delta^{1,7}$-DA and 3α-OH-Δ^7-DA) (Mahanti et al., 2014)and another one was postulated (Δ^4-DA) (Motola et al., 2006). All were shown to act on DAF-12 and restore *daf-36(k114)* suppressed gonadal longevity. Pointing at a high potential to have

overlapping and redundant functions in longevity promotion. Still it is possible that they have additional distinct sets of genes that are regulated via DAF-12 to promote longevity. Moreover, regarding the role of amino acid balance in CR longevity, it might also be a certain ratio of the different DAs that is needed to promote lifespan. Owed to the only recent discovery of most DAs, not much is known about spatial synthesis and regulation.

A long these lines it is also possible that 7-KC inhibition of DAF-36 leads to upregulation of other DA syntheses pathways - HSD-1 might be such a target, which is proposed to convert 4-cholesten-3-one into Δ^4-DA. Resulting Δ^4-DA then might extend lifespan. Notably, it might was well be *vice versa* - feeding of 7-KC blocks HSD-1 and the resulting Δ^4-DA depletion light lead to longevity.

Taken together, we are only beginning to unravel the role of the different DAs in longevity and also dauer formation and 7-keto-DA might turn out to be of particular interest since it seems to be the only DA that inhibits DAF-12 activator activity.

12.2 7-Ketocholesterol in mammals

We found that 7-KC feeding induced dauer formation and longevity in *C. elegans*. But what are the roles of 7-KC in mammals? The steroid metabolism is intensively studied in mammals. Cholesterol is the precursor for bile acids, all steroid hormones and oxysterols like 7-KC. It is known that it is rather the oxidized forms of cholesterol, than cholesterol itself that has signaling functions in many cellular processes like bile acid syntheses and cholesterol homeostasis (Björkhem et al., 1994; Otaegui-Arrazola et al., 2010). Although synthesis pathways have been described for 7-KC recently (Mitić et al., 2013)in mammals it is mostly produced by free radical attack (Hodis et al., 1991; Jessup and Brown, 2005). First cholesterol is oxidized to 7-hyperoxide (cholesten-5-ene-3-b-ol-7-hyperoxide) that in turn is converted to 7-KC and 7-hydroxycholesterols (α and β), the most prominent oxysterols in all tissues (Vejux and Lizard, 2009). As cholesterol is unstable in presence of light and air 7-KC it is also spontaneously formed *in vitro*. Thus, apart from the endogenous sources, 7-KC is also taken up with diet- and is especially present in cholesterol rich food (Nielsen et al., 1995). After uptake it is transported via chylomicrons and oxidized low-density lipoprotein. Despite its global presence in food and almost all living organisms no actual physiological function was unambiguously assigned to it. In brief, 7KC is widely regarded as a mainly toxic oxidation product of cholesterol that needs to be eliminated by cells. Accordingly, enzymes

that potentially reduce 7-ketocholesterol to less toxic products (Schweizer, 2004) or to feed it into bile acid synthesis pathway have been suggested (Jessup and Brown, 2005).

12.3 The role of 7-ketocholesterol in atherosclerosis

It is known that increased cholesterol is a risk factor for atherosclerosis and that such an increase can be induced e.g. by high fat diet. Interestingly, in atherosclerotic plaques not only cholesterol but also oxysterols are predominantly found and 7-KC is the second most abundant (Björkhem et al., 1994). Notably, over the last decade evidence emerged that it is rather 7-KC along with other oxysterols like 25-hydroxycholesterol than cholesterol itself that might have a promoting effect on proceeding atherosclerosis. Thus, oxysterols in general and 7-KC in particular became the most intensively studied molecules in pathophysiology of atherosclerosis (Lyons and Brown, 1999). And indeed multiple effects of 7-KC were described that potentially promote atherosclerosis. It was shown, that 7KC along with 7-β-hydroxycholesterol induces apoptosis via caspases (Luthra et al., 2008), nuclear factor kappa B (Huang et al., 2010; Larrayoz et al., 2010), and via NOX4 (Pedruzzi et al., 2004). Moreover it was shown to promote inflammation via interleukin-6 (Amaral et al., 2013; Buttari et al., 2013; Vejux and Lizard, 2009). In other cell culture experiments ROS was found to be increased after 7KC treatment. Lycopene and Vitamin-E were reported to partially abolish deleterious effects of 7-KC (Palozza et al., 2010; Wong and Wang, 2013). Notably, studies of 7-KC are mostly done in cell lines of multiple origins and many of these studies use concentrations that are rather high compared to physiological levels, which make a physiological role questionable. Owed to the fact that at least apoptosis, inflammation and ROS imbalance were shown to promote atherosclerosis - 7KC is a promising candidate to play an important role in the development of cardiovascular diseases. And understanding this role might help to develop drugs against this age-related diseases. Still, up to date the exact role of 7-KC and whether it really has a causative or only supporting effect in formation of atherosclerotic plaques remains elusive. Interestingly, in the context of atherosclerosis not only the mechanisms remain elusive but in general also direct targets of 7-KC to promote described effects are missing. The only targets that were found are in the context of cholesterol metabolism.

12.4 Potential function of 7-ketocholesterol in mammals

7-KC was shown to inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-COA reductase) (Saucier et al., 1989). HMG-CoA reductase is one of the key enzymes in cholesterol *de novo* synthesis. It is the target of statins - drugs that are used to counteract hypercholesterolemia. Moreover 7-KC was shown to inhibit 7-α-hydroxylase (CYP7A1) (Tamasawa et al., 1997; 1994), the first enzyme of bile acid pathway converting cholesterol to 7-α-hydroxycholesterol. Notably, feeding of 7-KC in rats was compensated by higher CYP7A1 activity, and intravenous injection was compensated by higher CYP7A1 expression (Tamasawa et al., 1994; 1997). Thus, in long term 7-KC does not affect bile acid synthesis. Thus, it is questionable if the inhibition of either of these targets is of actual physiological relevance.

In brief, the current understanding is that 7-KC is a toxic inadvertent oxysterol that has to be disposed or converted by the cell in order to prevent deleterious effects. It accumulates together with other oxysterols in the early onset of atherosclerosis – still this effect lacks a direct target for 7-KC and an underlying mechanism. Despite the direct targets in cholesterol metabolism, in mammals no physiological function of 7-KC was shown. Moreover, if 7-KC induced apoptosis, ROS and inflammation play a physiological role in healthy cells was not addressed so far.

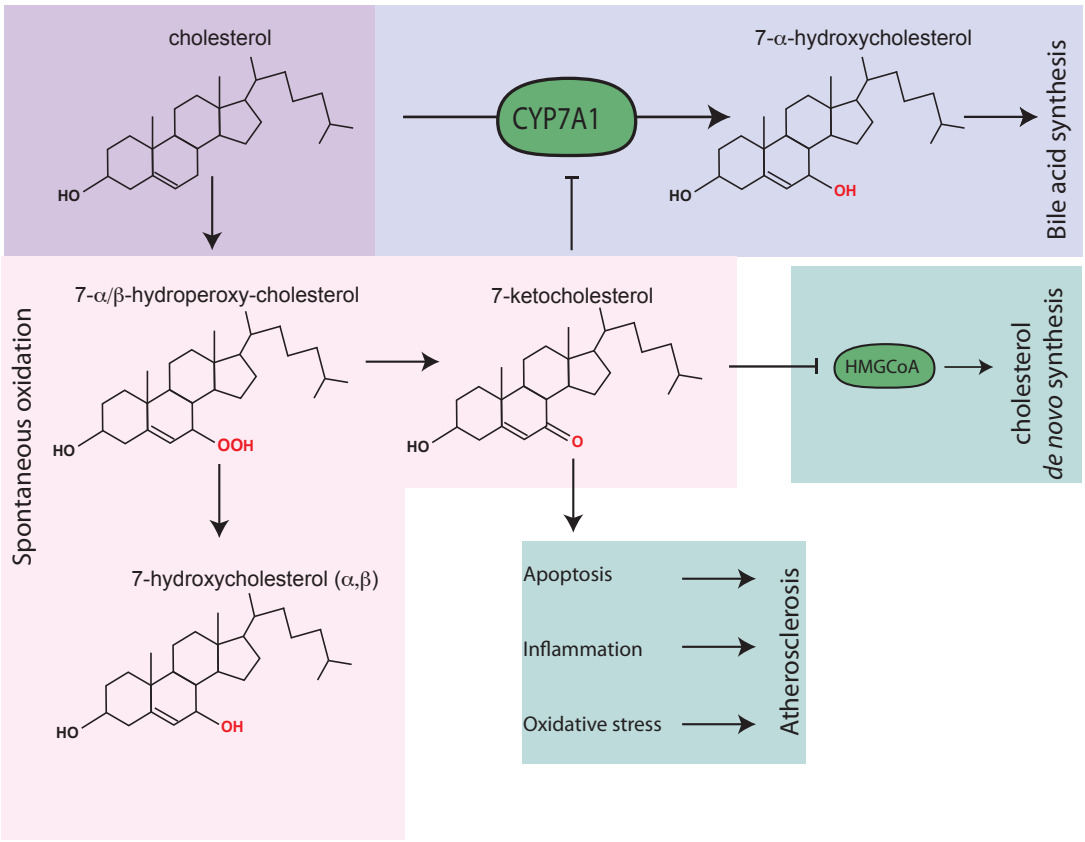


Figure 28 7-ketocholesterol in mammals

7-KC is formed spontaneously by ROS attack of cholesterol via 7-α/β- hydroperoxy-cholesterol. *In vitro* it was shown to inhibit HMGCoA reductase activity involved in cholesterol *de novo* synthesis. *In vivo* it was shown to inhibit CYP7A1 first step of bile acid synthesis converting cholesterol to 7-α-hydroxycholesterol. Moreover it was shown to increase apoptosis, inflammation and oxidative stress atherosclerosis promoting functions.

12.5 Potential of our study to understand function of 7-ketocholesterol in mammals

To our knowledge, the presented study is the first study investigating functions of 7-KC in *C. elegans*. We think it is of particular interest since this is also the first study describing a nuclear hormone receptor (DAF-12) as a possible direct target of 7-KC, promoting global phenotypes - dauer formation and longevity.

Available data are not sufficient to compare mammalian and nematode function of 7-KC. In a next step it should be approached whether some of the known functions in mammals are also found in worms, e.g. by measuring ROS levels and apoptosis. Especially the role of ROS might be interesting, since it might provide a link between mammalian findings and findings in *C. elegans*. ROS were shown to play a role in lifespan determination. A conceivable model is that 7-KC might function as a signaling molecule to reflect ROS levels and at slightly increased concentrations in worms this has a lifespan extending effect. In

higher concentrations in cells it would induce apoptosis at a certain concentration. It might actually involve a hormetic effect, in which 7-KC activates cell clearance. These ideas are very speculative and need to be validated in *C. elegans* with according assays.

The cholesterol auxotroph *C. elegans* provides a suitable system for further studies on 7-KC, since it excludes its statin effects. Moreover it lacks direct homolog of some genes found essential in mammalian studies, like CYP7A1 and NOX-4. Excluding these mediators of known mammalian effects might help to identify novel targets of 7-KC. These novel targets in turn could potentially help to understand 7-KC function in mammalian systems – in healthy cells and in the onset of atherosclerosis.

We think in cell culture and mammalian systems certain effects of 7-KC might have been overlooked due to the toxic effects of the high 7-KC levels that were used and the focus on atherosclerotic models mainly analyzing cells of the vascular system. Moreover, to our knowledge no systematic approach to investigate 7-KC as potential ligand for mammalian NHRs was performed. Regarding our findings this might be of particular interest since DAF-12 has homology to VDR and LXR and VDR activation was shown to counteract atherosclerotic events (Pilz et al., 2013).

12.6 Future experiments

So far the question if 7-KC regulation of DAF-12 is of any physiological relevance in *C. elegans* was not addressed. We have only shown that it can be used as a drug to extend lifespan. The first approach to clarify if 7-KC is a physiological regulator of ageing would be to determine whether levels of 7-KC are regulated during the ageing process in N2. If it has physiological relevance in lifespan modulation we would expect lower levels of 7-KC with increasing age. This regulation could be impaired in long-lived mutants.

Moreover, we could not unambiguously show that 7-keto-C/DA directly binds to DAF-12, to address this question we are planning in collaboration with the Schroeder lab (Cornell University, Ithaca, New York) to synthesize 7-keto-DA and test it in parallel to 7-KC regarding its capability to outcompete the binding of several DAs to DAF-12 in a cell free competition assay. With this assay, we hope to show, first, if there is a direct interaction, second, if 7-KC or 7-keto-DA is the biological active enzyme and third with this assay it is possible to quantify the dynamics of the competitive inhibition. Another issue that needs to be addressed is the question whether 7-keto-C/DA might act as a general quencher of NHR

activity. Thus, we are planning to investigate inhibitory potential of 7-KC on other NHRs, like VDR, in cell culture assays.

To unravel a potential role of 7-KC in gonadal longevity, we are planning to feed it to germline ablated animals and *glp-1(ts)* mutants. This approach could be combined with determination of *mir* expression profiles in 7-KC fed worms since we only addressed the microRNA expression on a global mRNA level. To get a better understanding of where and when 7-KC acts precisely we are planning to look at *mir-48::gfp* reporter to analyze DAF-12 regulation. Fluorescence markers might help to better understand the role of 7-KC. Moreover, tissue specific knockouts and timed knockdowns of *daf-12* and DA biosynthetic enzymes could help to shed light on tissue specific DA pathway function. For visualization of 7-KC distribution, we are planning to use a commercial available 7-KC fluorescent molecule.

We have shown that the effect of 7-KC on lifespan determination is mediated after development since we started feeding in late L4, it would be of further interest to narrow down the time window. According to their steroid hormonal character we would expect DAs to act in a highly coordinated and precisely timed time window. We therefore might expect 7-KC to also be a required at a certain time point we thus planning to prepare a time course, providing 7-KC during post-reproductive life in pulses.

Moreover we are planning to follow up, our candidates from the dauer screen (glycin, arachidonic acid). In a first step they will be tested for lifespan phenotype. But we are also planning to follow up the dauer phenotype and as a first step in this matter we will test for daf-16/FOXO dependence.

Finally we have started to test if 7-KC-phenotypes, dauer formation and longevity are recapitulated by the 7-KC regulated genes found in RNAseq and could not identify candidates in a first repeat.

13 Future perspectives

Our approach to use dauer as a primary readout to identify lifespan modulators has high potential to identify more small molecules that modulate the network of longevity pathways. With 7-KC we identified a steroid that either directly or after conversion to 7-keto-DA counteracts the known DAs to modulate dauer formation and lifespan in *C. elegans*. But it also will be interesting to follow up our other candidates that effected dauer in the screen - to further investigate their role in lifespan and identify targeted pathways. Interestingly, 7-KC induced lifespan extension is independent of IIS, underlining that although we used a mutant of this pathway, we not only identified modulators of IIS. This indicates a high potential to identify small molecules acting in other and maybe even novel longevity pathways.

The identification of a new regulator of DAF-12 that might actually work as the first competitive inhibitor is of particular interest to the field of ageing research, since DAF-12 is not only the key-regulator of dauer formation and a modulator of lifespan but has several other functions like developmental timing. It is one of the major transcription factors that highlights a hormonal link between developmental and lifespan decision in *C. elegans*. So far, it was only investigated how it promotes developmental specific expression profiles in its activated - DA–bound status compared to the repressed, DA unbound status. 7-ketocholesterol most certainly opposes effects of other DAs, and thus adds another level of regulation to DAF-12. 7-KC therefore provides a valid tool to study other DAF-12 regulated processes as well.

Unraveling the interaction of all known DAs will certainly gain exciting new insights to answer the following questions: What is the temporal and spatial pattern of the different DAs? What is the role of DAs in longevity of reproductive and germline-less *C. elegans*? What is the role of the potential inhibitor 7-keto-DA in this context? Is a steroid hormonal regulation of lifespan conserved in higher organisms as well? Can these small molecules be used to develop drugs against age related diseases?

Materials and methods

14 Materials

14.1 Strains

The following *C. elegans* strains from Antebi Lab collection were used in this thesis:

N2 bristol, DR1572 *daf-2 (e1368)*, AA431 *daf-36(k114); daf-9::gfp*, AA2646 *dhIs64(Pdaf-9::daf-9::GFP; lin-15[+])*, JT10800 *npc-2(nr2023)III;npc-1(nr2022)X,* CB1372 *daf-7(e1372ts)*III, *CB1386 daf-5(e1386)*, AA580 *daf-5(e1386)II;daf-36(k114)V,* GR1311 daf-3(mgDf90)X, AA373 *daf-3(mgDf90)*X; *daf-36(k114)*V, GR1307 *daf-16(mgDf50)*I, AA374 *daf-16(mgDf50)*I;*daf-36(k114)*V, AA967 *dhs-16(tm1890)*V, AA009 *daf-9(k182)X*, AA1053 *daf-40(hd100)*II, AA003 *daf-12(rh61rh411)*, AA3433 *daf-2(e1368);daf-12(rh61rh411)*, AA420 *din-1(dh127)*II; *daf-9(dh6)*X, AA408 *din-1(dh127)*II, AA161 *daf-9(dh6);daf-12(rh61rh411)*X, AA968 *nhr-8(hd117)*IV.

14.2 Chemicals, buffers and media

Unless stated otherwise chemicals were purchased from Sigma. All chemicals used for the screen including distributor, CAS-number and MW are listed below.

List of screened compounds

Compound	Distributor	CAS-number
sugars		
D-glucose	Sigma	50-99-7
glycerol	Sigma	56-81-5
D-galactose	Sigma	59-23-4
D-fructose	Sigma	57-48-7
sucrose	Sigma	57-50-1
trehalose	Sigma	6138-23-4

Amino acids

L-serine	Sigma	56-45-1
L-threonine	Sigma	72-19-5
L-cysteine	Sigma	52-90-4
L-glycine	Sigma	56-40-6
L-alanine	Sigma	56-41-7
L-asparagine	Sigma	70-47-3
L-glutamine	Sigma	56-85-9
L-phenylalanine	Sigma	63-91-2
L-tyrosine	Sigma	60-18-4
L-tryptophane	Sigma	73-22-3
L-histidine	Sigma	71-00-1
L-lysine	Sigma	56-87-1
L-arginine	Sigma	74-79-3
L-arginine-HCL	Sigma	n.a
L-valine	Sigma	72-18-4
L-leucine	Sigma	61-90-5
L-isoleucine	Sigma	73-32-5
L-methionine	Sigma	63-68-3
L-proline	Sigma	147-85-3
L-aspartic acid	Sigma	56-84-8
L-glutamic acid	Sigma	56-86-0

Fatty acids

linoleic acid	Nu chek Prep, Inc	60-33-3
oleic acid	Nu chek Prep, Inc	112-80-1
palmitic acid	Nu chek Prep, Inc	57-10-3
γ-linolenic acid	Nu chek Prep, Inc	506-26-3
homo-γ-linolenic acid	Nu chek Prep, Inc	1783-84-2
stearic acid	Nu chek Prep, Inc	57-11-4

palmitoleic acid	Nu chek Prep, Inc	373-49-9
α-linoleic-acid	Nu chek Prep, Inc	463-40-1
myristic acid	Nu chek Prep, Inc	544-63-8
arachidonic acid	Nu chek Prep, Inc	506-32-1
eicosaptenoic acid	Nu chek Prep, Inc	10417-94-4
tridecanoic acid	Nu chek Prep, Inc	638-53-9

Steroids and others

aldosterone	Sigma	52-39-1
11-deoxycortisol	Nu cheak prep	152-58-9
17-α-hydroxypregnenolone	Nu cheak prep	387-79-1
testosterone	Sigma	58-22-0
17-α–hydroxyprogesterone	Nu cheak prep	68-96-2
corticosterone	Sigma	50-22-6
β-estradiol	Sigma	50-28-2
progesterone	Sigma	57-83-0
dehydrocholic acid	Sigma	475-31-0
cholic acid	Sigma	81-25-4
ursodeoxycholic acid	Nu cheak prep	128-13-2
deoxycholic acid	Sigma	83-44-3
desmosterol	Sigma	313-04-2
5-pregnen-3β-ol-20-one	Sigma	145-13-1
α-ecdysone	Sigma	3604-87-3
stigmasterol	Sigma	83-48-7
eticholan-3 α-ol 17-one	sigma	571-31-3
ergosterol	Sigma	57-87-4
retinoic acid	Sigma	302-79-4
9-cis retinoic acid	Sigma	5300_03-8
7-dehydrocholesterol	Sigma	434-16-2
lathosterol	Sigma	80-99-09

lathosterone	Corey Lab	15459_85-5
4-cholesten-3-one	Sigma	601-57-0
Δ dafachronic acid	Corey Lab	n.a.
22(R)-hydroxycholesterol	Avanti Polar Lipids	17954-9-28
22(S-)hydroxycholesterol	Avanti Polar Lipids	22348_64_7
25 hydroxycholesterol	Avanti Polar Lipids	2140_46_7
7-ketocholesterol	Sigma	566-28-9
lophenol	Research Plus, Inc	n.a.
7-β-hydroxycholesterol	Sigma	566-27-8

14.3 Primers

Primers for validation of RNA sequencing results by quantitative real time PCR were designed with the online tool of Integrated DNA Technologies. Primers for microRNA analyses were used as described previously in Shen et al. 2013:

mRNA		
Target gene	**primer**	**Sequence (5´-3´)**
lys-8	lys-8_for	CGCTGTTGATTTGTCTGTTCC
	lys8_rev	AGTATTGCAGGAGTTGGTGTC
gba-1	gba1_for	TGTGAAACTTCTGATTCTGGACG
	gba1_rev	TCTTGATATGCGTGAACCCC
clec-67	clec67_for	GAACTCTCAACCCGTAACAATTG
	clec67_rev	CTGTCTCCGAATCCCAATGTAG
prk-2	prk2_for	TTCAGAATCGTGTACTTCATAGGG
	prk2_rev	CAATAGAGTCGGGTTCCTTGG
T16G12.1	T16G12.1_for	AAAATCCAGACGAAGGAGACG

	T16G12.1_rev	AACATGGGAGAAGTGAACGG
vha-6	vha6_for	TGCTTCATACCTCCGTCTTTG
	vha6_rev	AGAACCATTGTCCAGAGAACG
Y32B6.1	Y32B6.1_for	TCAGGATCATGCTCTGTTTACG
	Y32B6.1_ rev	CTTTGTGAAAATCCTTGGTCCG
ama-1	ama-1_for	GGATGGAATGTGGGTTGAGA
	ama-1_rev	CGGATTCTTGAATTTCGCGC
microRNA		
U18	U18-f	GGCAGTGATGATCACAAATC
	U18-r	TGGCTCAGCCGGTTTTCTAT
mir-1	mir-1-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACATA
	mir-1-f	CGCCCTGGAATGTAAAGAAGT
mir-241	mir-241-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATTT
	mir-241 -f	CGCTGAGGTAGGTGCGAG
All *microRNAs*	mir-r	GTGCAGGGTCCGAGGT

14.4 Plasmids

Some plasmids used in this thesis have been published previously (Bethke et al., 2009) and were available in Antebi Lab collection. Plasmids have been construct bei Dr. Axel Bethke as follows: DAF-12 cDNA was cloned into pCMVTag2b (Stratagene #211172). *Mir-84* promotor was PCR amplified and inserted into ptk luc reporter vector (4) upstream of a thymidine kinase (tk) minima l promoter followed by firefly luciferase cDNA. Another plasmids available from Antebi collections are unpublished and were constructed by Dr. Dan Magner as follows: GAL-4(DBD) and DAF-12(LBD) cDNA were inserted to pCMX backbone.

14.5 Buffers and media

M9	
Material	**Amount per liter**
Na2HPO4	6 g
KH2PO4	3 g
NaCl	5 g
autoclave	
1M MgSO4	1 ml

NG agar plates	
Material	**Amount per liter**
NaCl	3 g
Agarose	25 g
Peptone	2.25 g
1M KPO4	25 ml
autoclave	
1M Cacl2	1 ml
1M MgSO4	1 ml
cholesterol 5mg/ml	1 ml

Cell culture

Gal- buffer (1L)	
Material	**Final conc**
Na2HPO4	60 mM
NaH2PO4	40 mM
KCl	10 mM
1M MgCl2	1 mM
Fill upto 1 l (ddH2O), filtrate sterile	

2X HBS (500 ml)	
Material	**Final conc**
NaCl	280 mM
KCl	10 mM
Na2HPO4	1.5 mM
Glucose	12 mM
HEPES	5g
adjust ph 7.05 and fill to 500 ml (ddH2O)	

10X CORE bffer (200 ml)	
Material	**Final concentration**
Tricine, PH 7.8	30 mM
Mg Acetate	80 mM
EDTA	800 ul
Fill upto 200 ml (ddH20), filtrate sterile	

ONPG buffer (250 ml)	
Material	**Final concentration**
Na2HPO4	60 mM
NaH2PO4	40 mM
Fill upto 250 ml (ddH2O), filtrate sterile	

15 Methods

15.1 *C. elegans* handling and physiological assays

15.1.1 Worm handling

To maintain worm populations either a part of a plate containing a growing or starved population was “chunked” with a sterilized spatula to a fresh NGM seeded with OP50. Or single worms of a growing population were transferred with a worm pick (platinum wire) to a fresh NGM seeded with OP50.

15.1.2 Bacterial handling

Bacteria (*E. coli*) from glycerol stock were streaked to petri dishes with soild LB medium containing corresponding antibiotics. Plates were incubated overnight at 37 °C and the next day a single colony was picked to incubate liquid LB containing corresponding antibiotics. Plates were kept at 4 °C and reused to pick single colonies for no longer than a month. Liquid culture was allowed to grow overnight but at least 16 h, to reach stationary growth phase. 400 μl, 200 μl or 100 μl of liquid overnight culture was then used to seed 10 cm, 6 cm and 3 cm plates.

15.1.3 Cleaning and synchronization Populations with bleaching solution

To clean contaminated plates depending on the population´s stage worms were either washed directly of the plate with M9 or a small chunk was transferred to a new plate. They were incubated than at appropriate temperature until enough animals had reached adulthood and eggs were visible in the germline and then washed of the plate with M9. For synchronization an uncontaminated plate with Worms were allowed to either settle in the tube (1, 5 eppendorf or 15 ml falcon tube) or spinned down for 2 min at 2000 rpm. Supernatant was removed and M9 and bleaching solution (2:3) was added worms were vortexed at RT for around 8 min until only eggs visible under the dissecting microscope. Eggs were then washed 3 times in M9 (2 min, 2000 rpm) and transferred to fresh plates.

15.1.4 Synchronization by egg lay

To achieve a higher degree of synchronization day two adults of a synchronized population were placed on fresh plates an allowed to lay eggs for two hours at 20 °C.

15.1.5 Dauer assay (hydrophobic compounds)

3.5 cm Petri dishes containing 3 ml nematode growth medium (NGM) where incubated with 50 µl *E.coli* OP50 in LB (saturated) and incubated over night at RT. 12 µl of 10 mM hydrophobic compound solved in ethanol (EtOH) was added to the surface to a final concentration of 40 µM. EtOH was allowed to evaporate for 1 h before eggs were transferred or pipetted to plates. A synchronized adult *daf-2(e1368)* population was bleached to receive eggs and approximately 70 eggs were pipetted to prepared plates. After 60 h and 48 h incubation at 22.5°C and 25 °C, respectively the fraction of animals that formed dauer larvae was scored with a microscope.

For dauer assays on RNAi parental generation was grown from egg on, on *E. coli* HT115 bacteria expressing according RNAi. All clones were picked from Ahringer library , only RNAi clones E02C12.6, C02A12.1, ZK455.4, Y39B6A.13 and Y39B6A.20 were picked from Vidal library (Kamath, 2003; Rual et al., 2004).

15.1.6 Dauer assay (hydrophilic compounds)

Powders of hydrophilic compound were weighed out and disolved in liquid NGM (60°C) to a final concentration of 10 mM or 100 mM. 3.5 cm NGM compound Petri dishes (3 ml) where incubated with 50 µl *E. coli* strain OP50 in LB (saturated) and incubated over night room temperature (RT). One batch was radiated with UV light (6,000 J/cm^2) to prevent further division of the bacteria. A synchronized adult *daf-2(e1368)* population was treated by bleaching to synchronize eggs. Around 70 eggs were pipetted to prepared plates. After 60 h incubation at 22.5°C the fraction of animals that formed dauer larvae was scored.

15.1.7 Gonadal migration defect

3.5 cm Petri dishes containing 3 ml nematode growth medium (NGM) where incubated with 50 µl *E.coli* OP50 in LB (saturated) and incubated over night at RT. 12 µl of 10 mM hydrophobic compound solved in ethanol (EtOH) was added to the surface to a final concentration of 40 µM. EtOH was allowed to evaporate for 1 h before eggs were transferred or pipetted to plates. A synchronized adult *daf-2(e1368)* population was bleached to receive

eggs and approximately 70 eggs were pipetted to prepared plates. After 60 h incubation at 26°C the fraction of animals that had gonadal migration defect was scored with a microscope.

15.1.8 Statiscical Analyzes of dauer and mig phenotype

At least three technical replicates per sample were scored to create one of at least three biological replicate. Fractions of three technical replicates were averaged and at least three biological replicates were used to test for significance with Prism GraphPad.

15.1.9 DAF-9 hypodermal expression

3.5 cm Petri dishes containing 3 ml nematode growth medium (NGM) where incubated with 50 µl *E.coli* OP50 in LB (saturated) and incubated over night at RT. 12 µl of 2.5 mM hydrophobic compound or compound mix solved in ethanol (EtOH) was added to the surface to a final concentration of 10 µM. For DA a final concentration of 100 nM was used. Worms were synchronized by bleaching and around 2000 eggs were placed on each plate and incubated at 20 °C. L3 animals were then staged and either analyzed via Microscopy () or with the COPA Biosorter.

15.1.10 Lifespan analyzes

In brief, synchronized populations of desired strains were grown reproductively at 20°C for at least two generations. Adults were either allowed to lay eggs on experimental conditions or eggs were kept on NGM till L4 larval stage and then transferred to experimental conditions. Worms were transferred every 24 h during the egg laying period and every other day thereafter. Worms that did not move after being tipped gently with a worm pick were considered as dead and removed from the plate. All lifespan assays were performed at 20°C. All animals that crawl off the plate, showed phenotype of internally hatched larvae (bagged) or rupture of vulva (exploded) were censored and excluded from experiment.

For lifespan assay on UV killed bacteria plates were prepared as described above. In parallel regular 10 cm NGM plates were seeded with OP50 grown over night and subsequently UV treated (12,000 J/cm^2). Dead bacteria were washed off of 10 cm plates with M9 and transferred to experimental plates. To exclude contamination with bacteria coming from worms gut. Worms were bleached and grown to L4 larvae on plates with UV killed bacteria before being transferred to experimental plates.

For lifespan assays on RNAi parental generation was grown from egg on, on *E. coli* HT115 bacteria expressing according RNAi. All clone were picked from Ahringer library (Kamath, 2003), only RNAi clones E02C12.6, C02A12.1, ZK455.4, Y39B6A.13 and Y39B6A.20 were picked from Vidal library (Rual et al., 2004). Statistical analyses were performed using the log-rank (Mantel-Cox) method with GraphPad Prism software.

15.1.11 Brood size assay

Populations were synchronized by bleaching and incubated at 20 °C till day two of adulthood to make sure parental generation of experimental generation arose from eggs laid by animals at the same age. Parental generation was allowed to lay eggs for 2 h on fresh NGM plates seeded with OP50. After 48 hours at 20 °C L4 animals were singled to at least 20 x 6 cm NGM plates containing either 10 μM 7-KC or appropriate volume EtOH. After 24 h at 20 °C each animals was transferred to a fresh plate and the old plate was incubated another 24 hours before it was either scored with dissecting microscope or stored at 4 °C till scoring (but never longer than 3 days). This procedure was repeated for ten days or till no living eggs were laid. Only eggs that developed at least to L1 larvae were scored as living progeny.

15.1.12 Pharyngeal pumping rate

A population reproductively growing for at least 3 generations was synchronized by 2 h egg lay. After 48 h at 20 °C around 100 L4 worms were transferred to experimental plates containing either 10 μM 7-KC or appropriate volume EtOH. After 24 h (day 1) the number of pumps of the posterior pharyngeal bulb per 20 s was scored under dissecting microscope and multiplied by 3 to determine pumps/min. Animals were transferred every 24 hours during egg lay period and every other day thereafter. Pharyngeal pumping was scored on day 1, day 6 and day 10.

15.2 Quantitative Real time PCR and RNA sequencing

15.2.1 Sample collection for RT qPCR and RNA seq analyzes

A population that was grown reproductively for at least three generations without bleaching was synchronized by egg lay. Precisely 48 h later around 300 L4 worms were transformed to plates containing either appropriate compound or vehicle control and were incubated at 20 °C for 24 h. Adults then picked into 200 μl QIAzol (QUIAGEN) and directly

frozen in liquid nitrogen, samples were then either processed to directly isolate RNA or stored at – 80°C.

15.2.2 RNA isolation

Prior to total RNA preparation, samples were thawed in 37 °C waterbath and frozen in liquid nitrogen for at least seven cycles and afterwards shook (50 hz) with around 100 μl of glass-beads using TissueLyser LT (QIAGEN) at 4 °C. Subsequently chloroform was added and samples were spinned down. Supernatant was used for mRNA or microRNAs preparation according to the manual of RNeasy Mini Kit (QIAGEN). For mRNA isolation 70 % EtOH was used and for microRNA isolation 100 % EtOH was used. The optional step of DNAase treatment was performed each time with RNase- Free DNase (QUIAGEN) for 15 min at RT.

15.2.3 Quantity and quality measurement of RNA isolations

All RNA isolations were measured with NanoDrop 2000c (peqLab) to validate pureness and determine quantity. Additionally RNAseq samples were prepared and loaded onto a agilent chip and quality was checked with Tape Station 2200 (Agilent Technologies).

15.2.4 cDNA preparation with reverse transcriptase

From microRNA isolations cDNA was generated with Superscript III First Strand Synthesis System with random hexamers (Invitrogen). 150 ng of mRNA isolations were reverse transcribed according to iSCRIPT protocol and subsequently diluted with ddH2O to 1ng/ μl related to mRNA input.

15.2.5 qPCR measurements

For RNA measurement Power SYBR Green (Applied Biosystems) was used. 384 microRNA plates were prepared manually and technical quadruplicates of all samples were analyzed with 7900HT FastReal-Time PCR System. For preparation of 384 well mRNA plates JANUS automated workstation (PerkinElmer) was used. cDNA Power SYBR Green mix and primers in 1,5 ml tubes were manually prepared for the robot. A standard program for comparative CT values including Melting curve was used on both qPCR machines. For primer validation standard curve program was used.

Quantitavie real time PCR program		
	2 min	50 °C
	10 min	95 °C
40 cycles	15 s	95 °C
	1 min	60 °C
Melt curve	15 s	95 °C
	1 min	60°C
	15 s	95 °C

15.2.6 Statistic analyzes of qPCR data

Data were normalized to an internal housekeeping standard gene - *ama-1* for mRNA samples and the microRNA snoRNA U18 for microRNA samples. Each analyzed set contained a wild type control. Significance was tested with ANOVA using Prism GraphPad.

15.2.7 RNAseq analyses

As input we had three replicates per sample with a library size in the range of ~33-46 Mill reads. Before mapping to the genome, the reads were trimmed at both ends with the FASTA/Q Trimmer of the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit by Hannon Lab). The preprocessed reads were mapped to the genome with tophat2 (v2.0.7) (Ref) allowing only unique mapping. On average 99.9% of the reads could be aligned. Subsequently, differential gene expression was performed with DESeq REF. Genes with a q-value < 0.05 and fold change > |1.5| were classified as differentially expressed. (This part of the analysis was peformed by Dr. Corinna Klein of the MPI Bioinformatics Faicility). We then used DAVID online-tool (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) to determine GO term enrichments. For visualization REVIGO was used (**revigo**.irb.hr).

15.3 Steroid profile analyzes

15.3.1 Sample collection

A population that was grown reproductively for at least three generations without bleaching was synchronized by egg lay. Eggs were placed on experimental plates containing either 10 μM 7-KC or appropriate volume EtOH. L3 larvae were harvested quickly and washed in M9 twice and finally harvested in a volume of 200 μl M9. Especially for samples that are used for analyses of dafachronic acid it is important to not leave larvae to long in M9, since DA seems to diffuse to the medium (Dr. Magner personal communication).

15.3.2 Lipid extraction

L3 larvae in M9 buffer were frozen in liquid nitrogen and thawed in 37°C waterbath for at least seven cycles. Subsequently samples were sonicated on ice to lyse cuticle and tissues or they were vortexed (50 Hz) with glassbeads in TissueLyser LT (QIAGEN) at 4 °C. 100 ng internal standard (5β-cholanic acid and/or cholesterol-d_7) was added and for Bradford protein analyses 20 μl. Bradford analyzes was performed in cornwell 96 well plates according to Bio-Rad Protein Assay manual. Reads of reaction plates were performed at 595 nm with POLARstar Omega plate reader (BMG LABTECH). Subsequently lipids were extracted with chloroform: methanol (1:2, v/v) for 1 h at room temperature. Debris centrifuged down in GeneVac EZ2.3 Plus Evaporation System without vacuum. Supernatant containing extracts were divided into two approaches in GC glass vials- one for dafachronic acid (bile acid) analyzes and one for all other steroids.

15.3.3 Lipid Derivatization

Lipids were dried under N_2 stream or centrifuged down in GeneVac EZ2.3 Plus Evaporation System to dryness. For derivatization of steroid compounds other than DA, lipids were treated 15 min at 60°C with 15 μl N-methyl-N-Trimethylsilyltrifluoracetamid (MSTFA): NH_4I (1000:5, w/v) and subsequently 15 μl FLUKA III [TMSIM/BSTFA/TMCS(1-(trimethylsilyl)imidazole-N,Obis(trimethylsilyl) trifluoroacet-amide /trimethylchlorosilane), 3/2/2,(v/v/v) was added and incubated for 15 min at 60°C with (Metod adopted from (Magner and Antebi, 2008; Meunier-Solère et al., 2005)).

For derivatization of dafachronic acids, dried lipid extracts were treated with 50 μl toluene:methanol (1:1, v/v) and 200 μl (Trimethysilyl)diazomethane for 1 h at room

temperature. Afterwards lipids were dried under N_2 stream to dryness and resuspended in n-hexane (20 μl) prior to injection.

15.3.4 Preparation of standard curve

For later analyzes a standard curve for each compound was generated from powder stocks (Appendix Table). The highest concentration (100 ng) of the standard curve was prepared from a higher stock solution containing all compounds that should be analyzed (1 μg in MeOH) and repeatedly diluted 10 times in MeOH down to 0.01 ng. Internal standard was added to each dilution curve sample and from thereon samples were derivatized and analyzed in GC/MS/MS like steroid samples from worms.

15.3.5 GC/MS/MS analyzes

Methods for analyzing steroids and dafachronic acid with gaschromatography and masspectrometry were described before. The gaschromagraph was 7890A GC from Agilent. 4 μl of sample was injected in pulsed- splitless mode to a HP5-ms UI column (15m x 0.25 mm I.D., 0.25 μm, Agilent) and helium was used as the carrier gas with a flow rate of 23.979 ml/min and 1.2 min hold before purging to split vent. The initial oven temeperature started at 180 °C for 1 min and was then ramped with 30 °C/min up to 300 °C and held for another 11 min before it was increased to 325 °C for 8.17 min. The MS/MS coupled to the GC was a triple quadrupole (7000A) from Agilent Technologies. It was equipped with an ESI source and samples were separated. For the analyses a multiple reaction monitoring (MRM) method was created that combined the detection of all analyzed steroids. For identification of the silyated steroids the following transitions were used:

Compound	Transition (m/z)
Δ^7- dafachronic acid	428.3 → 229.1
5-β-cholanic acid	374.3 → 264.0
cholesterol	458.3 → 353.3
cholesterol-d_7	465.3 → 359.3
7-dehydrocholesterol	350.2 → 195.0
lathosterol	458.4 → 229.1

lathosterone	456.4 → 314.3

15.3.6 GC/MS/MS Data analyzes

Data were analyzed in Quantitatvie Aanlyzes Software, which uses the standard curves of each analyzed compound to determine absolute steroid content of measured sample. These values were then normalized to the measured protein content using Microsoft Excel. Values of at least 5 biological repeats were than further analyzed in GraphPad Prism to create graphs and to test for statistical significance (ANalyses Of VAriances -ANOVA).

15.4 Cell culture assays

15.4.1 maintainance of HEK 293T Cells

Cells used in this study are HEK 293T cells. They were maintained in Dulbecco´s modified Eagle Medium (DMEM , GIBCO) provided with 10 % Fetal Bovine Serum (FBS, GIBCO) at 37 °C and 5 % CO_2. To prevent dying of the cells, at an estimated confluence of 90 % they were detached from culture dish Cells by treatment with Trypsin 5 % for 1 min at 37 °C (GIBCO) washed twice with DPBS (GIBCO) and a 10 time dilution in fresh DMEM +FBS was distributed to new plates.

15.4.2 Plasmid preparation

Bacteria were grown as described in (15.1.2.) Plasmids were isolated according to protocol of QIAGEN MiniPrep or MidiPrep for plasmid isolation Kit. Concentration was measured with NanoDrop and plasmids were diluted to a concentration of 100 ng/μl. Plasmids were stored at 4 °C for no more than 6 month.

15.4.3 DAF-12 actvity assay

Adapted from Dr. Dan Magner. Confluent cells (around 10.000 cells/cm^2) on a plate were detached from 10 cm cell culture plate with Trypsin 5 %, washed twice with DPBS and diluted 1:10 in DMEM+FBS. Subsequently 100 μl were distributed to each well of a 96-well plate. Cells were grown overnight at 37 °C and 5 % CO_2. After 16 h A $CaCl_2$ master-mix for transfection was prepared containing β-gal plasmid and stuffer DNA. In addition either

pCMX-DAF-12 or PCMX-FLAGG was co-transfected with plasmid carrying pCMX::mir84p::luciferase (Bethke et al., 2009). One well was transfected with eGFP to control for efficiency. Cells were allowed to recover and grow for at least 7 h at 37 °C and 5 % CO_2. Compounds were concentrated 2000 times in EtOH and premixed with DMEM before they were provided to transfected cells. Cells were incubated after compound treatment for another 16 h at 37°C, 5 % CO_2.

15.4.4 Quantification of luciferase activty

First the luciferase activity was assayed. To induce luciferase illuminiscence cells were incubated with Luciferase Buffer (containing Core buffer, TritonX-100, D-Luciferine, ATP, Coenzyme A and β-mercaptoethanol see also Appendix) for 1 min at RT. Next Luciferase activity was determined with POLARstar Omega plate reader (BMG LABTECH) using a program that detects illumination. Subsequently ß-gal activity was assayed. To induce β-gal activity cells were incubated with β-gal induction buffer (containing β-gal buffer, OPNG buffer, OPNG and β-mercaptoethanol see Appendix) for 10 min at RT. Afterwards β-gal activity was measured with POLARstar Omega plate reader (BMG LABTECH) using a program that measures absorbance of light with λ 405 nm.

References

16 References

Ackerman, D., and Gems, D. (2012). The mystery of C. elegans aging: an emerging role for fat. Distant parallels between C. elegans aging and metabolic syndrome? Bioessays *34*, 466–471.

Ailion, M., and Thomas, J.H. (2000). Dauer formation induced by high temperatures in Caenorhabditis elegans. Genetics *156*, 1047–1067.

Albert, P.S., and Riddle, D.L. (1988). Mutants of Caenorhabditis elegans that form dauer-like larvae. Developmental Biology *126*, 270–293.

Amaral, J., Lee, J.W., Chou, J., Campos, M.M., and Rodríguez, I.R. (2013). 7-Ketocholesterol induces inflammation and angiogenesis in vivo: a novel rat model. PLoS ONE *8*, e56099.

Anisimov, V.N. (2010). Metformin for aging and cancer prevention. Aging (Albany NY) *2*, 760–774.

Anisimov, V.N., Egormin, P.A., Piskunova, T.S., Popovich, I.G., Tyndyk, M.L., Yurova, M.N., Zabezhinski, M.A., Anikin, I.V., Karkach, A.S., and Romanyukha, A.A. (2010). Metformin extends life span of HER-2/neu transgenic mice and in combination with melatonin inhibits growth of transplantable tumors in vivo. Cell Cycle *9*, 188–197.

Antebi, A., Yeh, W.H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes & Development *14*, 1512–1527.

Antebi, A. (2013). Steroid regulation of C. elegans diapause, developmental timing, and longevity. Curr. Top. Dev. Biol. *105*, 181–212.

Apfeld, J. (2004). The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in C. elegans. Genes & Development *18*, 3004–3009.

Arantes-Oliveira, N., Apfeld, J., Dillin, A., and Kenyon, C. (2002). Regulation of life-span by germ-line stem cells in Caenorhabditis elegans. Science *295*, 502–505.

Aston, L.M. (2006). Glycaemic index and metabolic disease risk. Proc Nutr Soc *65*, 125–134.

Bass, T.M., Weinkove, D., Houthoofd, K., Gems, D., and Partridge, L. (2007). Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. Mechanisms of Ageing and Development *128*, 546–552.

Bauer, J.H., Goupil, S., Garber, G.B., and Helfand, S.L. (2004). An accelerated assay for the identification of lifespan-extending interventions in Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.a. *101*, 12980–12985.

Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., Prabhu, V.V., Allard, J.S., Lopez-Lluch, G., Lewis, K., et al. (2006). Resveratrol improves health and survival of mice on a high-calorie diet. Nature *444*, 337–342.

Beher, D., Wu, J., Cumine, S., Kim, K.W., Lu, S.-C., Atangan, L., and Wang, M. (2009). Resveratrol is not a direct activator of SIRT1 enzyme activity. Chem Biol Drug Des *74*, 619–624.

Behm, C.A. (1997). The role of trehalose in the physiology of nematodes. Int. J. Parasitol. *27*, 215–229.

Benedetti, M.G., Foster, A.L., Vantipalli, M.C., White, M.P., Sampayo, J.N., Gill, M.S., Olsen, A., and Lithgow, G.J. (2008). Experimental Gerontology. Exg *43*, 882–891.

Berman, J.R., and Kenyon, C. (2006). Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. Cell *124*, 1055–1068.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D.J., and Antebi, A. (2009). Nuclear Hormone Receptor Regulation of MicroRNAs Controls Developmental Progression. Science *324*, 95–98.

Birnby, D.A., Link, E.M., Vowels, J.J., Tian, H., Colacurcio, P.L., and Thomas, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in caenorhabditis elegans. Genetics *155*, 85–104.

Björkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R.J., Duan, C., and Lund, E. (1994).

Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc. Natl. Acad. Sci. U.S.a. *91*, 8592–8596.

Bokov, A., Chaudhuri, A., and Richardson, A. (2004). The role of oxidative damage and stress in aging. Mechanisms of Ageing and Development *125*, 811–826.

Brack, C., Bechter-Thüring, E., and Labuhn, M. (1997). N-acetylcysteine slows down ageing and increases the life span of Drosophila melanogaster. Cell. Mol. Life Sci. *53*, 960–966.

Braeckman, B.P., Houthoofd, K., and Vanfleteren, J.R. (2001). Insulin-like signaling, metabolism, stress resistance and aging in Caenorhabditis elegans. Mechanisms of Ageing and Development *122*, 673–693.

Bratic, A., and Larsson, N.-G. (2013). The role of mitochondria in aging. J. Clin. Invest. *123*, 951–957.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71–94.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., and Schreiber, S.L. (1994). A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature *369*, 756–758.

BROWN, M., EVANS, J., and LUO, Y. (2006). Beneficial effects of natural antioxidants EGCG and α-lipoic acid on life span and age-dependent behavioral declines in Caenorhabditis elegans. Pharmacology Biochemistry and Behavior *85*, 620–628.

Brown-Borg, H.M., Borg, K.E., Meliska, C.J., and Bartke, A. (1996). Dwarf mice and the ageing process. Nature *384*, 33.

Buttari, B., Segoni, L., Profumo, E., D'Arcangelo, D., Rossi, S., Facchiano, F., Businaro, R., Iuliano, L., and Riganò, R. (2013). 7-Oxo-cholesterol potentiates pro-inflammatory signaling in human M1 and M2 macrophages. Biochem. Pharmacol. *86*, 130–137.

Cabreiro, F., Au, C., Leung, K.-Y., Vergara-Irigaray, N., Cochemé, H.M., Noori, T., Weinkove, D., Schuster, E., Greene, N.D.E., and Gems, D. (2013). Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. Cell *153*, 228–239.

Cafferkey, R., Young, P.R., McLaughlin, M.M., Bergsma, D.J., Koltin, Y., Sathe, G.M., Faucette, L., Eng, W.K., Johnson, R.K., and Livi, G.P. (1993). Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol. Cell. Biol. *13*, 6012–6023.

Cantó, C., Gerhart-Hines, Z., Feige, J.N., Lagouge, M., Noriega, L., Milne, J.C., Elliott, P.J., Puigserver, P., and Auwerx, J. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature *458*, 1056–1060.

Carrell, R.W., and Lomas, D.A. (1997). Conformational disease. Lancet *350*, 134–138.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental Biology *46*, 326–342.

Cava, E., and Fontana, L. (2013). Will calorie restriction work in humans? Aging (Albany NY) *5*, 507–514.

Chapman, T., and Partridge, L. (1996). Female fitness in Drosophila melanogaster: an interaction between the effect of nutrition and of encounter rate with males. Proc. Biol. Sci. *263*, 755–759.

Chen, S., Whetstine, J.R., Ghosh, S., Hanover, J.A., Gali, R.R., Grosu, P., and Shi, Y. (2009). The conserved NAD(H)-dependent corepressor CTBP-1 regulates Caenorhabditis elegans life span. Proc. Natl. Acad. Sci. U.S.a. *106*, 1496–1501.

Cheng, I.H., Scearce-Levie, K., Legleiter, J., Palop, J.J., Gerstein, H., Bien-Ly, N., Puoliväli, J., Lesné, S., Ashe, K.H., Muchowski, P.J., et al. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. J. Biol. Chem. *282*, 23818–23828.

Chiang, J.Y.L. (2013). Bile acid metabolism and signaling. Compr Physiol *3*, 1191–1212.

Chui, D.H., Tanahashi, H., Ozawa, K., Ikeda, S., Checler, F., Ueda, O., Suzuki, H., Araki, W., Inoue, H., Shirotani, K., et al. (1999). Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat. Med. *5*, 560–564.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science *292*, 104–106.

Cohen, E., Bieschke, J., Perciavalle, R.M., Kelly, J.W., and Dillin, A. (2006). Opposing activities protect against

age-onset proteotoxicity. Science *313*, 1604–1610.

Collins, J.J., Huang, C., Hughes, S., and Kornfeld, K. (2008). The measurement and analysis of age-related changes in Caenorhabditis elegans. WormBook 1–21.

Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W., et al. (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. Science *325*, 201–204.

Copeland, J.M., Cho, J., Lo, T., Hur, J.H., Bahadorani, S., Arabyan, T., Rabie, J., Soh, J., and Walker, D.W. (2009). Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain. Curr. Biol. *19*, 1591–1598.

Cuervo, A.M. (2008). Autophagy and aging: keeping that old broom working. Trends Genet. *24*, 604–612.

Denzel, M.S., Storm, N.J., Gutschmidt, A., Baddi, R., Hinze, Y., Jarosch, E., Sommer, T., Hoppe, T., and Antebi, A. (2014). Hexosamine pathway metabolites enhance protein quality control and prolong life. Cell *156*, 1167–1178.

Dillin, A., and Cohen, E. (2011). Ageing and protein aggregation-mediated disorders: from invertebrates to mammals. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *366*, 94–98.

Douglas, P.M., and Dillin, A. (2010). Protein homeostasis and aging in neurodegeneration. J. Cell Biol. *190*, 719–729.

Duan, W., and Mattson, M.P. (1999). Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. J. Neurosci. Res. *57*, 195–206.

Dumas, K.J., Guo, C., Wang, X., Burkhart, K.B., Adams, E.J., Alam, H., and Hu, P.J. (2010). Developmental Biology. Developmental Biology *340*, 605–612.

Edwards, C.B., Copes, N., Brito, A.G., Canfield, J., and Bradshaw, P.C. (2013). Malate and fumarate extend lifespan in Caenorhabditis elegans. PLoS ONE *8*, e58345.

Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., Ring, J., Schroeder, S., Magnes, C., Antonacci, L., et al. (2009). Induction of autophagy by spermidine promotes longevity. Nat. Cell Biol. *11*, 1305–1314.

Ewbank, J.J., Barnes, T.M., Lakowski, B., Lussier, M., Bussey, H., and Hekimi, S. (1997). Structural and functional conservation of the Caenorhabditis elegans timing gene clk-1. Science *275*, 980–983.

Feng, J., Bussière, F., and Hekimi, S. (2001). Mitochondrial electron transport is a key determinant of life span in Caenorhabditis elegans. Developmental Cell *1*, 633–644.

Fielenbach, N., and Antebi, A. (2008). C. elegans dauer formation and the molecular basis of plasticity. Genes & Development *22*, 2149–2165.

Fisher, A.L., and Lithgow, G.J. (2006). The nuclear hormone receptor DAF-12 has opposing effects on Caenorhabditis elegans lifespan and regulates genes repressed in multiple long-lived worms. Aging Cell *5*, 127–138.

Flatt, T., Min, K.-J., D'Alterio, C., Villa-Cuesta, E., Cumbers, J., Lehmann, R., Jones, D.L., and Tatar, M. (2008). Drosophila germ-line modulation of insulin signaling and lifespan. Proc. Natl. Acad. Sci. U.S.a. *105*, 6368–6373.

Fowler, D.M., Koulov, A.V., Alory-Jost, C., Marks, M.S., Balch, W.E., and Kelly, J.W. (2006). Functional amyloid formation within mammalian tissue. PLoS Biol *4*, e6.

Friedman, D.B., and Johnson, T.E. (1988a). Three mutants that extend both mean and maximum life span of the nematode, Caenorhabditis elegans, define the age-1 gene. J Gerontol *43*, B102–B109.

Friedman, D.B., and Johnson, T.E. (1988b). A mutation in the age-1 gene in Caenorhabditis elegans lengthens life and reduces hermaphrodite fertility. Genetics *118*, 75–86.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., and Riddle, D.L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics *150*, 129–155.

Gems, D., and Partridge, L. (2013). Genetics of longevity in model organisms: debates and paradigm shifts. Annu. Rev. Physiol. *75*, 621–644.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., and Antebi, A. (2001). A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Developmental Cell *1*, 841–851.

Gerisch, B., Rottiers, V., Li, D., Motola, D.L., Cummins, C.L., Lehrach, H., Mangelsdorf, D.J., and Antebi, A. (2007). A bile acid-like steroid modulates Caenorhabditis elegans lifespan through nuclear receptor signaling. Proc. Natl. Acad. Sci. U.S.a. *104*, 5014–5019.

Ghazi, A., Henis-Korenblit, S., and Kenyon, C. (2009). A transcription elongation factor that links signals from the reproductive system to lifespan extension in Caenorhabditis elegans. PLoS Genet *5*, e1000639.

Golden, J.W., and Riddle, D.L. (1984). The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental Biology *102*, 368–378.

Goudeau, J., Bellemin, S., Toselli-Mollereau, E., Shamalnasab, M., Chen, Y., and Aguilaniu, H. (2011). Fatty acid desaturation links germ cell loss to longevity through NHR-80/HNF4 in C. elegans. PLoS Biol *9*, e1000599.

Grandison, R.C., Piper, M.D.W., and Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature 1–5.

Greer, E.L., and Brunet, A. (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. Aging Cell *8*, 113–127.

Greer, E.L., Dowlatshahi, D., Banko, M.R., Villen, J., Hoang, K., Blanchard, D., Gygi, S.P., and Brunet, A. (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr. Biol. *17*, 1646–1656.

Grönke, S., Clarke, D.-F., Broughton, S., Andrews, T.D., and Partridge, L. (2010). Molecular evolution and functional characterization of Drosophila insulin-like peptides. PLoS Genet *6*, e1000857.

Guarente, L. (2005). Calorie restriction and SIR2 genes--towards a mechanism. Mechanisms of Ageing and Development *126*, 923–928.

Haigis, M.C., and Guarente, L.P. (2006). Mammalian sirtuins--emerging roles in physiology, aging, and calorie restriction. Genes & Development *20*, 2913–2921.

Hansen, M., Hsu, A.-L., Dillin, A., and Kenyon, C. (2005). New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a Caenorhabditis elegans genomic RNAi screen. PLoS Genet *1*, 119–128.

Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S.-J., and Kenyon, C. (2007). Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. Aging Cell *6*, 95–110.

HARMAN, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol *11*, 298–300.

Harrison, D.E., Strong, R., Sharp, Z.D., Nelson, J.F., Astle, C.M., Flurkey, K., Nadon, N.L., Wilkinson, J.E., Frenkel, K., Carter, C.S., et al. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature *460*, 392–395.

Hars, E.S., Qi, H., Ryazanov, A.G., Jin, S., Cai, L., Hu, C., and Liu, L.F. (2007). Autophagy regulates ageing in C. elegans. Autophagy *3*, 93–95.

He, L.-Q., Lu, J.-H., and Yue, Z.-Y. (2013). Autophagy in ageing and ageing-associated diseases. Acta Pharmacol. Sin. *34*, 605–611.

Heidler, T., Hartwig, K., Daniel, H., and Wenzel, U. (2009). Caenorhabditis elegans lifespan extension caused by treatment with an orally active ROS-generator is dependent on DAF-16 and SIR-2.1. Biogerontology *11*, 183–195.

Held, J.M., White, M.P., Fisher, A.L., Gibson, B.W., Lithgow, G.J., and Gill, M.S. (2006). DAF-12-dependent rescue of dauer formation in Caenorhabditis elegans by (25S)-cholestenoic acid. Aging Cell *5*, 283–291.

Henderson, S.T., and Johnson, T.E. (2001). daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr. Biol. *11*, 1975–1980.

Hendrie, H.C., Ogunniyi, A., Hall, K.S., Baiyewu, O., Unverzagt, F.W., Gureje, O., Gao, S., Evans, R.M., Ogunseyinde, A.O., Adeyinka, A.O., et al. (2001). Incidence of dementia and Alzheimer disease in 2 communities: Yoruba residing in Ibadan, Nigeria, and African Americans residing in Indianapolis, Indiana. Jama *285*, 739–747.

Hertweck, M., Göbel, C., and Baumeister, R. (2004). C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. Developmental Cell *6*, 577–588.

Hieb, W.F., and Rothstein, M. (1968). Sterol requirement for reproduction of a free-living nematode. Science *160*, 778–780.

Hodis, H.N., Crawford, D.W., and Sevanian, A. (1991). Cholesterol feeding increases plasma and aortic tissue cholesterol oxide levels in parallel: further evidence for the role of cholesterol oxidation in atherosclerosis. Atherosclerosis *89*, 117–126.

Honda, Y., Tanaka, M., and Honda, S. (2010). Trehalose extends longevity in the nematode Caenorhabditis elegans. Aging Cell *9*, 558–569.

Honjoh, S., Yamamoto, T., Uno, M., and Nishida, E. (2009). Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. Nature *457*, 726–730.

Horn, M., Geisen, C., Cermak, L., Becker, B., Nakamura, S., Klein, C., Pagano, M., and Antebi, A. (2014). DRE-1/FBXO11-dependent degradation of BLMP-1/BLIMP-1 governs C. elegans developmental timing and maturation. Developmental Cell *28*, 697–710.

Houthoofd, K., and Vanfleteren, J.R. (2006). The longevity effect of dietary restriction in Caenorhabditis elegans. Exg *41*, 1026–1031.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.-L., et al. (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature *425*, 191–196.

Hsia, A.Y., Masliah, E., McConlogue, L., Yu, G.Q., Tatsuno, G., Hu, K., Kholodenko, D., Malenka, R.C., Nicoll, R.A., and Mucke, L. (1999). Plaque-independent disruption of neural circuits in Alzheimer's disease mouse models. Proc. Natl. Acad. Sci. U.S.a. *96*, 3228–3233.

Hsin, H., and Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of C. elegans. Nature *399*, 362–366.

Hu, P.J. (2007). Dauer. WormBook.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc *4*, 44–57.

Huang, Z., Liu, Q., Li, W., Wang, R., Wang, D., Zhang, Y., Zhang, F., Chi, Y., Liu, Z., Matsuura, E., et al. (2010). 7-ketocholesterol induces cell apoptosis by activation of nuclear factor kappa B in mouse macrophages. Acta Med. Okayama *64*, 85–93.

Hwang, J.-T., Kwak, D.W., Lin, S.K., Kim, H.M., Kim, Y.M., and Park, O.J. (2007). Resveratrol induces apoptosis in chemoresistant cancer cells via modulation of AMPK signaling pathway. Ann. N. Y. Acad. Sci. *1095*, 441–448.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.-L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648–657.

Inoki, K., Zhu, T., and Guan, K.-L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577–590.

Ishii, N., Fujii, M., Hartman, P.S., Tsuda, M., Yasuda, K., Senoo-Matsuda, N., Yanase, S., Ayusawa, D., and Suzuki, K. (1998). A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. Nature *394*, 694–697.

Ishii, N., Senoo-Matsuda, N., Miyake, K., Yasuda, K., Ishii, T., Hartman, P.S., and Furukawa, S. (2004). Coenzyme Q10 can prolong C. elegans lifespan by lowering oxidative stress. Mechanisms of Ageing and Development *125*, 41–46.

Jessup, W., and Brown, A.J. (2005). Novel Routes for Metabolism of 7-Ketocholesterol. Rejuvenation Research *8*, 9–12.

Jia, K. (2004). The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. Development *131*, 3897–3906.

Jia, K., Albert, P.S., and Riddle, D.L. (2002). DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development *129*, 221–231.

Jiang, J.C., Jaruga, E., Repnevskaya, M.V., and Jazwinski, S.M. (2000). An intervention resembling caloric restriction prolongs life span and retards aging in yeast. Faseb J. *14*, 2135–2137.

Johnson, S.C., Rabinovitch, P.S., and Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-

related disease. Nature *493*, 338–345.

Kaeberlein, M., and Powers, R.W. (2007). Sir2 and calorie restriction in yeast: a skeptical perspective. Ageing Research Reviews *6*, 128–140.

Kaeberlein, M., Hu, D., Kerr, E.O., Tsuchiya, M., Westman, E.A., Dang, N., Fields, S., and Kennedy, B.K. (2005). Increased life span due to calorie restriction in respiratory-deficient yeast. PLoS Genet *1*, e69.

Kaeberlein, T.L., Smith, E.D., Tsuchiya, M., Welton, K.L., Thomas, J.H., Fields, S., Kennedy, B.K., and Kaeberlein, M. (2006). Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging Cell *5*, 487–494.

N. Kagawa, M. Kuwayama, Y. Ikeda, H. Nagashima, S.J. Silber, O. Kato (2010). Increased longevity of old infertile mice after allo-transplantation of young mice ovaries. Fertility and Sterility, Volume 94, Issue 4,

Kalender, A., Selvaraj, A., Kim, S.Y., Gulati, P., Brûlé, S., Viollet, B., Kemp, B.E., Bardeesy, N., Dennis, P., Schlager, J.J., et al. (2010). Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. Cell Metabolism *11*, 390–401.

Kamath, R. (2003). Genome-wide RNAi screening in Caenorhabditis elegans. Methods *30*, 313–321.

Kampkötter, A., Gombitang Nkwonkam, C., Zurawski, R.F., Timpel, C., Chovolou, Y., Wätjen, W., and Kahl, R. (2007). Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism Caenorhabditis elegans. Arch Toxicol *81*, 849–858.

Kapahi, P., Chen, D., Rogers, A.N., Katewa, S.D., Li, P.W.-L., Thomas, E.L., and Kockel, L. (2010). With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metabolism *11*, 453–465.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V., and Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr. Biol. *14*, 885–890.

Keaney, M., Matthijssens, F., Sharpe, M., Vanfleteren, J., and Gems, D. (2004). Superoxide dismutase mimetics elevate superoxide dismutase activity in vivo but do not retard aging in the nematode Caenorhabditis elegans. Free Radical Biology and Medicine *37*, 239–250.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461–464.

Kenyon, C.J. (2010). The genetics of ageing. Nature *464*, 504–512.

Kimura, K.D. (1997). daf-2, an Insulin Receptor-Like Gene That Regulates Longevity and Diapause in Caenorhabditis elegans. Science *277*, 942–946.

Klass, M., and Hirsh, D. (1976). Non-ageing developmental variant of Caenorhabditis elegans. Nature *260*, 523–525.

Klass, M.R. (1977). Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mechanisms of Ageing and Development *6*, 413–429.

Kojima, T., Kamei, H., Aizu, T., Arai, Y., Takayama, M., Nakazawa, S., Ebihara, Y., Inagaki, H., Masui, Y., Gondo, Y., et al. (2004). Association analysis between longevity in the Japanese population and polymorphic variants of genes involved in insulin and insulin-like growth factor 1 signaling pathways. Exg *39*, 1595–1598.

Komander, D., and Rape, M. (2012). The ubiquitin code. Annu. Rev. Biochem. *81*, 203–229.

Kumar-Singh, S., Dewachter, I., Moechars, D., Lübke, U., De Jonghe, C., Ceuterick, C., Checler, F., Naidu, A., Cordell, B., Cras, P., et al. (2000). Behavioral disturbances without amyloid deposits in mice overexpressing human amyloid precursor protein with Flemish (A692G) or Dutch (E693Q) mutation. Neurobiol. Dis. *7*, 9–22.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R., and Hall, M.N. (1993). Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell *73*, 585–596.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., et al. (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell *127*, 1109–1122.

Lakowski, B., and Hekimi, S. (1998). The genetics of caloric restriction in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U.S.a. *95*, 13091–13096.

Lamitina, S.T. (2005). Transcriptional targets of DAF-16 insulin signaling pathway protect C. elegans from extreme hypertonic stress. AJP: Cell Physiology *288*, C467–C474.

Lamming, D.W., Ye, L., Katajisto, P., Goncalves, M.D., Saitoh, M., Stevens, D.M., Davis, J.G., Salmon, A.B., Richardson, A., Ahima, R.S., et al. (2012). Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. Science *335*, 1638–1643.

Lapierre, L.R., Gelino, S., Meléndez, A., and Hansen, M. (2011). Autophagy and Lipid Metabolism Coordinately Modulate Life Span in Germline-less C. elegans. Current Biology *21*, 1507–1514.

Larrayoz, I.M., Huang, J.-D., Lee, J.W., Pascual, I., and Rodríguez, I.R. (2010). 7-ketocholesterol-induced inflammation: involvement of multiple kinase signaling pathways via NFκB but independently of reactive oxygen species formation. Invest. Ophthalmol. Vis. Sci. *51*, 4942–4955.

Larsen, P.L., Albert, P.S., and Riddle, D.L. (1995). Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics *139*, 1567–1583.

Larsen, P.L., and Clarke, C.F. (2002). Extension of life-span in Caenorhabditis elegans by a diet lacking coenzyme Q. Science *295*, 120–123.

Lee, C.-K., Pugh, T.D., Klopp, R.G., Edwards, J., Allison, D.B., Weindruch, R., and Prolla, T.A. (2004). The impact of alpha-lipoic acid, coenzyme Q10 and caloric restriction on life span and gene expression patterns in mice. Free Radical Biology and Medicine *36*, 1043–1057.

Lee, R.Y., Hench, J., and Ruvkun, G. (2001). Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr. Biol. *11*, 1950–1957.

Lee, S.-J., Murphy, C.T., and Kenyon, C. (2009). Glucose Shortens the Life Span of C. elegans by Downregulating DAF-16/FOXO Activity and Aquaporin Gene Expression. Cell Metabolism *10*, 379–391.

Li, J. (2004). NCR-1 and NCR-2, the C. elegans homologs of the human Niemann-Pick type C1 disease protein, function upstream of DAF-9 in the dauer formation pathways. Development *131*, 5741–5752.

Li, W., Gao, B., Lee, S.-M., Bennett, K., and Fang, D. (2007). RLE-1, an E3 ubiquitin ligase, regulates C. elegans aging by catalyzing DAF-16 polyubiquitination. Developmental Cell *12*, 235–246.

Lin, K., Hsin, H., Libina, N., and Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat. Genet. *28*, 139–145.

Liu, B.-L., Zhang, X., Zhang, W., and Zhen, H.-N. (2007). New enlightenment of French Paradox: resveratrol's potential for cancer chemoprevention and anti-cancer therapy. Cancer Biol. Ther. *6*, 1833–1836.

López-Otín, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*, 1194–1217.

Lucanic, M., Held, J.M., Vantipalli, M.C., Klang, I.M., Graham, J.B., Gibson, B.W., Lithgow, G.J., and Gill, M.S. (2011). N-acylethanolamine signalling mediates the effect of diet on lifespan in Caenorhabditis elegans. Nature *473*, 226–229.

Lucanic, M., Lithgow, G.J., and Alavez, S. (2013). Pharmacological lifespan extension of invertebrates. Ageing Research Reviews *12*, 445–458.

Luchsinger, J.A., Tang, M.-X., Shea, S., and Mayeux, R. (2002). Caloric intake and the risk of Alzheimer disease. Arch. Neurol. *59*, 1258–1263.

Luciani, G.M., Magomedova, L., Puckrin, R., Urbanus, M.L., Wallace, I.M., Giaever, G., Nislow, C., Cummins, C.L., and Roy, P.J. (2011). Dafadine inhibits DAF-9 to promote dauer formation and longevity of Caenorhabditis elegans. Nat. Chem. Biol. *7*, 891–893.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes & Development *18*, 2120–2133.

Luthra, S., Dong, J., Gramajo, A.L., Chwa, M., Kim, D.W., Neekhra, A., Kuppermann, B.D., and Kenney, M.C. (2008). 7-Ketocholesterol activates caspases-3/7, -8, and -12 in human microvascular endothelial cells in vitro. Microvasc. Res. *75*, 343–350.

Lyons, M.A., and Brown, A.J. (1999). 7-Ketocholesterol. Int. J. Biochem. Cell Biol. *31*, 369–375.

Magner, D.B., and Antebi, A. (2008). Caenorhabditis elegans nuclear receptors: insights into life traits. Trends in Endocrinology & Metabolism *19*, 153–160.

Magner, D.B., Wollam, J., Shen, Y., Hoppe, C., Li, D., Latza, C., Rottiers, V., Hutter, H., and Antebi, A. (2013). The NHR-8 nuclear receptor regulates cholesterol and bile acid homeostasis in C. elegans. Cell Metabolism *18*,

212–224.

Mahanti, P., Bose, N., Bethke, A., Judkins, J.C., Wollam, J., Dumas, K.J., Zimmerman, A.M., Campbell, S.L., Hu, P.J., Antebi, A., et al. (2014). Comparative Metabolomics Reveals Endogenous Ligands of DAF-12, a Nuclear Hormone Receptor, Regulating C. elegans Development and Lifespan. Cell Metabolism *19*, 73–83.

Mair, W., Morantte, I., Rodrigues, A.P.C., Manning, G., Montminy, M., Shaw, R.J., and Dillin, A. (2011). Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. Nature *470*, 404–408.

McDonald, R.B., and Ramsey, J.J. (2010). Honoring Clive McCay and 75 years of calorie restriction research.

McElwee, J., Bubb, K., and Thomas, J.H. (2003). Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. Aging Cell *2*, 111–121.

Mehta, R., Steinkraus, K.A., Sutphin, G.L., Ramos, F.J., Shamieh, L.S., Huh, A., Davis, C., Chandler-Brown, D., and Kaeberlein, M. (2009). Proteasomal regulation of the hypoxic response modulates aging in C. elegans. Science *324*, 1196–1198.

Meléndez, A. (2009). Autophagy in C. elegans. WormBook 1–26.

Meléndez, A., Tallóczy, Z., Seaman, M., Eskelinen, E.-L., Hall, D.H., and Levine, B. (2003). Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science *301*, 1387–1391.

Melov, S., Ravenscroft, J., Malik, S., Gill, M.S., Walker, D.W., Clayton, P.E., Wallace, D.C., Malfroy, B., Doctrow, S.R., and Lithgow, G.J. (2000). Extension of life-span with superoxide dismutase/catalase mimetics. Science *289*, 1567–1569.

Meunier-Solère, V., Maume, D., André, F., and Le Bizec, B. (2005). Pitfalls in trimethylsilylation of anabolic steroids. New derivatisation approach for residue at ultra-trace level. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. *816*, 281–288.

Mihaylova, V.T., Borland, C.Z., Manjarrez, L., Stern, M.J., and Sun, H. (1999). The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. Proc. Natl. Acad. Sci. U.S.a. *96*, 7427–7432.

Miller, R.A., Harrison, D.E., Astle, C.M., Baur, J.A., Boyd, A.R., de Cabo, R., Fernandez, E., Flurkey, K., Javors, M.A., Nelson, J.F., et al. (2011). Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. The Journals of Gerontology Series a: Biological Sciences and Medical Sciences *66*, 191–201.

Miller, R.A., Harrison, D.E., Astle, C.M., Fernandez, E., Flurkey, K., Han, M., Javors, M.A., Li, X., Nadon, N.L., Nelson, J.F., et al. (2013). Rapamycin-Mediated Lifespan Increase in Mice is Dose and Sex-Dependent and Appears Metabolically Distinct from Dietary Restriction. Aging Cell.

Mitić, T., Shave, S., Semjonous, N., McNae, I., Cobice, D.F., Lavery, G.G., Webster, S.P., Hadoke, P.W.F., Walker, B.R., and Andrew, R. (2013). 11β-Hydroxysteroid dehydrogenase type 1 contributes to the balance between 7-keto- and 7-hydroxy-oxysterols in vivo. Biochem. Pharmacol. *86*, 146–153.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. Nature *451*, 1069–1075.

Morris, J.Z., Tissenbaum, H.A., and Ruvkun, G. (1996). A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature *382*, 536–539.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A., et al. (2006). Identification of Ligands for DAF-12 that Govern Dauer Formation and Reproduction in C. elegans. Cell *124*, 1209–1223.

Murakami, M., Koga, M., and Ohshima, Y. (2001). DAF-7/TGF-beta expression required for the normal larval development in C. elegans is controlled by a presumed guanylyl cyclase DAF-11. Mech. Dev. *109*, 27–35.

Murphy, C.T. (2006). The search for DAF-16/FOXO transcriptional targets: approaches and discoveries. Exg *41*, 910–921.

Murphy, C.T., McCarroll, S.A., Bargmann, C.I., Fraser, A., Kamath, R.S., Ahringer, J., Li, H., and Kenyon, C. (2003). Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature *424*, 277–283.

Müller, R.-U., Fabretti, F., Zank, S., Burst, V., Benzing, T., and Schermer, B. (2009). The von Hippel Lindau tumor suppressor limits longevity. J. Am. Soc. Nephrol. *20*, 2513–2517.

Nielsen, J.H., Olsen, C.E., Duedahl, C., and Skibsted, L.H. (1995). Isolation and quantification of cholesterol oxides in dairy products by selected ion monitoring mass spectrometry. J. Dairy Res. *62*, 101–113.

Nomura, T., Horikawa, M., Shimamura, S., Hashimoto, T., and Sakamoto, K. (2009). Fat accumulation in Caenorhabditis elegans is mediated by SREBP homolog SBP-1. Genes Nutr *5*, 17–27.

O'Rourke, E.J., Kuballa, P., Xavier, R., and Ruvkun, G. (2013). -6 Polyunsaturated fatty acids extend life span through the activation of autophagy. Genes & Development *27*, 429–440.

Oesterreicher, T.J., Markesich, D.C., and Henning, S.J. (2001). Cloning, characterization and mapping of the mouse trehalase (Treh) gene. Gene *270*, 211–220.

Ogg, S., and Ruvkun, G. (1998). The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol. Cell *2*, 887–893.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G.I., Lee, L., Tissenbaum, H.A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994–999.

Onken, B., and Driscoll, M. (2010). Metformin Induces a Dietary Restriction–Like State and the Oxidative Stress Response to Extend C. elegans Healthspan via AMPK, LKB1, and SKN-1. PLoS ONE *5*, e8758.

Otaegui-Arrazola, A., Menéndez-Carreño, M., Ansorena, D., and Astiasarán, I. (2010). Oxysterols: A world to explore. Food Chem. Toxicol. *48*, 3289–3303.

Palozza, P., Simone, R., Catalano, A., Boninsegna, A., Böhm, V., Fröhlich, K., Mele, M.C., Monego, G., and Ranelletti, F.O. (2010). Lycopene prevents 7-ketocholesterol-induced oxidative stress, cell cycle arrest and apoptosis in human macrophages. J. Nutr. Biochem. *21*, 34–46.

Pan, K.Z., Palter, J.E., Rogers, A.N., Olsen, A., Chen, D., Lithgow, G.J., and Kapahi, P. (2007). Inhibition of mRNA translation extends lifespan in Caenorhabditis elegans. Aging Cell *6*, 111–119.

Paradis, S., and Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes & Development *12*, 2488–2498.

Paradis, S., Ailion, M., Toker, A., Thomas, J.H., and Ruvkun, G. (1999). A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes & Development *13*, 1438–1452.

Partridge, L., Piper, M.D.W., and Mair, W. (2005). Dietary restriction in Drosophila. Mechanisms of Ageing and Development *126*, 938–950.

Patel, D.S., Fang, L.L., Svy, D.K., Ruvkun, G., and Li, W. (2008). Genetic identification of HSD-1, a conserved steroidogenic enzyme that directs larval development in Caenorhabditis elegans. Development *135*, 2239–2249.

Pearson, K.J., Baur, J.A., Lewis, K.N., Peshkin, L., Price, N.L., Labinskyy, N., Swindell, W.R., Kamara, D., Minor, R.K., Perez, E., et al. (2008). Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metabolism *8*, 157–168.

Pedruzzi, E., Guichard, C., Ollivier, V., Driss, F., Fay, M., Prunet, C., Marie, J.-C., Pouzet, C., Samadi, M., Elbim, C., et al. (2004). NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. Mol. Cell. Biol. *24*, 10703–10717.

Pérez, V.I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q., Ikeno, Y., and Richardson, A. (2009a). Is the oxidative stress theory of aging dead? Biochim. Biophys. Acta *1790*, 1005–1014.

Pérez, V.I., Van Remmen, H., Bokov, A., Epstein, C.J., Vijg, J., and Richardson, A. (2009b). The overexpression of major antioxidant enzymes does not extend the lifespan of mice. Aging Cell *8*, 73–75.

Pierce, S.B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S.A., Buchman, A.R., Ferguson, K.C., Heller, J., Platt, D.M., Pasquinelli, A.A., et al. (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes & Development *15*, 672–686.

Pilz, S., Gaksch, M., O'Hartaigh, B., Tomaschitz, A., and März, W. (2013). The role of vitamin D deficiency in cardiovascular disease: where do we stand in 2013? Arch Toxicol *87*, 2083–2103.

Potter, C.J., Pedraza, L.G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat. Cell Biol. *4*, 658–665.

Pyo, J.-O., Yoo, S.-M., Ahn, H.-H., Nah, J., Hong, S.-H., Kam, T.-I., Jung, S., and Jung, Y.-K. (2013).

Overexpression of Atg5 in mice activates autophagy and extends lifespan. Nat Commun *4*, 2300.

Ramsey, J.J., Harper, M.E., and Weindruch, R. (2000). Restriction of energy intake, energy expenditure, and aging. Free Radical Biology and Medicine *29*, 946–968.

Redman, L.M., and Ravussin, E. (2011). Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes. Antioxid. Redox Signal. *14*, 275–287.

Renaud, S., and de Lorgeril, M. (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet *339*, 1523–1526.

Reznick, R.M., Zong, H., Li, J., Morino, K., Moore, I.K., Yu, H.J., Liu, Z.-X., Dong, J., Mustard, K.J., Hawley, S.A., et al. (2007). Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis. Cell Metabolism *5*, 151–156.

Riddle, D.L., Swanson, M.M., and Albert, P.S. (1981). Interacting genes in nematode dauer larva formation. Nature *290*, 668–671.

Rine, J., and Herskowitz, I. (1987). Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. Genetics *116*, 9–22.

Robida-Stubbs, S., Glover-Cutter, K., Lamming, D.W., Mizunuma, M., Narasimhan, S.D., Neumann-Haefelin, E., Sabatini, D.M., and Blackwell, T.K. (2012). TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. Cell Metabolism *15*, 713–724.

Rottiers, V., Motola, D.L., Gerisch, B., Cummins, C.L., Nishiwaki, K., Mangelsdorf, D.J., and Antebi, A. (2006). Hormonal Control of C. elegans Dauer Formation and Life Span by a Rieske-like Oxygenase. Developmental Cell *10*, 473–482.

Rual, J.-F., Ceron, J., Koreth, J., Hao, T., Nicot, A.-S., Hirozane-Kishikawa, T., Vandenhaute, J., Orkin, S.H., Hill, D.E., van den Heuvel, S., et al. (2004). Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. Genome Res. *14*, 2162–2168.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S.H. (1994). RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell *78*, 35–43.

Sabers, C.J., Martin, M.M., Brunn, G.J., Williams, J.M., Dumont, F.J., Wiederrecht, G., and Abraham, R.T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. J. Biol. Chem. *270*, 815–822.

Saucier, S.E., Kandutsch, A.A., Gayen, A.K., Swahn, D.K., and Spencer, T.A. (1989). Oxysterol regulators of 3-hydroxy-3-methylglutaryl-CoA reductase in liver. Effect of dietary cholesterol. J. Biol. Chem. *264*, 6863–6869.

Schaedel, O.N., Gerisch, B., Antebi, A., and Sternberg, P.W. (2012). Hormonal signal amplification mediates environmental conditions during development and controls an irreversible commitment to adulthood. PLoS Biol *10*, e1001306.

Schlotterer, A., Kukudov, G., Bozorgmehr, F., Hutter, H., Du, X., Oikonomou, D., Ibrahim, Y., Pfisterer, F., Rabbani, N., Thornalley, P., et al. (2009). C. elegans as Model for the Study of High Glucose- Mediated Life Span Reduction. Diabetes *58*, 2450–2456.

Schulz, T.J., Zarse, K., Voigt, A., Urban, N., Birringer, M., and Ristow, M. (2007). Glucose Restriction Extends Caenorhabditis elegans Life Span by Inducing Mitochondrial Respiration and Increasing Oxidative Stress. Cell Metabolism *6*, 280–293.

Schweizer, R.A.S. (2004). Rapid Hepatic Metabolism of 7-Ketocholesterol by 11 -Hydroxysteroid Dehydrogenase Type 1: SPECIES-SPECIFIC DIFFERENCES BETWEEN THE RAT, HUMAN, AND HAMSTER ENZYME. Journal of Biological Chemistry *279*, 18415–18424.

Selkoe, D.J. (2003). Folding proteins in fatal ways. Nature *426*, 900–904.

Selman, C., Tullet, J.M.A., Wieser, D., Irvine, E., Lingard, S.J., Choudhury, A.I., Claret, M., Al-Qassab, H., Carmignac, D., Ramadani, F., et al. (2009). Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. Science *326*, 140–144.

Shen, Y., Wollam, J., Magner, D., Karalay, O., and Antebi, A. (2012). A steroid receptor-microRNA switch regulates life span in response to signals from the gonad. Science *338*, 1472–1476.

Shorter, J., and Lindquist, S. (2005). Prions as adaptive conduits of memory and inheritance. Nat. Rev. Genet. *6*, 435–450.

Stan, R., McLaughlin, M.M., Cafferkey, R., Johnson, R.K., Rosenberg, M., and Livi, G.P. (1994). Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue. J. Biol. Chem. *269*, 32027–32030.

Steffen, K.K., MacKay, V.L., Kerr, E.O., Tsuchiya, M., Hu, D., Fox, L.A., Dang, N., Johnston, E.D., Oakes, J.A., Tchao, B.N., et al. (2008). Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. Cell *133*, 292–302.

Steinkraus, K.A., Smith, E.D., Davis, C., Carr, D., Pendergrass, W.R., Sutphin, G.L., Kennedy, B.K., and Kaeberlein, M. (2008). Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in Caenorhabditis elegans. Aging Cell *7*, 394–404.

Strong, R., Miller, R.A., Astle, C.M., Floyd, R.A., Flurkey, K., Hensley, K.L., Javors, M.A., Leeuwenburgh, C., Nelson, J.F., Ongini, E., et al. (2008). Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. Aging Cell *7*, 641–650.

Suh, Y., Atzmon, G., Cho, M.-O., Hwang, D., Liu, B., Leahy, D.J., Barzilai, N., and Cohen, P. (2008). Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proc. Natl. Acad. Sci. U.S.a. *105*, 3438–3442.

Takano, A., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M., and Kobayashi, M. (2001). Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol. Cell. Biol. *21*, 5050–5062.

Tamasawa, N., Hayakari, M., Murakami, H., Matsui, J., and Suda, T. (1997). Reduction of oxysterol levels up-regulates HMG-CoA reductase activity in rat liver. Atherosclerosis *131*, 237–242.

Tamasawa, N., Tamasawa, A., Takebe, K., and Hayakari, M. (1994). The effect of dietary 7-ketocholesterol, inhibitor of sterol synthesis, on hepatic microsomal cholesterol 7 alpha-hydroxylase activity in rat. Biochim. Biophys. Acta *1214*, 20–26.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., and Garofalo, R.S. (2001). A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science *292*, 107–110.

Thomas, J.H., Birnby, D.A., and Vowels, J.J. (1993). Evidence for parallel processing of sensory information controlling dauer formation in Caenorhabditis elegans. Genetics *134*, 1105–1117.

Timmers, S., Auwerx, J., and Schrauwen, P. (2012). The journey of resveratrol from yeast to human. Aging (Albany NY) *4*, 146–158.

Tomiyama, T., Matsuyama, S., Iso, H., Umeda, T., Takuma, H., Ohnishi, K., Ishibashi, K., Teraoka, R., Sakama, N., Yamashita, T., et al. (2010). A mouse model of amyloid beta oligomers: their contribution to synaptic alteration, abnormal tau phosphorylation, glial activation, and neuronal loss in vivo. J. Neurosci. *30*, 4845–4856.

True, H.L., Berlin, I., and Lindquist, S.L. (2004). Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits. Nature *431*, 184–187.

Tullet, J.M.A., Hertweck, M., An, J.H., Baker, J., Hwang, J.Y., Liu, S., Oliveira, R.P., Baumeister, R., and Blackwell, T.K. (2008). Direct Inhibition of the Longevity-Promoting Factor SKN-1 by Insulin-like Signaling in C. elegans. Cell *132*, 1025–1038.

Vanfleteren, J.R. (1993). Oxidative stress and ageing in Caenorhabditis elegans. Biochem. J. *292 (Pt 2)*, 605–608.

Vejux, A., and Lizard, G. (2009). Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. Mol. Aspects Med. *30*, 153–170.

Vellai, T., Takács-Vellai, K., Zhang, Y., Kovács, A.L., Orosz, L., and Müller, F. (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. Nature *426*, 620.

Venn, B.J., and Green, T.J. (2007). Glycemic index and glycemic load: measurement issues and their effect on diet-disease relationships. Eur J Clin Nutr *61 Suppl 1*, S122–S131.

Viswanathan, M., Kim, S.K., Berdichevsky, A., and Guarente, L. (2005). A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. Developmental Cell *9*, 605–615.

Wadsworth, W.G., and Riddle, D.L. (1989). Developmental regulation of energy metabolism in Caenorhabditis elegans. Developmental Biology *132*, 167–173.

Wang, J., Ho, L., Qin, W., Rocher, A.B., Seror, I., Humala, N., Maniar, K., Dolios, G., Wang, R., Hof, P.R., et al. (2005). Caloric restriction attenuates beta-amyloid neuropathology in a mouse model of Alzheimer's disease.

Faseb J. *19*, 659–661.

Wang, M.C., O'Rourke, E.J., and Ruvkun, G. (2008). Fat metabolism links germline stem cells and longevity in C. elegans. Science *322*, 957–960.

Wang, Y., Oh, S.W., Deplancke, B., Luo, J., Walhout, A.J.M., and Tissenbaum, H.A. (2006). C. elegans 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. Mechanisms of Ageing and Development *127*, 741–747.

Watts, J.L., and Browse, J. (2006). Dietary manipulation implicates lipid signaling in the regulation of germ cell maintenance in C. elegans. Developmental Biology *292*, 381–392.

Weindruch, R., Walford, R.L., Fligiel, S., and Guthrie, D. (1986). The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J. Nutr. *116*, 641–654.

Williams, D.S., Cash, A., Hamadani, L., and Diemer, T. (2009). Oxaloacetate supplementation increases lifespan in Caenorhabditis elegansthrough an AMPK/FOXO-dependent pathway. Aging Cell *8*, 765–768.

Wilson, M.A., Shukitt-Hale, B., Kalt, W., Ingram, D.K., Joseph, J.A., and Wolkow, C.A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in Caenorhabditis elegans. Aging Cell *5*, 59–68.

Wolff, S., Ma, H., Burch, D., Maciel, G.A., Hunter, T., and Dillin, A. (2006). SMK-1, an essential regulator of DAF-16-mediated longevity. Cell *124*, 1039–1053.

Wolkow, C.A., Muñoz, M.J., Riddle, D.L., and Ruvkun, G. (2002). Insulin receptor substrate and p55 orthologous adaptor proteins function in the Caenorhabditis elegans daf-2/insulin-like signaling pathway. J. Biol. Chem. *277*, 49591–49597.

Wollam, J., and Antebi, A. (2011). Sterol regulation of metabolism, homeostasis, and development. Annu. Rev. Biochem. *80*, 885–916.

Wollam, J., Magner, D.B., Magomedova, L., Rass, E., Shen, Y., Rottiers, V., Habermann, B., Cummins, C.L., and Antebi, A. (2012). A novel 3-hydroxysteroid dehydrogenase that regulates reproductive development and longevity. PLoS Biol *10*, e1001305.

Wollam, J., Magomedova, L., Magner, D.B., Shen, Y., Rottiers, V., Motola, D.L., Mangelsdorf, D.J., Cummins, C.L., and Antebi, A. (2011). The Rieske oxygenase DAF-36 functions as a cholesterol 7-desaturase in steroidogenic pathways governing longevity. Aging Cell *10*, 879–884.

Wong, D., and Wang, M. (2013). Inhibitory activities of some vitamins on the formation of cholesterol oxidation products in beef patties. J. Agric. Food Chem. *61*, 8471–8476.

Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., Tatar, M., and Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature *430*, 686–689.

Yamawaki, T.M., Berman, J.R., Suchanek-Kavipurapu, M., McCormick, M., Gaglia, M.M., Lee, S.-J., and Kenyon, C. (2010). The Somatic Reproductive Tissues of C. elegans Promote Longevity through Steroid Hormone Signaling. PLoS Biol *8*, e1000468.

Yang, W., and Hekimi, S. (2010). Two modes of mitochondrial dysfunction lead independently to lifespan extension in Caenorhabditis elegans. Aging Cell *9*, 433–447.

Yoshiyama-Yanagawa, T., Enya, S., Shimada-Niwa, Y., Yaguchi, S., Haramoto, Y., Matsuya, T., Shiomi, K., Sasakura, Y., Takahashi, S., Asashima, M., et al. (2011). The conserved Rieske oxygenase DAF-36/Neverland is a novel cholesterol-metabolizing enzyme. Journal of Biological Chemistry *286*, 25756–25762.

You, Y.-J., Kim, J., Raizen, D.M., and Avery, L. (2008). Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in C. elegans: a model for satiety. Cell Metabolism *7*, 249–257.

Yu, B.P., Masoro, E.J., and McMahan, C.A. (1985). Nutritional influences on aging of Fischer 344 rats: I. Physical, metabolic, and longevity characteristics. J Gerontol *40*, 657–670.

Zhang, Y., Shao, Z., Zhai, Z., Shen, C., and Powell-Coffman, J.A. (2009). The HIF-1 hypoxia-inducible factor modulates lifespan in C. elegans. PLoS ONE *4*, e6348.

Zid, B.M., Rogers, A.N., Katewa, S.D., Vargas, M.A., Kolipinski, M.C., Lu, T.A., Benzer, S., and Kapahi, P. (2009). 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell *139*, 149–160.

ZOU, S., SINCLAIR, J., WILSON, M., CAREY, J., LIEDO, P., OROPEZA, A., KALRA, A., DECABO, R., INGRAM, D., and LONGO, D. (2007). Comparative approaches to facilitate the discovery of prolongevity

interventions: Effects of tocopherols on lifespan of three invertebrate species. Mechanisms of Ageing and Development *128*, 222–226.

Appendix

Table 1 Lifespan statistics of at least 3 biological replicates

	median	SEM	t-test	max	SEM	t-test
N2 EtOH	22,4	0,37		32,4	0,6	
N27-KC	26,1	0,82	0,0007	32,9	1,1	0,25
daf-12(rh61rh411) EtOH	18,8	1,158		28,8	0,8	
daf-12(rh61rh411) 7KC	18,4	0,2449	0,74	28,4	1,2	0,78
daf-16(mgDf50) EtOH	16,4	0,24		24,6	0,87	
daf-16(mgDf50) 7-KC	18,2	0,2	0,0005	24,6	0,87	1

Table 2 Lifespan table

If not noted differently treatmaent was start from late L4, p-value is based on log-rank (Mantel-Cox) method to compare curves. (n>60)

	treatment	**median**	**med lifespan difference(%)**	**max**	**p-value**	**referenced to (EtOH)**
N2	EtOH	24		33		
	10 µM	31	29,17	42	0,0001	N2
daf-2(e1368)	EtOH	42		52		
	10 µM	44	4,76	53	n.s.	*daf-2(e1368)*
N2	EtOH	21		29		
	5 µM	26	19,23	29	0,0001	N2
	10 µM	26	19,23	29	0,0001	N2
	40 µM	24	12,50	29	n.s.	N2
N2	EtOH	24		31		
	5 µM	26	7,69	30	0,0005	N
	10 µM from egg	26	7,69	31	0,0005	N2
	40 µM from egg	25	4,00	29	n.s	N2
N2	EtOH	23		32		
	0.01 µM	25	8,00	30	n.s.	N2
	0.1 µM	25	8,00	34	n.s.	N2
	1 µM	25	8,00	36	0,0005	N2
	10 µM	28	17,86	34	0,0001	N2
	40 µM	28	10,71	32	0,002	N2
N2	EtOH	23		31		
	0.01 µM	24	4,17	30	n.s	N2
	0.1 µM	25	8,00	33	n.s	N2

	1 µM	26	11,54	35	0,005	N2
	10 µM	28	17,86	34	0,0001	N2
	40 µM	27	11,11	32	n.s	N2

N2	EtOH	21		32		
	10 µM L4	26	19,23	32	0,0019	N2
daf-16(mgDf50)	EtOH	17		23		
	10 µM L4	19	10,53	23	n.s.	*daf-16(mgDf50)*
daf-12(rh61rh411)	EtOH	23		26		
	10 µM L4	19	-21,05	28	0,0002	*daf-12(rh61rh411)*

N2	EtOH	21		32		
	10 µM L4	21	0,00	32	0,42	N2
daf-16(mgDf50)	EtOH	16		16		
	10 µM L4	18	11,11	16	0,045	*daf-16(mgDf50)*
daf-12(rh61rh411)	EtOH	16		28		
	10 µM L4	18	11,11	24	n.s.	*daf-12(rh61rh411*)
daf-2(e1368)	EtOH	32		na		
	10 µM L4	34	5,88	na	0,01	*daf-2(e1368)*
N2		22		35		
	10 µM L4	25	13,64	36	0,009	N2
daf-16(mgDf50)	EtOH	16		26		
	10 µM L4	18	12,50	26	0,0001	*daf-16(mgDf50)*
daf-12(rh61rh411)	EtOH	18		30		
	10 µM L4	18	0,00	30	n.s.	*daf-12(rh61rh411*)
daf-2(e1368)	EtOH	35		na		
	10 µM L4	39	11,43	na	0,0001	*daf-2(e1368)*

N2	EtOH	22		35		
	10 µM L4	25	13,64	35	0,009	N2
daf-16(mgDf50)	EtOH	16		26		
	10 µM L4	18	12,50	26	0,0001	*daf-16(mgDf50)*
daf-12(rh61rh411)	EtOH	19		30		
	10 µM L4	19	0,00	30	n.s.	*daf-12(rh61rh411*)
daf-2(e1368)	EtOH	35		47		
	10 µM L4	39	11,43	48	0,0001	*daf-2(e1368)*
daf-36(k114)	EtOH	22		35		
	10 µM L4	26	18,18	36	0,01	daf-36(k114) EtOH
daf-12(rh61rh411); daf-9(dh6)	EtOH	18		29		
	10 µM L4	17	-5,56	28	n.s.	*daf-12(rh61rh411); daf-9(dh6)*
din-1(dh127); daf-9(dh6)	EtOH	23		33		
	10 µM L4	24	4,35	34	n.s.	*din-1(dh127);daf-9(dh6)*

din-1(dh127)	EtOH	22		32		
	10 µM L4	23	4,55	33	0,04	*din-1(dh127)*
N2	EtOH	23		34		
	10 µM L4	25	8,70	34	0,01	N2 EtOH
daf-16(mgDf50)	EtOH	17		26		
	10 µM L4	18	5,88	26	0,01	*daf-16(mgDf50) EtOH*
daf-12(rh61rh411)	EtOH	18		30		
	10 µM L4	18	0,00	30	n.s.	*daf-12(rh61rh411)* EtOH
daf-2(e1368)	EtOH	35		na		
	10 µM L4	38	8,57	na	0,0001	*daf-2(e1368) EtOH*
daf-36(k114)	EtOH	22		35		
	10 µM L4	26	18,18	36	0,001	daf-36(k114) EtOH
daf-12(rh61rh411); daf-9(dh6)	EtOH	18		29		
	10 µM L4	16	-11,11	28	0,001	*daf-12(rh61rh411); daf-9(dh6)*
din-1(dh127);daf-9(dh6)	EtOH	23		33		
	10 µM L4	23	0,00	34	ns	*din-1(dh127);daf-9(dh6)*
din-1(dh127)	EtOH	24		32		
	10 µM L4	25	4,17	33	0,01	*din-1(dh127)*
N2 post dauer	EtOH	23		32		
	10 µM L4	30	23,33	34	0,0001	N2 post dauer EtOH
daf-12(rh273) ost dauer	EtOH	20		32		
	10 µM L4	23	13,04	32	n.s.	*rh273* EtOH
N2 post dauer	EtOH	22		32		
	10 µM L4	27	18,52	33	0,0001	N2 post dauer EtOH
daf-12(rh273) post dauer	EtOH	20		32		
	10 µM L4	21	4,76	32	n.s	*rh273* EtOH
N2 dead OP50	EtOH	21		35		
	10 µM	26	19,23	37	0.0002	N2 on dead Op50 EtOH
N2 dead OP50	EtOH	21		34		
	10 µM	25	16,00	35	0.0005	N2 on dead Op50 EtOH

Table 3 7-KC regulated genes (p adj < 0.05) - independent of *daf-12*

Gene-Id	Gene-name	Fold Change		Description

		N2	*daf-12(rh61rh411)*	
Y49G5A.1		0,18	0,22	
Y48G9A.10	**ctp-3**	0,37	0,46	Carnitine Palmitoyl Transferase
F33D4.1	**nhr-8**	0,66	0,63	
C10C5.4		0,69	0,64	aminoacylase
D1009.1	**asc-2**	0,76	0,75	Fatty acid transport protein
T18H9.2	**asp-2**	1,32	1,33	aspartic protease
F54F11.2	**nep-17**	1,32	1,38	thermolysin-like zinc metallopeptidase
K10C2.1		1,34	1,41	
Y39D8C.1	**abt-4**	1,34	1,53	predicted ATP-binding cassette transporter
H22K11.1	**asp-3**	1,43	1,40	aspartyl protease homolog
Y39B6A.20	**asp-1**	1,47	1,43	cathepsin D aspartic protease
F28B4.3		1,52	1,47	
Y39B6A.1		1,52	1,44	
W08D2.4	**fat-3**	1,55	1,49	
F38A5.3	**lec-11**	1,56	1,67	predicted member of galectin fammily binds sugar in vitro
T01D3.6		1,60	1,45	
ZK112.1	**pcp-1**	1,64	1,67	polycarboxypeptidase (lysosmal; serine-type)
C35D10.14	**clec-5**	1,70	1,50	C-type lectin
F42A10.6		1,72	1,36	
Y54G2A.9	**clec-81**	1,72	1,75	C-type lectin
F58B4.5		1,75	1,45	
F08G5.6		1,88	1,78	
C50B6.7		2,25	1,74	
ZK455.4	**asm-2**	2,54	2,42	
C02A12.1	**gst-33**	2,68	2,66	glutathione S-tranferase
F14F7.3		2,79	3,24	cytochrome p450
K11D2.2	**asah-1**	2,87	1,93	N-Acylsphingosine Amidohydrolase
F21F8.4		3,42	3,90	aspartyl protease

E02C12.6		3,67	2,73	
T16G1.6		4,24	2,41	
T16G1.4		4,27	2,27	
C44H9.1	**ugt-15**	4,40	4,64	UDP-GlucuronosylTransferase
T16G1.7		5,72	3,00	DLC tumor supressor gene
Y46C8AL.3	**clec-70**	7,84	7,18	c-type lectin

Table 4 7-KC regulated genes (p adj < 0.05) - dependent of *daf-12*

Gene-id	Gene-name	Fold Change N2	Description
F45H7.4	**prk-2**	0,61	Pim (mamalian oncogen) Related Kinase
Y32F6B.1		0,68	
Y66A7A.6	**gly-8**	0,71	predicted polypeptide N-acetylgalactosaminyl transferase
C17G10.5	**lys-8**	1,29	lysozyme required for regualr longevity
VW02B12L.1	**vha-6**	1,32	vacuolar proton-translocating ATPase
C33C12.3	**gba-1**	1,39	β-GlucocereBrosidAse
T16G12.1		1,42	
F56D6.2	**clec-67**	2,27	C-type lectin

Table 5 Genes down-regulated in *daf-12(rh61rh41)*

Gene-ID	Fold Change	padj
M05B5.6	0,02	8,4E-11
Y48E1B.8	0,03	7,0E-11
C49G7.1	0,03	2,9E-03

Gene-ID	Fold Change	padj
ZK563.7	0,67	4,8E-02
H04D03.6	0,67	1,0E-02
Y39B6A.27	0,67	4,2E-02

T28F2.1	0,03	2,9E-15
T04C12.23	0,05	4,2E-04
Y41D4A.1	0,05	2,4E-02
C46A5.1	0,07	3,0E-23
F11G11.10	0,07	4,7E-02
Y69A2AR.14	0,08	4,1E-02
B0511.4	0,12	6,1E-07
C17D12.6	0,12	8,1E-05
T08B6.4	0,14	1,3E-04
C55C3.7	0,14	1,5E-12
F14F9.6	0,14	2,1E-03
C55C3.6	0,15	8,4E-24
B0511.3	0,15	2,4E-04
C03E10.5	0,15	1,0E-91
C12D8.18	0,16	2,7E-07
B0511.11	0,16	4,2E-35
Y47D3A.30	0,18	1,5E-05
M106.2	0,19	2,3E-115
F48C1.9	0,19	5,0E-05
F42G4.2	0,21	2,6E-10
T28F4.6	0,22	3,5E-21
T16H12.6	0,25	2,1E-02
K10G9.2	0,26	1,1E-06
C55C3.3	0,26	1,0E-25
F14E5.5	0,27	2,4E-24
F19C7.7	0,27	4,8E-02
F23H12.8	0,27	1,6E-05
T08B2.15	0,28	4,0E-02
F54C9.4	0,28	1,8E-14
Y73B6BL.7	0,28	2,7E-06

Y106G6D.5	0,67	7,5E-07
C37A2.5	0,67	2,2E-07
Y38C1AA.12	0,68	2,1E-05
Y46H3B.1	0,68	4,5E-02
T25B9.8	0,68	7,5E-05
C36F7.2	0,68	4,1E-07
T23D8.5	0,69	1,8E-02
C53B7.1	0,69	2,0E-03
T08B2.11	0,69	1,5E-03
K10G9.3	0,69	5,6E-03
C29E4.15	0,69	1,4E-02
C05C8.7	0,70	2,7E-02
M02F4.7	0,70	4,3E-02
R07B7.5	0,70	1,1E-02
Y95B8A.6	0,71	1,8E-02
K06B9.4	0,71	3,5E-03
W06A11.4	0,72	1,9E-02
Y22D7AL.9	0,72	4,0E-03
C04F6.5	0,72	1,4E-02
F55D10.1	0,73	1,1E-02
B0212.3	0,74	3,9E-03
ZK792.8	0,74	4,3E-03
F44E7.5	0,74	2,3E-02
Y37E11AR.3	0,74	6,0E-03
T10B10.3	0,74	3,6E-04
F28F8.7	0,75	3,7E-02
Y105C5B.5	0,75	6,1E-03
F54F7.2	0,76	4,2E-02
K10H10.2	0,76	1,2E-02
M110.3	0,76	2,6E-03

Y73B6BL.12	0,28	2,2E-16
C12D8.19	0,29	1,5E-02
R03D7.5	0,29	2,8E-02
Y43C5A.7	0,29	1,9E-03
T28F4.3	0,30	8,6E-05
B0238.15	0,30	4,4E-07
T05D4.5	0,31	4,7E-03
Y39G10AR.3	0,31	7,5E-09
C17H1.7	0,31	4,7E-02
T23F6.3	0,32	4,4E-04
F42G8.4	0,32	4,6E-14
F42G8.3	0,33	2,3E-08
C04C3.5	0,33	1,1E-18
W02A2.4	0,33	5,5E-03
K08D8.12	0,34	1,8E-04
F02E9.8	0,35	2,4E-02
R90.4	0,35	1,4E-02
C09G5.7	0,35	1,5E-08
F17E9.11	0,35	1,2E-02
T04D3.4	0,36	1,5E-08
F45D11.16	0,36	1,1E-07
T19C4.6	0,37	1,2E-05
F46F2.3	0,37	8,7E-05
F45D11.14	0,37	6,1E-04
C42C1.9	0,37	2,4E-05
Y71H2AM.10	0,38	1,1E-07
T21C12.8	0,39	1,8E-04
F08B4.2	0,39	5,9E-05
Y48G1BL.4	0,40	1,8E-04
F45D11.15	0,40	1,5E-02

T28F3.3	0,77	6,8E-03
Y39B6A.38	0,78	2,0E-02
ZK829.5	0,78	2,8E-02
C18H2.2	0,78	9,7E-03
M01D7.7	0,78	7,2E-03
T02G5.11	0,79	2,7E-02
C37H5.8	0,79	1,1E-02
T01C3.9	0,79	1,2E-02
K08E4.2	0,79	4,8E-02
F58H1.3	0,80	3,2E-02
M01E11.7	0,80	2,7E-02
F54E2.1	0,80	4,7E-02
F54C8.4	0,81	1,7E-02
C25A8.4	0,81	1,4E-02
T10C6.8	0,81	3,8E-02
C25H3.6	0,82	3,2E-02
B0414.3	0,82	4,1E-02
Y39B6A.17	0,54	6,5E-10
F33A8.6	0,54	5,0E-03
Y54F10BM.3	0,54	4,7E-03
H36L18.2	0,54	2,6E-06
M04F3.3	0,54	1,4E-09
R01H10.4	0,55	9,4E-05
T05G5.1	0,55	1,8E-04
Y24D9B.1	0,55	2,2E-04
Y53G8AL.1	0,55	2,2E-02
F23F1.3	0,56	4,9E-03
F35B12.7	0,56	1,8E-04
Y50D4A.6	0,56	1,7E-02
Y75B8A.35	0,56	4,9E-10

Y53G8B.3	0,41	1,9E-02
T28F2.5	0,41	6,2E-22
F42G8.5	0,42	5,8E-13
R08E5.3	0,43	6,4E-27
F23C8.8	0,43	9,4E-05
F55H2.4	0,43	3,5E-06
F53B6.4	0,44	3,7E-03
C26D10.3	0,44	1,8E-14
T15B7.16	0,45	2,3E-03
R10F2.6	0,45	1,5E-17
M106.8	0,45	4,3E-17
Y55F3AM.11	0,45	1,2E-12
B0334.9	0,46	1,4E-03
F54A3.4	0,46	1,7E-02
F42G9.7	0,47	2,5E-24
C24G6.4	0,47	6,9E-10
Y94H6A.4	0,48	1,9E-03
R01H10.5	0,48	3,8E-04
ZK596.1	0,48	2,0E-14
F23C8.7	0,48	1,4E-04
T28F4.4	0,48	5,0E-09
F22F4.5	0,50	1,6E-03
T15B7.1	0,50	1,2E-22
F11A6.2	0,50	5,9E-05
B0222.4	0,51	4,5E-06
F14H12.3	0,51	3,2E-03
C39F7.5	0,52	1,9E-08
B0218.3	0,52	2,4E-05
C38D4.10	0,52	1,4E-04
ZK507.4	0,53	2,5E-03

F28F8.10	0,56	5,3E-03
C17F4.6	0,57	1,7E-02
C28A5.6	0,57	8,0E-03
F30F8.2	0,57	3,0E-04
F28F8.2	0,58	1,6E-03
D2045.7	0,58	2,8E-05
F22H10.2	0,58	3,7E-06
F54D11.3	0,58	3,8E-09
T12F5.4	0,58	1,1E-14
F45B8.2	0,59	6,1E-04
ZK520.3	0,59	3,8E-03
Y66D12A.20	0,60	5,2E-05
F47G6.4	0,60	1,2E-07
ZK897.1	0,61	1,7E-03
F58G6.9	0,61	1,0E-06
C25D7.3	0,61	8,6E-07
M88.1	0,61	2,0E-06
ZC204.14	0,62	2,6E-04
F35C5.9	0,62	7,3E-03
F36F2.2	0,62	2,7E-04
T10D4.6	0,62	2,3E-02
T04D3.5	0,62	2,0E-05
Y57G11C.8	0,62	1,3E-03
F52E4.4	0,62	1,1E-02
F22D6.9	0,63	2,9E-02
T02G5.4	0,63	8,4E-08
C48B6.9	0,64	1,3E-04
C45G9.6	0,64	2,0E-02
W09C3.1	0,64	4,3E-03
C26H9A.2	0,64	2,3E-02

C25G4.6	0,53	3,6E-02
W05F2.8	0,53	1,7E-02
F15B9.1	0,53	1,2E-02
F13H8.8	0,53	2,2E-02
Y75B8A.28	0,53	1,6E-05
F55G1.15	0,54	3,0E-02
Y39B6A.17	0,54	6,5E-10
F33A8.6	0,54	5,0E-03
Y54F10BM.3	0,54	4,7E-03
H36L18.2	0,54	2,6E-06
M04F3.3	0,54	1,4E-09
R01H10.4	0,55	9,4E-05
T05G5.1	0,55	1,8E-04
Y24D9B.1	0,55	2,2E-04
Y53G8AL.1	0,55	2,2E-02
F23F1.3	0,56	4,9E-03
F35B12.7	0,56	1,8E-04
Y50D4A.6	0,56	1,7E-02
Y75B8A.35	0,56	4,9E-10
F28F8.10	0,56	5,3E-03
C17F4.6	0,57	1,7E-02
C28A5.6	0,57	8,0E-03
F30F8.2	0,57	3,0E-04
F28F8.2	0,58	1,6E-03
D2045.7	0,58	2,8E-05
F22H10.2	0,58	3,7E-06
F54D11.3	0,58	3,8E-09
T12F5.4	0,58	1,1E-14
F45B8.2	0,59	6,1E-04
ZK520.3	0,59	3,8E-03

R01B10.6	0,64	3,8E-09
C54D1.4	0,65	1,4E-06
F45G2.2	0,66	1,1E-02
B0336.4	0,66	2,4E-05
Y38C1AA.4	0,66	1,7E-05
C44E4.8	0,66	1,2E-02
F55G1.2	0,66	1,0E-03
ZK563.7	0,67	4,8E-02
H04D03.6	0,67	1,0E-02
Y39B6A.27	0,67	4,2E-02
Y106G6D.5	0,67	7,5E-07
C37A2.5	0,67	2,2E-07
Y38C1AA.12	0,68	2,1E-05
Y46H3B.1	0,68	4,5E-02
T25B9.8	0,68	7,5E-05
C36F7.2	0,68	4,1E-07
T23D8.5	0,69	1,8E-02
C53B7.1	0,69	2,0E-03
T08B2.11	0,69	1,5E-03
K10G9.3	0,69	5,6E-03
C29E4.15	0,69	1,4E-02
C05C8.7	0,70	2,7E-02
M02F4.7	0,70	4,3E-02
R07B7.5	0,70	1,1E-02
Y95B8A.6	0,71	1,8E-02
K06B9.4	0,71	3,5E-03
W06A11.4	0,72	1,9E-02
Y22D7AL.9	0,72	4,0E-03
C04F6.5	0,72	1,4E-02
F55D10.1	0,73	1,1E-02

Y66D12A.20	0,60	5,2E-05
F47G6.4	0,60	1,2E-07
ZK897.1	0,61	1,7E-03
F58G6.9	0,61	1,0E-06
C25D7.3	0,61	8,6E-07
M88.1	0,61	2,0E-06
ZC204.14	0,62	2,6E-04
F35C5.9	0,62	7,3E-03
F36F2.2	0,62	2,7E-04
T10D4.6	0,62	2,3E-02
T04D3.5	0,62	2,0E-05
Y57G11C.8	0,62	1,3E-03
F52E4.4	0,62	1,1E-02
F22D6.9	0,63	2,9E-02
T02G5.4	0,63	8,4E-08
C48B6.9	0,64	1,3E-04
C45G9.6	0,64	2,0E-02
W09C3.1	0,64	4,3E-03
C26H9A.2	0,64	2,3E-02
R01B10.6	0,64	3,8E-09
C54D1.4	0,65	1,4E-06
F45G2.2	0,66	1,1E-02
B0336.4	0,66	2,4E-05
Y38C1AA.4	0,66	1,7E-05
C44E4.8	0,66	1,2E-02
F55G1.2	0,66	1,0E-03
B0212.3	0,74	3,9E-03
ZK792.8	0,74	4,3E-03
F44E7.5	0,74	2,3E-02
Y37E11AR.3	0,74	6,0E-03
T10B10.3	0,74	3,6E-04
F28F8.7	0,75	3,7E-02
Y105C5B.5	0,75	6,1E-03
F54F7.2	0,76	4,2E-02
K10H10.2	0,76	1,2E-02
M110.3	0,76	2,6E-03
T28F3.3	0,77	6,8E-03
Y39B6A.38	0,78	2,0E-02
ZK829.5	0,78	2,8E-02
C18H2.2	0,78	9,7E-03
M01D7.7	0,78	7,2E-03
T02G5.11	0,79	2,7E-02
C37H5.8	0,79	1,1E-02
T01C3.9	0,79	1,2E-02
K08E4.2	0,79	4,8E-02
F58H1.3	0,80	3,2E-02
M01E11.7	0,80	2,7E-02
F54E2.1	0,80	4,7E-02
F54C8.4	0,81	1,7E-02
C25A8.4	0,81	1,4E-02
T10C6.8	0,81	3,8E-02
C25H3.6	0,82	3,2E-02
B0414.3	0,82	4,1E-02

Table 6 Genes up-regulated in *daf-12(rh61rh411)*

Gene-ID	Fold Change	padj

Gene-ID	Fold Change	padj

D1005.1	1,22	4,2E-02
T08H10.1	1,22	4,1E-02
Y39G8B.1	1,23	4,8E-02
R57.1	1,23	4,9E-02
Y76A2B.3	1,24	2,9E-02
Y71H2AM.13	1,24	2,9E-02
Y43F8C.13	1,25	4,6E-02
T23F2.5	1,25	2,3E-02
K07E3.3	1,25	3,4E-02
D1009.1	1,25	1,8E-02
W02A11.3	1,25	3,0E-02
M6.1	1,26	4,2E-02
K09F5.3	1,26	9,2E-03
T08A9.9	1,26	7,7E-03
F11A3.1	1,26	3,8E-02
F52E4.1	1,26	8,0E-03
K07E3.8	1,26	2,1E-02
C26B9.5	1,27	2,4E-02
F59A3.1	1,27	3,0E-02
C15C7.5	1,27	3,4E-02
F59F4.4	1,27	8,6E-03
T07C12.7	1,27	9,0E-03
Y55B1AR.1	1,28	2,8E-03
T13H2.5	1,28	7,8E-03
H06O01.3	1,28	1,6E-02
B0286.3	1,28	1,3E-02
F18E2.1	1,28	8,4E-03
T03D8.6	1,29	4,9E-02
Y37E3.16	1,29	4,3E-02
K12B6.1	1,29	1,5E-02
Y53F4B.33	1,64	5,9E-05
Y54G2A.29	1,65	4,0E-04
Y34F4.2	1,65	5,2E-03
F22E10.1	1,65	1,9E-05
T05E7.1	1,66	9,5E-03
W07G9.2	1,66	5,9E-06
F25H8.5	1,66	3,9E-03
K04H4.6	1,66	7,9E-05
C44F1.1	1,66	1,1E-03
T15B7.3	1,66	2,2E-06
F57B1.4	1,67	1,2E-02
R09B5.4	1,67	8,2E-03
C06G4.5	1,67	3,8E-02
Y45F10A.4	1,67	1,1E-07
C08E8.10	1,68	3,1E-02
F43C1.5	1,68	2,5E-02
C25E10.10	1,68	4,5E-05
F37H8.3	1,69	3,8E-03
F31C3.4	1,69	1,9E-14
M153.2	1,70	5,8E-03
T02B5.3	1,72	6,7E-04
ZC84.3	1,72	2,6E-06
R11G11.2	1,72	3,1E-02
F48C1.1	1,73	4,5E-05
C39E9.7	1,73	3,3E-03
Y55F3C.9	1,74	2,0E-02
F38B7.3	1,74	5,3E-04
C32D5.7	1,74	2,2E-03
Y19D10A.11	1,74	9,5E-03
T28H10.2	1,75	8,0E-03

K10C2.1	1,29	1,4E-02
C17G10.5	1,29	3,8E-03
T07C4.5	1,29	3,2E-02
F49E12.2	1,29	7,4E-03
C16B8.3	1,29	8,0E-03
T01E8.3	1,29	4,7E-02
Y6G8.3	1,29	2,6E-02
F36F12.8	1,29	1,7E-02
R12E2.5	1,29	2,0E-02
C17G1.2	1,30	1,0E-02
T04B2.5	1,30	2,3E-02
W10G6.2	1,30	1,9E-02
B0034.3	1,30	9,2E-03
C49C3.4	1,31	6,9E-03
F42A10.6	1,31	1,9E-02
R07E4.3	1,31	3,3E-02
F22F4.4	1,31	1,1E-02
F28D1.5	1,31	1,2E-02
F31C3.3	1,31	9,3E-04
T07C4.4	1,31	6,8E-03
Y37E11AM.2	1,32	6,2E-03
C49C3.1	1,32	3,3E-02
K02F3.4	1,32	9,8E-04
K04C1.5	1,32	1,9E-03
K06G5.1	1,32	1,1E-03
H34I24.2	1,32	8,7E-04
T08B1.6	1,32	4,2E-02
F55A8.1	1,32	4,0E-02
W01A11.4	1,32	3,9E-04
F56F10.1	1,32	1,5E-03

W01F3.2	1,75	1,6E-09
F28D1.9	1,75	4,3E-02
F26G1.2	1,75	4,5E-04
F22E5.1	1,75	3,0E-05
ZK652.8	1,76	1,9E-02
Y56A3A.16	1,76	3,7E-16
R10H10.5	1,76	3,5E-03
T16G1.7	1,76	5,1E-03
C01B4.9	1,76	1,3E-08
Y19D10A.12	1,76	1,2E-07
K11G12.1	1,77	3,0E-12
T25B9.7	1,78	2,6E-02
H17B01.3	1,78	1,5E-02
Y69H2.14	1,79	4,5E-02
F08G5.5	1,79	2,7E-02
K02D7.3	1,79	6,6E-15
R07E3.1	1,80	5,7E-15
C23F12.1	1,80	3,9E-07
T11F9.3	1,81	2,8E-11
K07A1.6	1,82	3,1E-04
F41E6.2	1,82	1,5E-04
F16B4.12	1,82	8,3E-03
Y69A2AR.12	1,84	4,8E-02
R06B9.6	1,84	3,8E-17
F38A1.1	1,85	2,8E-02
F39E9.2	1,85	4,8E-02
F55G11.8	1,86	4,2E-02
F47C10.6	1,86	6,1E-03
Y19D10B.6	1,87	7,0E-03
E02H4.7	1,87	1,1E-04

F32A5.3	1,33	3,7E-03
JC8.14	1,33	1,3E-03
R10H10.3	1,33	1,7E-03
Y54G2A.6	1,33	1,2E-02
C13B7.6	1,33	2,0E-03
C02D5.4	1,33	3,3E-03
C35C5.10	1,33	1,1E-02
Y82E9BR.16	1,33	6,5E-04
Y48C3A.4	1,33	4,1E-02
F55A4.8	1,33	4,2E-02
F37C4.6	1,34	3,4E-02
T01D3.6	1,34	1,6E-03
Y58A7A.3	1,34	4,9E-02
T02C5.1	1,34	5,0E-04
F17A9.4	1,34	1,4E-02
ZK112.1	1,34	6,2E-03
F20D6.11	1,35	1,5E-02
F36G3.2	1,35	4,1E-02
T07C4.12	1,35	1,2E-02
T26C5.1	1,35	4,0E-03
K08F8.1	1,35	1,0E-04
F01D4.2	1,35	1,4E-03
Y66H1A.5	1,35	2,8E-02
Y69F12A.2	1,35	3,5E-04
D1037.3	1,36	7,8E-05
C01B10.6	1,36	2,0E-04
Y45G12C.16	1,36	9,2E-04
Y53F4B.45	1,36	2,5E-02
T27A10.7	1,36	5,1E-03
C06H5.6	1,36	1,9E-03

F56B3.1	1,88	3,4E-02
T06E6.10	1,88	7,3E-11
C46F2.1	1,88	1,6E-05
ZC455.6	1,88	2,6E-06
F09B12.3	1,89	2,1E-11
C30G7.4	1,89	3,6E-03
Y110A2AL.9	1,89	3,5E-02
F56A4.12	1,90	5,0E-03
C30G12.2	1,90	7,7E-03
C56A3.2	1,91	2,0E-08
F59A1.7	1,92	7,7E-07
C29F3.7	1,92	5,0E-10
C05E4.1	1,93	2,7E-05
F33H2.6	1,93	2,2E-20
W05E7.1	1,94	8,0E-05
C52D10.1	1,94	1,0E-05
ZC455.5	1,95	1,4E-07
K10D3.6	1,95	4,1E-03
F46G11.3	1,95	3,4E-12
K07C6.5	1,96	1,2E-06
F35E12.7	1,97	1,4E-04
F56C3.9	1,97	8,0E-03
Y56A3A.19	1,97	1,2E-24
T12A7.4	2,01	1,4E-03
F14F7.1	2,02	3,0E-02
ZK1290.14	2,02	6,7E-06
B0454.5	2,02	1,7E-06
K10C2.3	2,03	1,4E-15
R06B10.2	2,05	1,7E-02
ZK455.4	2,05	1,7E-03

F26E4.12	1,36	1,4E-02
T28D6.7	1,36	1,5E-02
K11D12.3	1,36	5,1E-03
C51E3.6	1,37	4,6E-02
Y48B6A.6	1,37	1,5E-03
D1037.2	1,37	6,9E-03
K08C7.1	1,37	3,4E-02
R11D1.11	1,38	3,3E-03
Y71H9A.3	1,38	1,6E-03
T06A1.5	1,38	1,2E-02
R09H10.5	1,38	3,4E-04
F43H9.4	1,38	4,6E-02
T20B3.1	1,38	3,6E-02
C28D4.9	1,38	7,7E-03
Y105C5B.28	1,38	8,1E-03
K09E3.5	1,39	3,5E-02
R102.4	1,39	3,1E-04
R12B2.1	1,39	3,7E-03
Y4C6B.6	1,39	4,3E-02
C33A11.1	1,39	2,7E-02
T09A5.2	1,39	8,6E-03
F13H6.3	1,40	7,7E-04
Y54E10A.17	1,40	2,4E-02
E04F6.15	1,40	2,1E-02
M28.10	1,40	8,8E-05
ZK6.11	1,40	9,7E-05
T27E4.4	1,40	1,3E-03
Y5F2A.2	1,40	2,4E-04
Y54F10AM.8	1,41	2,0E-05
C28A5.3	1,41	1,5E-02

ZK337.2	2,06	3,1E-06
F45E1.4	2,07	1,3E-04
Y43D4A.10	2,07	4,2E-02
Y19D10A.4	2,08	1,1E-07
ZK337.5	2,08	3,0E-04
T01G5.8	2,09	1,2E-05
Y6G8.2	2,09	2,9E-03
C31B8.8	2,10	1,6E-15
F31C3.6	2,11	8,6E-07
K09E3.6	2,11	2,8E-03
M01A8.1	2,12	5,5E-04
ZC513.8	2,12	1,7E-03
C01B4.7	2,13	4,2E-08
C50B6.7	2,15	3,4E-15
C04H5.2	2,15	1,4E-08
K03H1.5	2,16	1,8E-11
F52F10.3	2,17	2,6E-02
F31C3.1	2,17	1,0E-33
T07A9.7	2,17	1,0E-02
K08C7.2	2,20	4,0E-07
F46H5.8	2,20	2,4E-09
C18H9.5	2,21	1,9E-03
F01D5.5	2,22	9,5E-03
H23N18.3	2,22	8,9E-03
Y52E8A.3	2,22	2,0E-02
F08H9.5	2,23	3,3E-02
T19D2.1	2,26	6,4E-03
C40H1.8	2,26	1,3E-03
F01D5.1	2,29	1,8E-04
C17H12.8	2,31	6,4E-28

F54F11.2	1,41	3,6E-05
C07B5.5	1,41	6,1E-03
F08A8.4	1,41	8,9E-05
ZK105.1	1,41	3,3E-04
F23B2.11	1,42	3,4E-04
F55B11.2	1,42	5,6E-03
F31C3.5	1,42	5,6E-03
W05H9.1	1,42	2,1E-02
AH9.3	1,42	2,7E-03
ZC376.2	1,42	3,5E-03
T01C8.5	1,42	4,7E-04
Y48A6B.7	1,42	6,8E-04
F55D12.2	1,43	8,4E-03
F13D11.1	1,43	3,0E-02
C33E10.4	1,44	3,6E-02
Y105C5A.15	1,44	3,3E-03
R08E3.1	1,44	4,5E-05
Y105C5A.24	1,44	1,7E-02
F32A5.5	1,44	3,2E-04
ZK121.2	1,44	1,3E-03
B0228.7	1,44	5,6E-05
F52H3.1	1,45	1,4E-03
VC5.3	1,45	3,5E-06
H32C10.2	1,45	3,7E-02
C12C8.2	1,45	1,6E-05
Y34B4A.5	1,45	6,3E-03
Y37A1B.17	1,46	4,3E-03
C05G6.1	1,46	1,5E-02
C44E12.1	1,47	4,2E-02
C01B7.4	1,47	8,8E-03

D2096.3	2,32	2,8E-24
F34D10.6	2,33	8,8E-04
F25A2.1	2,35	2,0E-02
T04C12.7	2,37	4,1E-02
T21E8.3	2,37	1,2E-02
D1044.7	2,40	3,5E-03
K11D2.2	2,42	1,6E-09
Y38E10A.14	2,43	4,1E-08
Y45G12C.2	2,45	6,1E-10
Y69A2AR.5	2,46	1,9E-03
B0331.1	2,48	2,1E-04
F09D12.1	2,54	4,3E-02
F15B9.6	2,55	3,6E-02
Y39B6A.47	2,57	1,4E-11
K04F1.9	2,57	9,0E-07
R11G11.14	2,59	9,0E-03
T22F7.4	2,59	1,1E-05
ZK1251.2	2,59	1,0E-02
C24B9.9	2,60	1,7E-05
Y19D10A.16	2,60	1,5E-25
ZK909.6	2,61	1,4E-19
Y41C4A.19	2,65	3,0E-05
F49F1.7	2,67	9,4E-04
C06E4.3	2,72	2,0E-03
Y53F4B.32	2,74	3,3E-02
Y75B12B.6	2,80	1,5E-34
Y17D7A.4	2,80	6,8E-03
C01B4.6	2,85	4,2E-30
T24B8.5	2,87	1,9E-02
C32H11.4	2,90	4,3E-07

T05C3.6	1,47	5,1E-03
F07D10.1	1,47	9,7E-03
T25B6.2	1,47	5,9E-06
F21D5.3	1,47	1,4E-04
F33H2.2	1,48	1,2E-07
F28B4.3	1,48	4,0E-08
ZK682.2	1,48	1,0E-04
B0238.1	1,48	1,3E-02
ZK813.2	1,48	9,2E-08
F46G10.2	1,49	2,1E-03
M60.5	1,49	1,3E-02
F41F3.2	1,49	2,6E-03
F59A1.10	1,50	8,1E-03
ZC239.6	1,50	4,4E-02
C18B2.5	1,51	4,3E-08
T26C12.6	1,52	3,0E-04
ZC455.4	1,52	4,9E-02
T07D3.4	1,52	3,4E-02
F55F3.2	1,52	9,8E-04
B0213.4	1,52	5,1E-03
C49F5.2	1,53	8,1E-04
T12B5.15	1,53	1,8E-02
Y44A6E.1	1,53	1,6E-02
C35C5.9	1,53	3,9E-02
M04G12.2	1,54	2,6E-08
F42A9.6	1,54	5,7E-03
C41C4.10	1,54	1,7E-04
F33H2.7	1,54	6,3E-06
C29F9.3	1,54	2,2E-03
F31D5.5	1,54	1,1E-02

H25K10.1	2,92	2,4E-06
C16D9.6	2,93	3,2E-17
ZK455.5	2,96	9,1E-06
F58F12.4	2,98	3,7E-06
F56A4.2	3,04	1,6E-34
F59A1.8	3,05	9,8E-16
Y19D10A.9	3,07	2,8E-29
ZK337.1	3,09	3,2E-09
C54F6.14	3,13	1,6E-03
F56D6.2	3,23	5,1E-11
T04H1.9	3,37	7,4E-13
F08D12.2	3,37	1,3E-10
F56A4.3	3,59	2,0E-10
R52.9	3,64	1,1E-04
T22B7.7	3,66	2,0E-13
W01B11.5	3,67	8,3E-15
F11A5.12	3,76	2,1E-03
T07A5.1	3,91	2,9E-05
T03D8.7	4,04	1,1E-07
Y46G5A.13	4,09	5,5E-81
C05C10.4	4,18	5,2E-70
Y38E10A.15	4,25	5,2E-05
F55G11.5	4,31	1,9E-07
C33B4.4	4,37	1,2E-34
Y48G8AL.11	4,38	1,3E-79
F35F10.13	4,40	3,8E-04
C06E1.7	4,41	2,1E-03
C24G7.2	4,42	8,7E-05
W09G12.7	4,51	6,4E-28
F37B1.3	4,51	1,4E-03

B0365.6	1,55	8,1E-09
F08A8.2	1,55	2,2E-03
F49E2.2	1,55	1,5E-07
C07H4.1	1,55	3,5E-02
F52H3.5	1,55	7,5E-04
F59E11.11	1,56	1,4E-02
K09A11.2	1,56	1,8E-02
Y37D8A.3	1,56	1,8E-05
Y37D8A.4	1,56	2,9E-08
T16G12.1	1,57	1,1E-08
T28C6.1	1,57	3,8E-09
T08B1.4	1,57	5,9E-03
C35D10.14	1,57	9,8E-04
F35B12.2	1,57	1,8E-03
Y22F5A.1	1,58	3,2E-02
F57B9.1	1,59	6,7E-07
F49E11.10	1,59	5,7E-03
F14B8.6	1,59	4,5E-05
ZK6.10	1,60	5,3E-11
M01F1.5	1,60	3,5E-07
C05D12.3	1,61	2,3E-03
R04E5.10	1,61	3,8E-04
C09G5.5	1,61	1,3E-02
ZK112.7	1,61	4,0E-05
F33H2.3	1,61	5,1E-11
T19H12.10	1,61	3,5E-03
C29F7.2	1,62	3,4E-07
C10C5.4	1,62	5,4E-08
T06C12.6	1,62	1,4E-02
F38B7.2	1,62	2,3E-02

Y41C4A.16	4,54	1,3E-25
F55G11.4	4,67	1,0E-06
T15B7.5	4,75	4,1E-07
F08E10.7	4,93	1,6E-03
W05E7.3	5,08	4,7E-10
F43E2.6	5,10	2,6E-18
K08H10.6	5,15	2,3E-04
F37F2.3	5,32	1,2E-03
F57G4.4	5,52	5,1E-12
F56D3.1	5,87	4,1E-15
F21C10.11	6,05	5,6E-20
F08G5.6	6,28	1,5E-54
C54D10.14	7,14	1,1E-02
F28G4.5	7,43	5,0E-02
T20D4.7	9,09	3,8E-04
C45B2.3	9,56	2,3E-03
ZC373.7	10,32	5,7E-11
K09C8.4	10,33	2,0E-19
F57G4.8	10,79	4,0E-56
F56A4.9	12,93	9,8E-15
M05B5.1	13,08	1,6E-15
F59A1.9	13,85	8,7E-70
F58D7.1	14,78	2,4E-04
Y19D10A.7	14,86	5,0E-06
F44C4.2	16,17	1,8E-05
C33G8.2	21,35	3,0E-93
Y5H2A.4	22,81	7,0E-08
Y38C1AB.6	35,43	1,8E-56
Y38C1AB.2	41,27	4,6E-57
F15E11.10	42,41	9,1E-10

T20D4.6	1,63	9,7E-03
C53D5.1	1,63	1,5E-08
F35E12.5	1,63	1,1E-03
M02D8.5	1,64	1,8E-02

C54F6.4	44,76	1,3E-10
Y5H2A.3	67,67	8,6E-09
F57H12.6	89,14	2,5E-24
C33G8.3	97,09	1,1E-126

Erklärung:

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Die von mir vorgelegte Dissertation ist von Prof. Dr. Adam Antebi betreut worden.

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