KLF-1 mediates mitohormesis by orchestrating a xenobiotic detoxification response essential for longevity in *C.elegans*

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To my family and friends

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Abbreviations

°C	degree Celsius
3'	three prime end of DNA sequence
5'	five prime end of DNA sequence
A	alanine
AA	Antimycin A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CGC	*Caenorhabditis* Genetics Center
cm	centimeter
CR	caloric restriction
D	aspartic acid
D1	day one of adulthood
D5	day five of adulthood
DCFDA	2',7' –dichlorofluorescin diacetate
DEPC	diethylpyrocarbonate
DME	drug metabolizing enzyme

DNA	desoxyribonucleic acid
dNTPs	deoxynucleotides
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
Fw	forward
g	g force
GFP	green fluorescent protein
GSH	glutathione
h	hour
H_2O_2	hydrogen peroxide
HCl	hydrochloric acid
$HO^{\bullet}$	hydroxyl radical
IIS	insulin/IGF-1 like signaling
IMM	inner mitochondrial membrane
IMS	inter-membrane space
IPTG	isopropyl-β-D-thiogalactopyranosid
K_2HPO_4	dipotassium phosphate
KH_2PO_4	monopotassium phosphate

KPI	phosphate buffer
L	liter
M	molar
MAPK	mitogen-activated protein kinase
MES	2- [N-morpholino] ehtanesulfonic acid
$MgCl_2$	magnesium chloride
$MgSO_4$	Magnesium sulfate
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MRC	mitochondrial respiratory chain
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtUPR	mitochondrial unfolded protein response
Na_2HPO_4	disodium phosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
nDNA	nuclear DNA
ng	nanogram

NGM	nematode growth medium
nm	nanometer
NOX	NADPH oxidases
NP-40	Nonidet P40 Biochemica
$O_2^{\bullet-}$	superoxide anion radical
OD595	optic density at 595 nm
OH^-	hydroxyl anion
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PQ	Paraquat (1,1'-Dimethyl-4,4'-bipyridinium dichloride)
PTP	protein tyrosine phosphatase
qPCR	quantitative polymerase chain reaction
RC	respiratory chain
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	revolutions per minute
Rv	reverse
S	serine

SDS	sodiumdodecylsulfate
SEM	standard error of the mean
t-Test	Students t-Test
TCA	tricarboxylic acid
TIM	translocator or the IMM
TOM	translocator of the OMM
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiole
UV	ultra violet
vol/vol	volume per volume
wt	wild type
µg	microgram
µl	microlitre
µm	micrometer

Abstract

Most manipulations that extend lifespan also increase resistance to stress in different organisms from yeast to mammals. In fact, mild mitochondrial dysfunction is able to extend lifespan in *C.elegans* by inducing the activation of an adaptive stress response, which results in increased stress resistance and fitness of the nematodes in a process called mitohormesis. However, little is known about the molecular mechanisms underlying the regulation of stress resistance during aging. Here, we identify Krüppel-like factor 1 (KLF-1) as the transcription factor responsible for mediating the mitohormetic activation of a cytoprotective response essential for longevity. Upon mild mitochondrial dysfunction and oxidative stress, KLF-1 is activated through redox regulated steps and a MAPK signaling cascade, leading to its translocation into the nucleus where it activates genes involved in the xenobiotic detoxification program. Xenobiotic detoxification is an important cytoprotective response upregulated by many lifespan-extending pathways across species. Here we show that phase I cytochrome P450 oxidases (CYPs) are major targets of KLF-1 in different longevity models like mitochondrial dysfunction or insulin/IGF-1 signaling disruption, and that CYPs are able to prime the activation of phase II SKN-1-regulated targets, which collectively result in enhanced cytoprotection. Our results underline the relevance of xenobiotic detoxification in longevity assurance and identify KLF-1 as a key novel factor orchestrating this response.

Zusammenfassung

Die meisten Manipulationen, welche in unterschiedlichen Organismen von Hefen bis Säugtieren die Lebensspanne verlängern, erhöhen gleichzeitig auch die Resistenz gegenüber vielen Stressfaktoren. Eine milde mitochondriale Dysfunktion etwa, ist durch Aktivierung einer adaptiven Stressantwort ausreichend, um die Lebensspanne von *C.elegans* zu verlängern. Dieser Signalweg, welcher zu einer erhöhten Stressresistenz und Fitness führt, wird Mitohormesis genannt. Die molekularen Mechanismen, die dieser Stressresistenz während des Alterns zu Grunde liegen, sind bislang unbekannt. In der vorliegenden Arbeit wurde KLF-1 als der Transkriptionsfaktor identifiziert, welcher verantwortlich ist für die hormetische Aktivierung der zellschützenden Antwort, welche essenziel für die Langlebigkeit ist. Bei einer milden mitochondrialen Dysfunktion und oxidativen Stress, wird KLF-1 durch eine redox-regulierte MAPK Signalkaskade aktiviert. Diese Aktivierung führt zu der Translokation von KLF-1 in den Zellkern, wodurch die Expression von xenobiotischen Detoxifikationsgene induziert wird. Proteine des xenobiotischen Detoxifikationsprogramms sind Teil eines wichtigen zellschützenden Mechanismus, deren Expression durch viele lebensverlängernde Maßnahmen in unterschiedlichen Spezies erhört ist. Hier zeigen wir, dass Phase I Zytochrom P450-Oxidasen (CYPs) Zielgene von KLF-1 in verschiedenen Langlebigkeitsmodellen wie z.B. mitochondriale Dysfunktion und Insulin/IGF-1 Signalisierung sind. Des Weiteren können CYPs die Aktivierung von Phase II Enzymen, deren Expression von SKN-1 reguliert wird, verstärken, sodass beide Signalwege gemeinsam den Zellschutz sicherstellen. Unsere Ergebnisse unterstreichen die Relevanz der xenobiotischen Detoxifikation in der Determinierung der Langlebigkeit und identifizieren KLF-1 als neuen und essentiellen Transkriptionsfaktor, der diese Antwort kontrolliert.

1. Introduction

1.1. The aging process

Medical advances have been able to reduce infant mortality, fight disease and infection altogether leading to a general increase of life expectancy and longevity. A consequence of this advance is an increasing senior population and a higher frequency of age-related diseases. The aging process is characterized by a progressive and generalized impairment of function of the organisms that renders them vulnerable to disease and ultimately to death.

Why do we age? When does the aging process start? Why do some people live longer than others? Why do some suffer from age related diseases while others seem to undergo a very healthy aging? Is there a limit for longevity? There is millions of question that one could ask regarding the intriguing topic of aging and there is not a unique answer to all of them. Aging appears to be determined by several genetic, epigenetic and environmental factors: despite of great advances made in the aging research field, little is known about the control and regulation of this fascinating process.

1.1.1. Aging theories

Historically, researches have postulated many different aging theories that can be mainly divided into two categories: programmed and damage/error: The programmed theories hypothesize that aging is determined by a biological timetable, similarly to the regulation of biological changes that happen during the growth and development of the organisms from childhood to adulthood. Hence this regulation would be dependent on gene-expression-changes over time. Conversely, the damage or error theories imply that the accumulation of damage on the organisms caused by various environmental assaults during life is the cause of aging. None of these theories have been able to fully explain the aging process but it is necessary to consider all the possible factors proposed to have an impact

on aging, in order to get a better understanding of it's function. Therefore, some of the most important aging theories are reviewed down bellow.

The programmed theories

a) Programmed Longevity: this theory postulates that aging is the result of the senescence of the sequential switching of gene-expression during life, which is characterized by the manifestation of age-associated pathologies. Hence the genomic (in)stability of each organism would be key for the progress of the aging process (reviewed in(Davidovic et al., 2010)).

b) Endocrine Theory: This other programmed theory proposes that aging is regulated by hormones that act as a biological clock. The pathway that has been most extensively studied is the insulin/IGF-1 signaling (IIS) pathway. It was first described in *C.elegans* 1988 with the identification of *age-1* mutation (Friedman and Johnson, 1988), followed by the description of *daf-2* and *daf-16* (Kenyon et al., 1993) and it has been shown to be evolutionary conserved (reviewed in(van Heemst, 2010)).

c) Immunological Theory: This third theory implies that the function of the immune system is programmed to decline over time, entailing an increased vulnerability to infection, disease and lastly death. The immune system has been extensively studied and it is well established that it undergoes a maturation process from a pre- and perinatal phase until reaching it's peak around puberty and declining gradually with age. The decrease of hematopoietic stem cells in the bone marrow and replacement by adipose tissue together with the thymic atrophy are some of the features characteristic of the age-associated decline of the immune system (reviewed in(Gruver et al., 2007)).

d) Telomere theory: It was already described in the early sixties that cells simply stop dividing after a certain number of divisions (Hayflick and Moorhead, 1961). Molecular explanation for this restrain of divisions was based on the telomeres, which have been experimentally shown to shorten in each successive division until reaching a critical length were cells stop replicating eventually leading to cell death and progressively the death of the organism (reviewed in(Campisi, 2000)). Certain cells can restore their telomeres thanks to the enzyme telomerase, whose discovery was awarded with the Nobel Prize for Physiology and Medicine in 2009 (Greider and Blackburn, 1985). However telomerase activity is found to be absent in most adult human somatic cells and present in cancerous and immortalized cell lines (Kim et al., 1994; Shay and Bacchetti, 1997). Moreover telomere shortening has been implicated with disrupted neuronal differentiation and neuritogenesis (Ferron et al., 2009).

The damage or error theories

a) Wear and tear theory: A. Weismann proposed this damage theory already in 1882. He postulated that the building blocks of the organisms (cells, tissues, organs) loose their vitality due to repeated use. At some point in life the components start wearing out leading to dysfunction, disease and death (Weismann, 1882)

b) Cross-linking theory: proposes that during aging there is an accumulation of crossed-liked proteins in the cells that leads to damage and dysfunction of the tissues (Bjorksten, 1968).

c) Rate of living theory: this theory is frequently summarized with the phrase “live fast- die young”. It is based on early work of Rubner, Loeb

and Northrop (Loeb and Northrop, 1917; Rubner, 1908) but it wasn't formally postulated as the rate-of-living theory until 1928 (Pearl, 1928). This theory postulates that metabolic rate and lifespan are inversely correlated because a "vital component" (energy) is consumed during life (Pearl, 1928). In agreement with this, the rate-of-living theory suggests that long-lived organisms should have a lower basal metabolic rate than their shorter-lived counterparts.

d) Free radicals theory: proposes that reactive oxygen species (ROS), like superoxide or hydrogen peroxide, can damage macromolecules resulting in an impairment of their function. Damage accumulation results eventually in the dysfunctions of the cells and organs (Gerschman et al., 1954; Harman, 1956). This theory has been closely related to the above-described rate-of-living theory, suggesting that the metabolic rate could determine the ROS production, thus affecting longevity. Understanding the role of ROS function is a key part of our research and therefore we will deepen this theory as well as other aspects of ROS in a forthcoming section.

e) Somatic DNA damage theory: DNA damage occurs continuously and at a very high rate in cells of living organisms (Lindahl and Nyberg, 1972). While most of these damages are repaired, some accumulate, mostly due to dysfunction of cellular DNA checkpoints (Bartek et al., 2007). Hence, DNA damage occurs and accumulates with increasing age, causing cells to deteriorate and malfunction.

1.1.2. Current hallmarks of aging

Neither of the above listed theories appears to be fully satisfactory explaining the aging process. This strict division in too distinct and practically

excluding theories is a quite outdated point of view and just a comprehensive and unifying approach can help us to really understand aging.

López-Otín and colleagues attempted to identify and categorize the cellular and molecular hallmarks of aging (Lopez-Otin et al., 2013). They propose nine hallmarks that are considered generally as contributors to the aging process and determiners of the aging phenotype.

All these hallmarks should ideally fulfill the following criteria (i) manifest during normal aging; (ii) its experimental intensification should accelerate aging; and (iii) its experimental amelioration should postpone aging and hence increase healthy lifespan. Of course the key concepts or ideas behind the definition of these hallmarks are nothing new but based on all the theories named above from a conciliatory point of view: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction (7) cellular senescence (8) stem cell exhaustion and (9) altered intercellular communication.

The most interesting fact about this approach for the understanding of aging is the fact that it considers the hallmarks to be extensively interconnected, implying that the intervention or amelioration of any of them will impact the others classifying them in three categories: primary, antagonistic and integrative hallmarks (Figure 1.1). The primary hallmarks are implicitly negative like DNA damage, mitochondrial DNA mutations, loss of proteostasis or telomere shortening. They could be considered as triggers of aging that accumulate over time. The antagonistic hallmarks, in contrast, have different effects based on their intensities: at low levels they may be beneficial but after reaching a certain threshold they become deleterious: ROS as signal molecules vs. ROS theory of aging, nutrient deprivation as a pro-longevity intervention or pathological if prolonged over time etc. They are initially beneficial but they become progressively negative influenced by the primary hallmarks. The third and last category includes the integrative hallmarks that affect tissue homeostasis and function. Based on the fact that all these hallmarks co-occur during aging and are intricately linked, understanding their exact causal network is the challenge of the

future aging research, so that specific medical and pharmaceutical interventions can ultimately lead to a healthier aging.

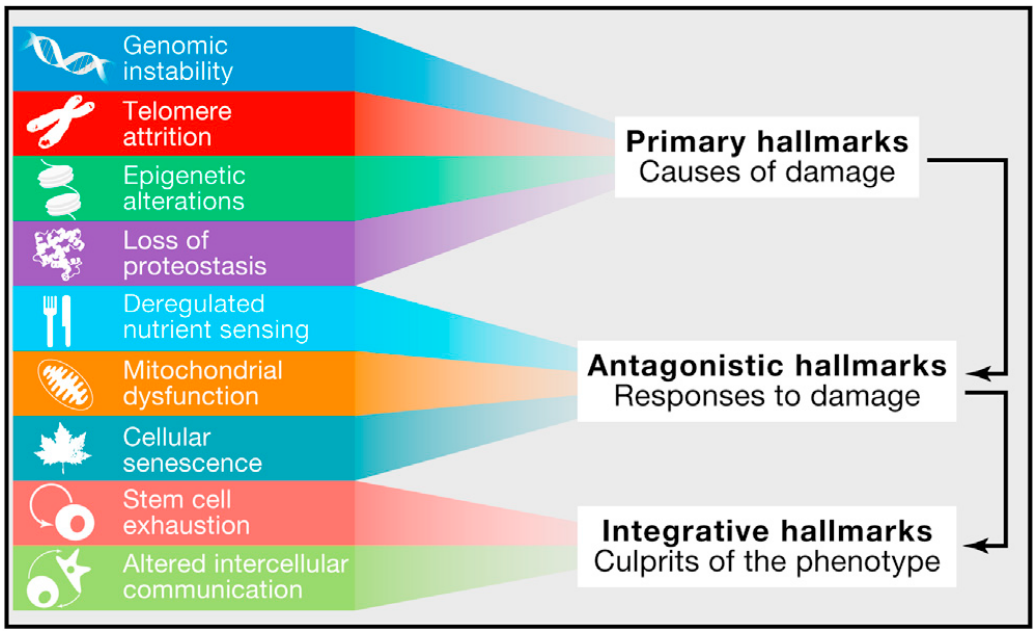

Figure 1.1. The hallmarks of aging
Model of the nine hallmarks of aging and their relationship proposed by López-Otín and colleagues (Lopez-Otin et al., 2013).

Being well aware of the complexity of the field we focus our research interest in understanding the role of mitochondria in the aging process, since they are implicated in a more or less direct way in many of the above introduced aging determining factors: they supply energy to the cells and regulate metabolism, control cell-cycle and cell death, are implicated in antiviral response and are major ROS among many other features (McBride et al., 2006). The following section will focus on dissecting the key features of mitochondria and their implication in the aging field.

1.2. Free radicals and ROS

Over a century ago, free radicals were first described (Gomberg, 1900) and for quite long time there were not even considered to occur in biological systems probably due to their high reactivity and therefore short half-life. A few decades later, free radicals were proposed to be mediators of all oxidation reactions involving organic molecules (Michaelis, 1939). Despite the fact that this statement was mostly wrong, it highly incremented the interest in understanding the role of free radicals in biological processes. As a result, in the early 1950s free radicals were found in "biological materials" (Commoner et al., 1954) and rapidly related to pathological processes and aging theories (Gerschman et al., 1954; Harman, 1956). Since then, our knowledge about free radicals in biological processes has grown considerably. Remarkably, free radicals have been mostly suggested to play deleterious roles as sources of molecular damage. The discovery of specialized protective enzymes like super oxide dismutases (McCord and Fridovich, 1969), robustly strengthened this idea. Nonetheless, this perspective was strikingly challenged by the implication of free radicals in beneficial processes like combating infection (Babior et al., 1975; Babior et al., 1973; Britigan et al., 1987; Rossi et al., 1985) or vasodilation (Furchgott and Zawadzki, 1980; Palmer et al., 1987) among others. It is clear now that free radicals are more than just damaging agents, but mediators of physiological processes. However detailed understanding of the molecular mechanisms in charge as well as to which extend and in how many processes they are involved are key questions that remain been studied.

Quite often the terms "free radicals" and "reactive oxygen species" (ROS) are used interchangeably, however, this is not always correct and discriminating both is important: The reduction of oxygen to water can generate different reactive intermediates (ROS), but not all of them are free radicals: under aerobic conditions most of the oxygen is reduced to water in the electron transport chain (ETC) via a four-electron mechanism without ROS production. Alternatively, a little percentage of oxygen can undergo successive one-electron reduction pathways resulting in the generation of superoxide anion radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) after accepting two protons (Figure 1.2.). The H_2O_2 molecules can

accept one more electron and generate hydroxyl radical ($HO^{\bullet}$) and hydroxyl anion (OH^-). Lastly, $HO^{\bullet}$ reacts with one more proton and electron forming a water molecule. Reactive oxygen species include not only mentioned above but also diverse peroxides, like lipid peroxides, and peroxides of proteins, and nucleic acids (Figure 1.2.)(reviewed in (Lushchak, 2014)).

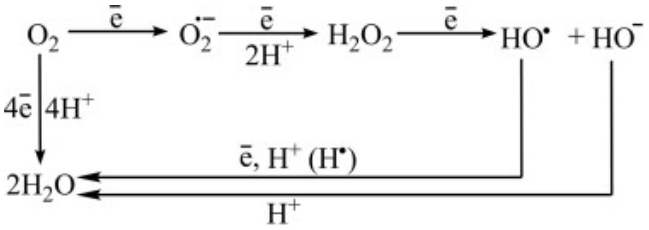

Figure 1.2. Reduction of molecular oxygen via four- and one-electron schemes
Oxygen reduction to water can happen through alternative reactions via four-electron or one-electron steps generates different types of ROS . Scheme from(Lushchak, 2014).

1.2.1. Intracellular sources of ROS

Mitochondria: These organelles are generally assumed to be the major ROS producers of the cells, with up to 90% of the total ROS production, consistent with the fact that mitochondria consume most of the cellular oxygen during oxidative phosphorylation (OXPHOS) (Balaban et al., 2005; Orrenius et al., 2007). Therefore mitochondria have been proposed to be main targets for oxidative damage and major contributors to aging (Harman, 1972). However there is some discrepancy about the role of mitochondria as main ROS source, and some reports question whether the experimental evidence available in the literature for this statement is convincing or not (Brown and Borutaite, 2012). Some studies suggest that between one and five percent of the total oxygen consumed is used to generated ROS (Chance et al., 1979), while others estimate a value around 0,2% (St-Pierre et al., 2002).

During the flow of electrons across the ETC, some can escape and reduce oxygen to form $O_2^{\bullet -}$, which can be further reduced to H_2O_2 (Figure 1.3). The magnitude of ROS generation in mitochondria and the physiological relevance of this process are still under debate. The analysis of isolated mitochondria can show that there are distinct molecular sites of superoxide production within the mitochondria (Brand,

2010). The main sites of $O_2^{\bullet-}$ production are within complex I and complex III of the ETC.

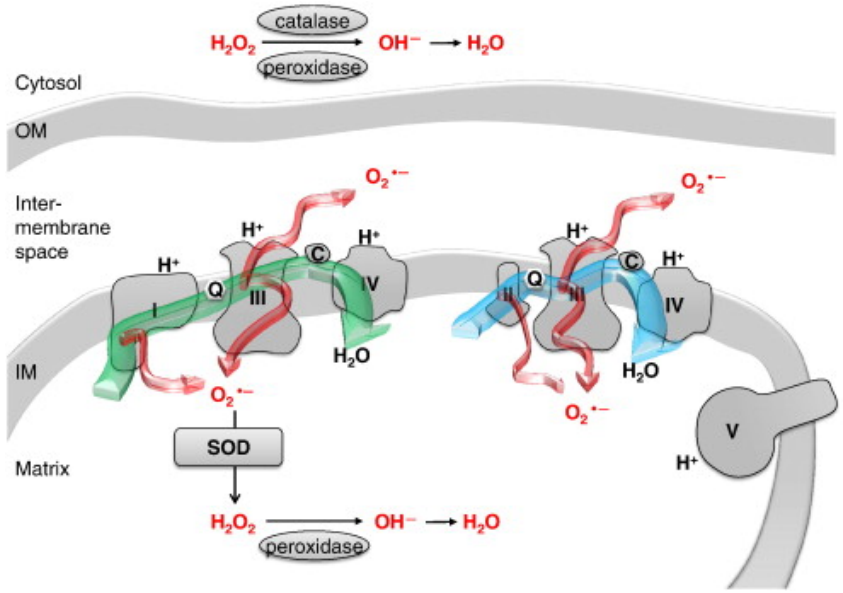

Figure 1.3. Mitochondrial ROS production
The electrons enter the ETC at complex I (green arrow) or complex II (blue arrow) and are reduced to water in complex IV. Some electrons leak (red arrows) into the matrix or IMS and react with oxygen generating superoxide. $O_2^{\bullet-}$ can be further transformed into H_2O_2 in mitochondria and be subsequently converted to hydroxide and water both inside and outside of mitochondria by different ROS scavenging enzymes. Image from (Dancy et al., 2014).

It remains unclear, which factors are responsible for regulating the rate of mitochondrial ROS production. Membrane potential is one of the main proposed mechanisms (Mailloux and Harper, 2012; Murphy, 2009): Generally an increased proton-motive force is associated with an increase in ROS, and a decrease in the proton-motive force is thought to decrease ROS production. This is based on the idea that high proton-motive force slows down the flow of electrons across the ETC and therefore the "leaking" probability increases, leading to an increase in $O_2^{\bullet-}$ production (Mailloux and Harper, 2012). However, the association of these two factors remains unclear. The same is true for other mechanisms proposed to be determinants of ROS production, like oxygen availability (Sena and Chandel, 2012). $O_2^{\bullet-}$ is thought not to cross mitochondrial membranes because of its charge, however small amounts of it may cross in its protonated form or trough anion

transporters, although the permeability of the inner mitochondrial membrane still remains unclear due to contradictory studies (reviewed in(Andreyev et al., 2005). However, superoxide can be produced to both sites of the inner mitochondrial membrane. This implies that superoxide stays in mitochondria until its consumption: it can oxidize mitochondrial molecules or be converted to H_2O_2 by superoxide dismutases. H_2O_2 can than freely diffuse outside of mitochondria and reach the cytosol or nucleus and cause oxidative damage in different cellular compartments.

But the ETC is not the only site of ROS production within mitochondria: seven other ROS producers have been described including cytochrome b5 reductase, monoamine oxidase, dihydroorotate dehydrogenase, dehydrogenase of α-glycerophosphate, succinate dehydrogenase, aconitase and α-Ketoglutarate dehydrogenase complex (Andreyev et al., 2005).

Peroxisomes: Oxygen consumption is not exclusively limited to mitochondria: in peroxisomes electrons are transferred from various metabolites to reduce molecular oxygen to H_2O_2, and then further reduced to water (De Duve and Baudhuin, 1966). Contrary to the respiratory chain in mitochondria, the electron transfer in peroxisomes is not coupled to adenosine triphosphate (ATP) production, and energy is released in form of heat. In hand with the high peroxisomal oxygen consumption, different sources of ROS, including H_2O_2, $O_2^{\bullet-}$ and $HO^{\bullet}$ as well as nitric oxide have been reported to be produced in peroxisomes (De Duve and Baudhuin, 1966; Elliott et al., 1986; Stolz et al., 2002; Zwacka et al., 1994). Peroxisomes play an important role in cellular ROS production and exhibit a great collection of ROS metabolizing enzymes, both producers and scavengers (Schrader and Fahimi, 2006). The peroxisomes are mostly considered sources of H_2O_2, being the β-oxidation of fatty acids, the enzymatic reactions of the flavin oxidases and the disproportionation of superoxide radicals the main metabolic processes contributing to this generation. Estimations performed in rat liver propose that about 35% of the total cellular H_2O_2 is generated by peroxisomal oxidases (Boveris et al., 1972). Whether this estimation is conserved among tissues and species remains unclear. Despite of being major producers of H_2O_2, peroxisomes posses powerful ROS scavenging mechanisms like catalase,

glutathione peroxidase, superoxide dismutase, epoxide hydrolase, peroxireodoxin 1 and Peroxisomal membrane protein 20 (Schrader and Fahimi, 2006).

Endoplasmic reticulum: The endoplasmic reticulum (ER) is a specialized organelle in charge of protein trafficking and folding. The folding of most membrane and secretory proteins is characterized by the presence of disulfide bonds between cysteines that stabilize their tree-dimensional structure. The disulfide bond formation is very sensitive to the redox state of the ER lumen that can be easily disrupted (Malhotra and Kaufman, 2007). The oxidative protein folding in eukaryotic cells is catalyzed by ER oxidorrecutases and the electron transfer between molecules can result in molecular oxygen receiving the electrons and therefore producing H_2O_2 as a byproduct of the process (Cao and Kaufman, 2014).

In addition to the enzymes involved in protein folding, Cytochrome P450 enzymes (CYPs) are involved in ER-ROS production. These monooxygenases are involved in xenobiotic detoxification as well as oxidation of metabolic intermediates. During CYP reaction cycles, electrons from heme iron are transferred to activated bound oxygen molecule in a NADPH-dependent manner, generating superoxide anions, hydrogen and hydroxyl radicals (Fleming, 2001). However the presence of CYPs is not limited to the ER and they are also present in other cellular compartments like cytosol or mitochondria where they can also act as sources of ROS (Kil et al., 2012).

Cytosol, plasma membrane and extracellular space: several enzymes can contribute to ROS generation in the cytosol. Among those the NOX family is remarkable. NOX enzymes are NADPH oxidases that were first described as ROS producers in neutrophils (Hampton et al., 1998). However, many other homologs of the neutrophil NOX have been found in different cells, showing that these enzymes are not restricted to this cell type. NOX enzymes transfer electron from cytosolic NADPH across biological membranes to molecular oxygen producing superoxide and other types of ROS (Bedard and Krause, 2007).

In addition to the well characterized NOX family, the cells posses a wide range of other ROS producing enzymes like nitric oxide synthase (Palmer et al., 1987),

lipooxygenases (O'Donnell and Azzi, 1996; Roy et al., 1994), cyclooxygenases and xanthine oxidase (McNally et al., 2003).

The increasing interest in understanding the role of ROS in biological processes, lead to the discovery that ROS is produced in several different cellular compartments: here we briefly reviewed the most important intracellular ROS sources according to their subcellular localization (Figure 1.4.).

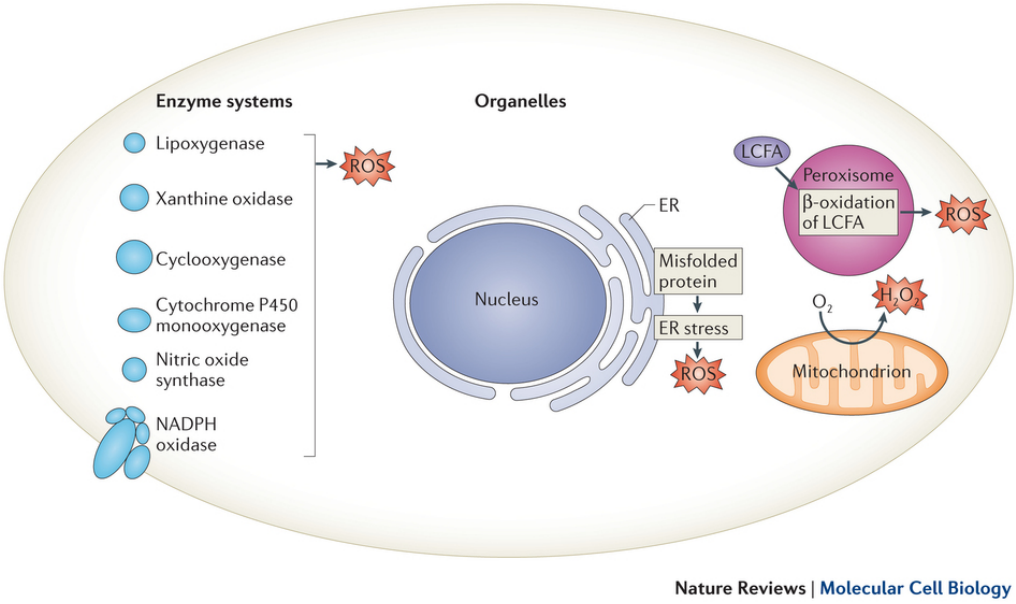

Figure 1.4. Cellular sources of ROS
ROS are produce inside the cell in various cellular compartments including mitochondria, peroxisomes and endoplasmic reticulum as well as the cytoplasm and the plasma membrane. Figure adopted from (Holmstrom and Finkel, 2014).

1.2.2. Antioxidant defense system

As above described, there are multiple cellular sources of ROS, but naturally, the cells are also well equipped with machinery to eliminate or balance this ROS production at different levels. Antioxidant proteins and molecules keep concentration of ROS at optimal concentration for cellular function and signaling. The antioxidant defense system includes different classes of compounds that can be broadly divided as following: (i) enzymatic ROS scavengers (ii) small antioxidant molecules and (iii) tight metal chelators. Additionally, if ROS is not eliminated prior to generating oxidative damage, there are also specialized

enzymes involved in DNA and protein damage repair as well as detoxification (Sirkorski and Kolakowska, 1938).

Enzymatic ROS scavengers

This group is composed by enzymes dedicated to the active degradation of different ROS species like hydrogen peroxide or superoxide. They are distributed throughout several cellular compartments, among which we emphasize the role of catalase ($2H_2O_2 \rightarrow O_2+H_2O$), superoxide dismutases ($O_2{\cdot}^- \rightarrow H_2O_2$), glutathione peroxidases ($H_2O_2+GSH \rightarrow GSSG+H_2O$) and peroxirredoxins ($H_2O_2+TrxS_2 \rightarrow Trx(SH)_2+H_2O$).

Small antioxidant molecules

Low-molecular-weight ROS scavengers can act at several levels in antioxidant defense (Kirsch and De Groot, 2001): (i) preventive action, intercepting ROS before it can react with biomolecules, (ii) interventive action, inhibiting the amplification of free-radical chain reactions and lastly (iii) repair action, repairing biomolecules that have been transformed into their free-radical form by ROS. These small antioxidant molecules include glutathione, ascorbic acid, NAD(P)H, uric acid, bilirubin, ubiquinol and others from vegetal origin like tocopherols, tocotrienols and carotenoids. Out of this large and diverse group, is worth highlighting the functions of glutathione and ubiquinol.

- **Glutathione** (GSH) is a tripeptide consisting of glycine, cysteine and glutamic acid, that participates in several cellular processes like cell proliferation, apoptosis, protein folding and cell signaling. GSH is the main hydrophilic intracellular antioxidant and it is present at millimolar concentrations. In addition to its antioxidant function, GSH can play antitoxic, modulator and prooxidant roles in the cell, being a substrate for glutathione peroxidases and glutathione S-transferases and a cofactor in the glutarredoxin system, but also acting in a nonenzymatic manner (reviewed in (Pompella et al., 2003)). Manteinance of an optimum GSH/GSSH ratio is therefore essential for cellular homeostasis, and a decreased ratio is a

common indicator of redox imbalance characteristic of pathologies like cancer, neurodegeneration or the aging process itself (Townsend et al., 2003).

- **Ubiquinol** is the fully reduced for of coenzyme Q or ubiquinone. While the ubisemiquinone form can be a major source of ROS in the ETC, the reduced ubiquinol is known to act as a lipid-soluble antioxidant that can avoid the initiation or prevent the propagation of lipid peroxidation in biological membranes and low-density serum lipoproteins (Ernster and Forsmark-Andree, 1993; Mellors and Tappel, 1966). Therefore the balance between the different oxidation forms of coenzyme Q and their correct enzymatic regulation are key determinants for mitochondrial redox homeostasis.

Metal ion chelators

Metal ions can aggravate oxidative damage due to their involvement in Fenton reactions and decomposition of lipid hydroperoxides as well as for autooxidation. However, proteins involved in transport and storage of transition metals, also known as metal sequestering proteins are therefore important for antioxidant defense. To this group belong proteins like ferritin, haptoglobin, hemopexin or transferrin.

Oxidative damage repair

When the above described mechanisms are not sufficient to avoid oxidative damage, a second level of cellular protection is involved in repairing DNA and protein oxidative damage (glycosylases, sulfiredoxins and methionine sulfoxide reductases) and detoxifying ROS byproducts (glutathione-S-transferase, cytochrome P450 enzymes).

1.2.3. The dual nature of ROS

The free radical theory of aging (Harman, 1956), has been determinant for the interpretation of the role of ROS in biological processes by the scientific community: for many years, ROS have been exclusively considered as damaging forces, whose production and impact is unregulated and random. The accumulation of ROS-dependent damage to lipids, proteins and DNA, was therefore

considered as a major to pathologies and the aging process itself (Halliwell, 1993; Markesbery, 1997; Stadtman and Berlett, 1998; Stich and Anders, 1989). Despite the fact that this nonspecific and apparently random and damaging aspect of ROS still persists, there is growing evidence that some oxidant species like superoxide or hydrogen peroxide play useful, beneficial and essential roles in cellular processes (Lo and Cruz, 1995; Palmer et al., 1987; Sundaresan et al., 1995). To date is broadly accepted that ROS play a role in cellular signaling. Redox signaling is based on reversible oxidation and reduction of crucial residues by ROS molecules. Both $O_2^{\bullet -}$ and H_2O_2 have been described as potential messenger, however, due to the greater stability of hydrogen peroxide it is more probable that it can act as a signaling intermediate. One of the biggest questions regarding ROS-mediated signaling is the regulation and specificity due to the simplicity of most ROS species and apparent "random" generation. It is proposed that subcellular localization of oxidants and targets may be key determinants of ROS-mediated signaling, however many aspects remain unknown and are object of current research.

The rapid and extensive study of reactive oxygen species in biology, like the study of any other biological process, led to the appearance and use of new subject-related terms that are worth mentioning. The term **oxidative stress** was first introduced in 1985 (Sies, 1985) and defined as "Imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage". Years latter the concept was updated to include the role of redox signaling (Jones, 2006; Sies and Jones, 2007) stepping back from "damage" and focusing on the signaling. However, there has been a tendency to spread and overuse of the term, phenomenon that the author himself will refer to as "over-stressing" the oxidative stress. There has been several attempts to classify oxidative stress or introduce an intensity scale (Lushchak, 2014); classification that for sure would lead to a more proper definition and more accurate understanding of oxidative stress. However, these terms are not (yet) broadly accepted or rooted in the scientific community.

If not the terms, the idea of an intensity scale is essential for understanding this dual role of ROS. A severe and/or prolonged oxidative stress can lead to irreversible and detrimental **oxidative damage** of cellular components that can accumulate and be involved in the pathophysiology of several diseases and aging,

in agreement with the oxidative stress theory of aging. However low levels of these same stressors are necessary for cellular **signaling.** At this point, and in hand with the role that stress in general can play in biological processes, it is important to highlight the concept of **hormesis**: it stems from the toxicology field but its scope has been broaden and refers to the beneficial effect of an intervention, that a higher dose or intensity is harmful. Or in other words; sublethal exposure to stressors (like ROS) induces a response that results in an increased stress resistance (Calabrese et al., 2011). This concept opens a new window for ROS implications in aging that has nothing to do with the free radical theory of aging, but rather considers ROS as a switch that activates resistance responses.

1.3. Mitochondria and their role in aging

Mitochondria are double-membrane-bounded eukaryotic organelles. The earliest reports of the existence of mitochondria date back to the 1840s, few years after the discovery of the nucleus (Ernster and Schatz, 1981). However, it was first in 1890 when Altman discovered the ubiquitous existence of these structures and called them “bioblasts”, as they were “elementary organisms" living inside cells and carrying out vital functions (Altmann, 1890). Altman didn’t know back then that his description was already implying the theory that decades later would explain the endosymbiotic origin of mitochondria (Margulis, 1971). The current name mitochondrion was introduced in 1898, and comes from the Greek “mitos” (thread) and “chondrion” (granule), as it refers to the appearance of these granules during spermatogenesis (Benda, 1898).

1.3.1. Mitochondrial genome

One of the most interesting features of the mitochondria, that make them such an exclusive and specialized organelle, is the fact that they possess their own genome, which is separated and distinct from the nuclear one. In agreement with the generally accepted endosymbiotic origin of mitochondria, these organelles posses, like bacteria, circular molecules of double-stranded mitochondrial DNA (mtDNA). The size of the mtDNA can vary considerably between species: for example the human mtDNA is about 16 kb, however there are substantially larger mitochondrial genomes like in yeasts (approximately 80 kb) or plants (more than 200 kb). Nevertheless, these larger mitochondrial genomes contain predominantly noncoding sequences and therefore do not appear to contain significantly more genetic information.

The human mitochondrial genome encodes 13 subunits of the ETC, two ribosomal RNA (16S and 12S rRNAs) and 22 tRNAs, which are required for mitochondrial protein synthesis. *C.elegans* mtDNA is very similar to the human one and it just differs in the lack of ATP8 and the arrangement of the genes (Figure 1.5.)(Ballard and Dean, 2001; Chomyn and Attardi, 2003; Larsson and Clayton, 1995; Lemire, 2005; Okimoto et al., 1992). The rest of the approximately 1500-mitochondrial

proteins are encoded in the nuclear DNA (nDNA), translated in the cytoplasm and imported into the mitochondria (Larsson and Clayton, 1995). This requires a regulated crosstalk between both genomes in order to coordinate mitochondrial biogenesis and assembly of the electron transport chain.

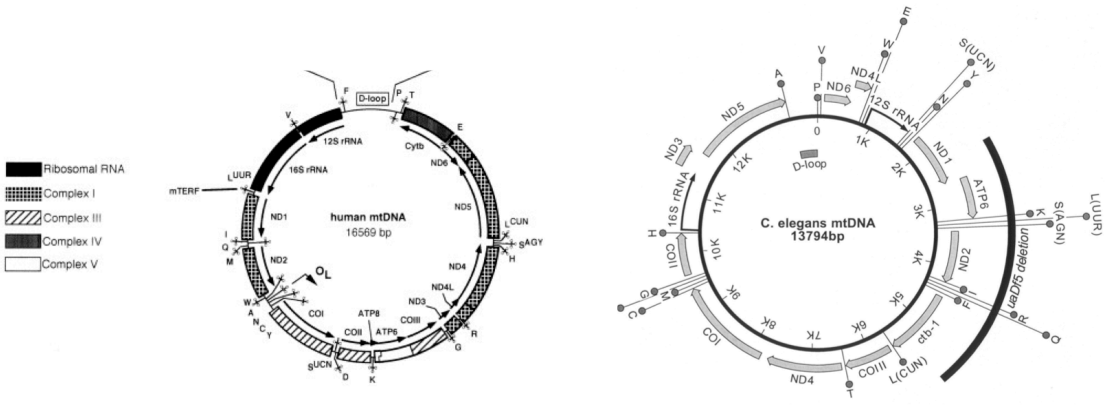

Figure 1.5. Mitochondrial genome
Left, human mtDNA encoding for 13 genes (ND1-ND6,ND4L, Cytb, Cox1,Cox2,Cox3, ATP6 and ATP8), 2 rRNAs(16s rRNA and 12 rRNA) and 22 tRNAs. Right, *C.elegans* mtDNA enconding for the same genes, excepting the ATP8 subunit. (Figures from(Garesse and Vallejo, 2001; Lemire, 2005)).

Another characteristic of mtDNA is its polyploidy, since it is present in the cell in multiple copies: each animal cell has between several hundreds and over a thousand mitochondria, each carrying between two and ten copies of mtDNA (Carugno et al., 2012), what results in 1000 to 10.000 mtDNA molecules per mammalian cell. The regulation of this number of copies of mtDNA seems to be strictly regulated during development since every stage is characterized by a specific mtDNA content (Bratic et al., 2010; Moraes, 2001).

mtDNA replication occurs randomly during the cell cycle, is independent of nDNA replication and not all the molecules are necessarily replicated (Bogenhagen and Clayton, 1977; Clayton, 1982). Nuclear genes encode the machinery necessary for mtDNA replication and the resulting proteins are imported into the mitochondria, where in contrast to nDNA, mtDNA replication and transcription happened as coupled events. Although the mode of mtDNA replication is still a subject of intense debate, the molecular mechanisms necessary for this process are well described. The replisome or core components needed for mtDNA replication are the DNA polymerase gamma (POLG-1) and the helicase Twinkle and MTSS-1 (mitochondrial

single stranded DNA binding protein). Further components are the mitochondrial transcription factor A (TFAM), RNA polymerase (POLRMT), RNA processing enzymes and topoisomerase (mtTOP1) (Falkenberg et al., 2007). Mitochondrial genes are inherited in a non-Mendelian manner and have been reported to be mostly maternally inherited and suffer a strong purifying selection (HutchisonIII et al., 1974; Stewart et al., 2008a), although some exceptions of paternal inheritance are known (Gyllensten et al., 1991; Hoeh et al., 1991; Kondo et al., 1992; Meusel and Moritz, 1993). The fact that mtDNA is polyploid and not every molecule is necessarily replicated (Clayton, 1982), plays an important role in the appearance of mitochondrial diseases. One can talk about homoplasmic or heteroplasmic cells, referring to cells carrying identical mtDNA copies or a mix of mutated and wild type molecules, respectively. Although the high number of mtDNA molecules reduces the impact of mutations in single copies, it cannot avoid the expansion of de novo mutations arising in a single mtDNA molecule (Holt et al., 1988; Wallace et al., 1988). Heteroplasmic mtDNA mutations segregate during cell division because, as commented above, there is no mechanism to ensure that every mtDNA molecule is replicated only once during each cell cycle (Clayton, 1982).Therefore random distribution of mtDNA molecules during cell division can lead to increased amounts of mutant mtDNA molecules in one of the daughter cells. Thus, a cell carrying low levels of mutated molecules can eventually give rise to a daughter cell with high mutation level.

1.3.2. Mitochondrial structure and function

Mitochondria form highly dynamic networks in the cells that undergo constant fusion and fission events (reviewed in (Chan, 2012)). These organelles are defined by a double-membrane system consisting of the outer (OMM) and inner membranes (IMM) that are separated by the so-called intermembrane space (IMS)(Figure 1.6.). The OMM is highly permeable to small molecules due to the presence of porins, which form channels that allow the free diffusion of molecules with low molecular weight. Because of this high permeability, the composition of the IMS is similar to the cytosol with respect to ions and small molecules. The IMM is folded forming cristae that extend towards the interior of the organelle or

matrix. The IMM has much higher protein content and is therefore less permeable, consequently acting as a functional barrier for small molecules between the cytosol and matrix (Cooper, 2000; Lodish et al., 2004). This compartmental structure is key for the distinct functional and numerous roles exerted by mitochondria.

As shortly described in the mitochondrial genome section, most of the proteins necessary for the mitochondrial function have to be imported from the cytosol. For this purpose, mitochondria posses specialized transport machinery. The translocation of the proteins is mediated by the multisubunit protein complexes TOM and TIM, which stand for translocator of the OMM or the IMM, respectively (Figure 1.6). The TOM complex is required for import of all nuclear-encoded mitochondrial proteins. It recognizes their mitochondrial specific targeting sequence and initially transports them into the IMS. Additionally TOM assists the incorporation of proteins into the OMM. TIM23 complex transports then some of these mitochondrial proteins into the matrix and helps inserting transmembrane proteins into the IMM. TIM22 complex completes the insertion of a selection of IMM proteins, including carries proteins responsible for the transport of ADP, ATP and phosphate (Cooper, 2000; Lodish et al., 2004).

Mitochondria are mostly recognized for their role in energy production. The matrix contains a great collection of enzymes responsible for the main reactions of the oxidative metabolism: oxidative breakdown of glucose is one of the main sources of metabolic energy. The first stages of this process, glycolysis, occurs in the cytosol, where glucose is converted to pyruvate. Pyruvate is then transported into the mitochondria where it is oxidize producing adenosine triphosphate (ATP). Initially, the pyruvate is oxidized into acetyl CoA that is then broken down to CO_2 in the tricarboxylic acid (TCA) cycle. The fatty acids, another major energy source, are also oxidized yielding acetyl CoA, that is similarly metabolized by the TCA cycle inside mitochondria or alternatively in the peroxisomes. The oxidation of acetyl CoA to CO_2 is accompanied by the reduction of NAD+ and FAD to NADH and FADH2, respectively. NADH and FADH2 then feed the ETC with their high-energy electrons that are carried to an electron acceptor (oxygen) after several redox reactions. The enzymes that compose the ETC are located in the IMM forming four

macromolecular complexes: complex I (CO I, NADH:ubiquinone oxidoreductase), complex II (CO II, Succinate dehydrogenase), complex III (CO III, Ubiquinol cytochrome-c reductase) and complex IV (CO IV, cytochrome c oxidase or COX). The energy resulting from this electron transfer is used to translocate protons from the matrix to the IMS, thus resulting in the generation of an electrochemical gradient across the IMM with a potential of ~150–180 mV (Saraste, 1999). The transmembrane proton gradient is used to drive the production of cellular energy in from of ATP by the complex V (CO V, ATP synthase) trough the process of oxidative phosphorylation (OXPHOS)(Figure 1.6.).

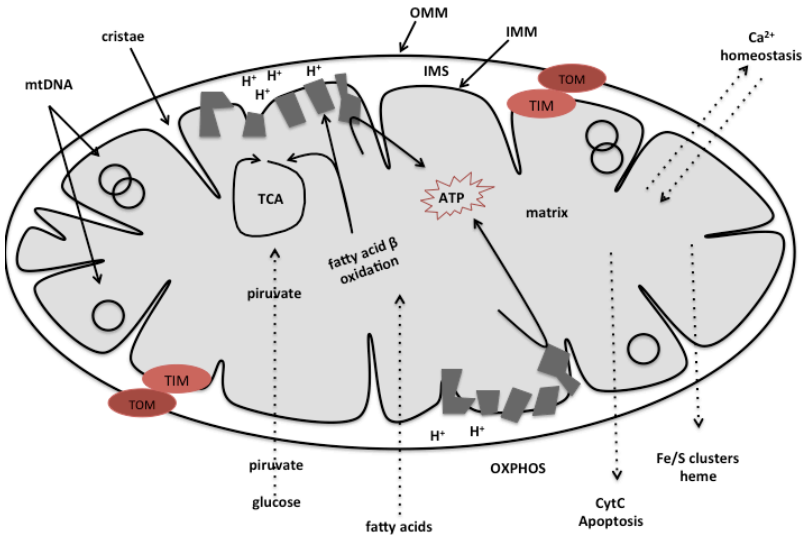

Figure 1.6. Mitochondrial structure and function
The image depicts on the one hand the main characteristics of mitochondrial structure, which is defined by a double membrane composed of OMM and IMM. Between both membranes we find the IMS. The IMM gets folded forming cristae and encloses the interior of the organelle or matrix, which contains multiple copies of mtDNA The protein translocators TOM and TIM are represented in red. On the other, some of the most important mitochondrial functions are highlight: calcium homeostasis, regulation of apoptosis, metabolisms of iron/sulfur clusters and heme and OXPHOS, which is fed with electrons by the TCA cycle and fatty acid β oxidation, and results in ATP production.

But mitochondria are far from being exclusively the "powerhouses of the cell". As shortly introduced above they are key for the metabolism of lipids and carbohydrates, they are the center for biosynthesis of several essential cellular compounds like heme, Fe-S clusters, coenzyme Q or cardiolipin, among others (Rensvold et al., 2013). Mitochondria are additionally essential for calcium

homeostasis, and play a key role in the regulation of cell death (apoptosis)(Figure 1.6.). Moreover they are one of the main cellular sources of ROS production, fact that is of specific interest for our research

1.3.3. Mitochondrial implication in aging

It has been long recognized that mitochondrial function declines during aging. Characteristic features of this decline are, for example, morphological alterations (Shigenaga et al., 1994), reduction in mitochondrial number in different organisms from mice to humans (Herbener, 1976; Stocco and Hutson, 1978; Tauchi and Sato, 1968; Yen et al., 1989) and a decrease in mtDNA copy number as well as a reduction in the levels of mitochondrial proteins (Stocco et al., 1977).

In addition to the decrease in mitochondrial number and mtDNA content, there have been numerous reports of reduction in the respiratory chain (RC) capacity (Ojaimi et al., 1999; Short et al., 2005). Nevertheless the reduction in RC capacity differs between tissues and it does not affect all complexes in the same way, and the reason for this variance remains unclear. The complexity and interconnectivity of the mitochondrial biology, hardly difficult the dissection of mitochondrial functions and the study of their isolated impact on aging. Here we focus on three main features of mitochondrial (dys)function and their impact on lifespan determination, although we are aware of they being closely related: (i) ATP production (ii) mtDNA mutations and (iii) mtROS.

Mitochondrial ATP production and aging

The electron transport and the ATP production are two usually very tightly coupled processes: inhibition of ATP synthase will therefore inhibit electron transport. However this two processes can be uncoupled by protonophores or uncoupling proteins that generate an alternative way for the protons to enter the mitochondrial matrix. As a consequence, uncoupling results in lower ATP production but a simultaneously high electron flow and respiration (Cannon et al., 2006). The link between mitochondrial respiration and ATP production with longevity has been extensively studied resulting in the appearance of conflicting results: different genetic and dietary interventions known to prolong lifespan can

both decrease and increase ATP production (reviewed in(Bratic and Trifunovic, 2010).

- **Caloric restriction (CR):** consists on reducing the caloric intake without malnutrition and it is the only dietary intervention that has been shown to extend lifespan from yeast to mammals: It was first described in rodents (McCay et al., 1989) and has been extensively studied in both mice and rats (Masoro, 1998, 2009; Weindruch and Walford, 1988). Additionally there have been numerous reports about the lifespan extension exerted by CR in yeast (Jiang et al., 2000; Sinclair, 2005), worms (Lakowski and Hekimi, 1998; Walker et al., 2005) and flies (Chapman and Partridge, 1996), suggesting that it could be evolutionary conserved. Despite the great advances made in the CR field, the molecular mechanisms responsible for it have not been fully determined. On the one hand, some reports suggest that reduced metabolic rate and ROS production are necessary for CR longevity: in an *in vitro* CR model, an increased number in mitochondria was linked to reduced O_2 consumption and membrane potential resulting in a decrease ROS production (Lambert and Merry, 2004). Reduced ROS would therefore ensure reduce oxidative damage during aging and hence lead to lifespan extension (Lopez-Lluch et al., 2006). Contrary to these reports, growing body of evidence suggests that an increase in the metabolic rate and oxidative metabolism are essential for CR-induced longevity (Houthoofd et al., 2002; Kharade et al., 2005; Schulz et al., 2007). Remarkably, the levels of energy production in from of ATP do not appear to differ in CR compared to control conditions (Khraiwesh et al., 2013). The controversy remains and the precise molecular mechanisms responsible for CR-induce longevity are still unknown, but it appears that mitochondrial energy metabolism is most likely going to stay as one of the essential players regulating this process

- **Mutations on the electron transport chain:** A decrease in mitochondrial bioenergetic capacity has been related with aging and disease. However, paradoxically several *C.elegans* mitochondrial mutants with defective mitochondrial respiratory chain function are longlived. *C.elegans*

mitochondrial mutants are in the spotlight of our research and will be more extensively reviewed in the section 1.4.1. *C.elegans* mitochondrial mutants.

mtDNA mutations and aging

The fact that mitochondria are major sites of ROS production and that they posses their own genome, led to the theory that, mtDNA would be highly exposed to ROS because of their close spatial proximity and therefore suffer and accumulate damage. The resulting mutation accumulation would impair respiratory chain function, therefore increasing ROS production in a vicious cycle that would be responsible for further damage to proteins, lipids and DNA consequently becoming the driving force of aging (Harman, 1972). After Harman's theory, the impact of mtDNA mutations on aging has been extensively studied (Bratic and Larsson, 2013; Park and Larsson, 2011). Since the eighties, increasing number of publications have reported the presence of mtDNA heterogeneity and rearrangements consistent with mtDNA deletions in aged animals including humans (Corral-Debrinski et al., 1992; Piko et al., 1988; Sato et al., 1989; Soong et al., 1992). To date it is widely accepted that mtDNA mutations increase with age in humans (Corral-Debrinski et al., 1992; Fayet et al., 2002; Taylor et al., 2003; Yen et al., 1991). But what is the origin of these mutations? Mutations occur when DNA damage remains unrepaired. In addition to ROS as possible damage agent, errors during replication and DNA synthesis are other putative explanations, which seem to be more important in determining the appearance of mtDNA mutations than ROS (Ameur et al., 2011; Larsson, 2010; Stewart et al., 2008b). Despite the great amount of reports relating mtDNA presence with disease and accumulation during aging, the first experimental evidence for a causative link between mtDNA mutation and aging was reported in 2004 with the mutator mouse (Trifunovic et al., 2004): Mutator mice are homozygous knock-in mice expressing a proofreading deficient version of the mitochondrial DNA polymerase. As a consequence, these mice exhibit a significantly higher rate of mitochondrial mutations including both point mutations and deletion. Despite their normal phenotype at birth, these mice rapidly exhibit symptoms of premature aging (Trifunovic et al., 2004). An interesting aspect of these mutator mice in regard to the role of ROS in aging

determination is, that despite the increase in mtDNA mutations they do not exhibit an increase in ROS production (Trifunovic et al., 2005), arguing against the direct role of ROS in the aging process.

ROS and aging

As we have seen in the previous sections, ROS always appears as a suspicious mitochondrial-aging-determinant either for causing mtDNA mutations and damage and/or for affecting mitochondrial bioenergetics. An oversimplified concept of ROS as "ying" and antioxidants or ROS scavengers as "yang" has led to a series of reports that have tried to establish unsatisfactorily a linear relationship between ROS and longevity. In agreement with this idea, if ROS are cause of senescence, the anti-oxidant system should be a major player of lifespan determination.

In *C.elegans* contradictory results have been publish regarding the role of superoxide dismutase (SOD) in lifespan determination: some maintain that mutational inactivation of *sod*s doesn't impact normal lifespan but leads to PQ hypersensitivity, with the exception of *sod-1* that reduces lifespan extension by cold (Yen et al., 2009), while other suggest that deletion of *sod-2* can extend lifespan (Van Raamsdonk and Hekimi, 2009). However, further research from the same lab, suggested that superoxide dismutases are dispensable for determination of normal lifespan (Van Raamsdonk and Hekimi, 2012). Additionally it has been shown that, lack of SODs does not result in increased oxidative damage or "vicious cycle" (Gruber et al., 2011), and that the lifespan extension observed in some SOD overexpression models is not caused by decreased oxidative damage (Cabreiro et al., 2011). Overexpression of human SOD1 in mice had no impact in lifespan despite the increased resistance to $O_2^{\bullet -}$-mediated brain injury (Huang et al., 2000b). Partial lack of SOD2 in mice (*Sod2*$^{+/-}$) results in increased mitochondrial oxidative damage (Li et al., 1995) but no detectable changes in damage to nuclear or cytoplasmic compartments and no changes in lifespan compared to wild type littermates (Van Remmen et al., 2003). An exhaustive study on the effect of overexpressing or suppressing several antioxidant enzymes, observed that out of the 18 genetic manipulations tested, just the deletion of Sod1 gene had an impact

in lifespan, seriously questioning the idea that anti-oxidant systems are major determinants of longevity (Perez et al., 2009a; Perez et al., 2009b).

Altogether these results suggest that the role of ROS in lifespan determination is not linear and is a more complex process that cannot be simply explained by the oxidative stress theory of aging.

1.4. *Caenorhabditis elegans* as a model for aging research

So far, different model organisms like yeast, worms, flies or mice have been successfully used to study aging and help understanding human age-related diseases. In this work we make use of the round worm *Caenorhabditis elegans* (*C.elegans*). Due to its rapid life cycle, short lifespan, small size, well annotated genome and the ease and low cost of its growth and maintenance, *C.elegans* is an optimal tool for aging studies.

C.elegans is a free-living organism found worldwide. It was back in the sixties when it was first described and used as a model organism for biological research, which was followed by an extensive characterization of its genetics and establishment of research methods (Brenner, 1974). *C.elegans* exists mostly as a self-fertilizing hermaphrodite (XX), but a male (XO) form also arises spontaneously with low frequency (0,1-2%) by non-disjunction of the hermaphrodite germline or with higher frequency trough mating. Males allow the isolation and maintenance of mutant strains as well as inter-strain crossings and mutation transmission. *C.elegans* has a life cycle of approximately three days under optimal conditions and undergoes four different larval stages until reaching the adult stage (Figure 1.7.). *C.elegans* hermaphrodites can lay up to 300 eggs what makes the propagation of isogenic populations very fast and useful for genetic studies (Brenner, 1973; Byerly et al., 1976; Sulston et al., 1983). The nematodes are grown on agar plates spread with *E.coli* and kept in incubators at constant temperatures. Another great feature of this worm it that it can be frozen, what facilitates its storage and conservation. Additionally, mutants can be quite easily generated by radiation or chemical mutagenesis (Anderson, 1995; Jorgensen and Mango, 2002) and a large library is available for research purposes (Caenorhabditis Genetic Center –CGC-, University of Minnesota).

In addition to theses advantages, it is worth highlighting that many key molecules, pathways, organelles, and tissues found in higher organisms like humans are conserved in these nematodes, which puts *C.elegans* at the forefront of genetic research on aging. Similarly to more complex animals, *C.elegans* exhibits aging features: they are not longer fertile, are more prone to infection, move slower,

reduce their eating rate and are less responsive to stimuli (Yanos et al., 2012). Other age related characteristics are the accumulation of age pigments, the change in shape from cellular nuclei and the redistribution of the heterochromatin (Gerstbrein et al., 2005; Haithcock et al., 2005). Evidently *C.elegans* also exhibits many differences in aspects, which are highly related to age related diseases in humans: their telomere seem to not shorten with age (Raices et al., 2005) they lack a cardiovascular and adaptive immune system as well as the senses of sight and hearing, among others.

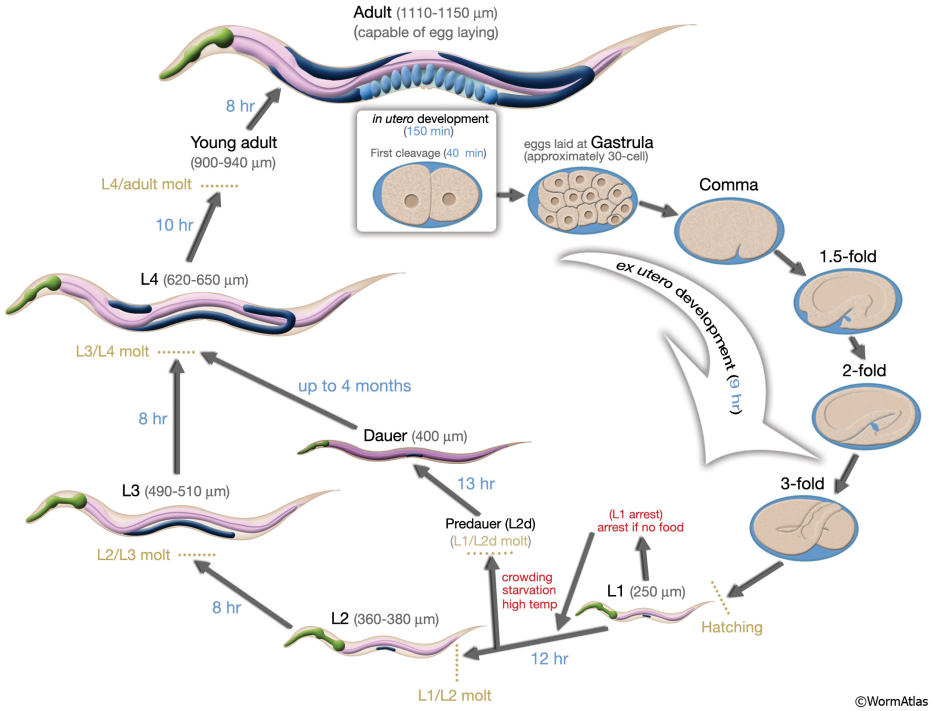

Figure 1.7. *C.elegans* life cycle
Once the embryonic development is completed the eggs hatch and pass through four different larval stages (L1 to L4) until reaching the gravid adult stage that is capable of egg laying, completing the cycle. Alternatively in unfavorable conditions L1 larvae can transform into resistance forms called dauer, that can live up to four months under starvation. If the conditions are favorable again, dauer larvae can exit this lethargus and complete their developmental cycle. Figure available at WormAtlas

1.4.1. *C.elegans* mitochondrial mutants

As we have already introduced above, our research focuses on understanding the mechanism underlying the, *a priory* paradoxical, lifespan extension caused by mitochondrial dysfunction in *C.elegans*: henceforth we will refer to this term as mito-induce longevity and to the worms carrying mutation in components of the

mitochondrial respiratory chain (MRC), mit-mutants. Although several both short- and longlived mit-mutants were described beforehand, the interest in understanding the role of mitochondrial chain dysfunction in lifespan determination of *C.elegans* greatly increased after the publication of two screen of chromosome I that detected that RNAi of subunits of all complexes of the MRC were able to extend lifespan (Dillin et al., 2002; Lee et al., 2003). The screen of the entire genome was conducted shorty after, again reinforcing the implication of mitochondria in lifespan determination due to the high representation of MRC genes (Hamilton et al., 2005; Hansen et al., 2005). Down below we review some remarkable mit-mutants covering examples of all MRC complexes and focusing on their mitochondrial dysfunction and ROS production (Dancy et al., 2014)

Complex I: *gas-1* and *nuo-6*

gas-1(general anesthetic sensitive) encodes a 49kDa subunit of complex I of the ETC (Kayser et al., 1999). *gas-1(fc21)* mutants have been shown to exhibit a complex-I dependent OXPHOS rate (with glutamate and malate as substrates) of approximately 70% of the wild type (Kayser et al., 2001). Complex-I activity is also reduced compared to wild type and interestingly they exhibit almost double values of complex II-dependent respiration (with succinate as substrate)(Kayser et al., 2001), which is believed to be a compensatory mechanism. *gas-1(fc21)* are shortlived at 20°C, produce less progeny, exhibit a delay development and are highly sensitive to oxidative stress (Hartman et al., 2001). Additionally *gas-1* mutants have been shown to have an increased mitochondrial ROS production (Kondo et al., 2005) and oxidative damage (Kayser et al., 2004a).

nuo-6 encodes the *C.elegans* orthologous of mammalian NUDFB4/B15 subunit of complex I (Yang and Hekimi, 2010b). *nuo-6(qm200)* have decreased complex I function and both *nuo-6(qm200)* mutation and *nuo-6* RNAi have been implicated in lifespan extension (Yang and Hekimi, 2010b). Yang and Hekimi proposed that the RNAi caused a stress-like response activating heat shock response and autophagy while the *nuo-6(qm200)* allele did not, and concluded that there are two different mechanisms activated by mitochondrial dysfunction able to extend lifespan. Additionally they detected increased mitochondrial ROS

production in the *nuo-6(qm200)* mutant essential for lifespan extension (Yang and Hekimi, 2010a).

Both *gas-1* and *nuo-6* are therefore examples of how mutations in the same complex can result and complete opposite phenotypes. In order to understand this paradox, previous work done in our lab tried to establish differences and similarities between the two models: surprisingly both short-lived *gas-1(fc21)* and long-lived *nuo-6(qm200)* activated longevity assurance pathways, shown to be important for lifespan showing similar dependence on alternative metabolic pathways, activation of hypoxia-inducible factor -1 (HIF-1) stress pathway and mitochondrial unfolded protein response (mtUPR). The main difference detected between the two mutants, was the massive loss of complex I accompanied by upregulation of complex II levels, only in short-lived, *gas-1(fc21)* mutant. This upregulation of complex II levels resulted in a decrease of complex I stability and lifespan reduction (Pujol et al., 2013).

Complex II: *mev-1*

mev-1 (methyl viologen sensitive) encodes the *C. elegans* ortholog of the succinate dehydrogenase cytochrome *b560* subunit, an integral membrane protein that is a subunit of mitochondrial respiratory chain complex II. *mev-1(kn1)* was the very first mutation affecting a mitochondrial protein described to impact lifespan (Ishii et al., 1998). *mev-1* mutants have a shorter lifespan, decreased fecundity, increased oxidative damage and hypersensitivity to oxidative stress (Ishii et al., 1998; Ishii et al., 2002; Yanase et al., 2002). Additionally, *mev-2* mutants exhibit reduced complex II-dependent OXPHOS rate and activity (Ishii et al., 1998; Kayser et al., 2004a). The steady state levels of superoxide as well as its production rate are elevated in *mev-1* mutants and this observation is accompanied by an overall increased oxidative damage but different from the one observed in *gas-1* (Senoo-Matsuda et al., 2001; Yanase et al., 2002). This suggested that the site of ROS production can determine different damage patterns and result in lifespan shortening, in contrast to the essential role of ROS for lifespan extension of *nuo-6* and *isp-1* (Yang and Hekimi, 2010a), again questioning the role of ROS in longevity

determination and suggesting that this can not be generalized to all mutant backgrounds.

Coenzyme Q: *clk-1*

clk-1 (clock) gene encodes the *C. elegans* ortholog of COQ7/CAT5 demethoxyubiquinone (DMQ) hydroxylase essential for conenzyme Q synthesis.

Like most mit-mutants, *clk-1* mutants have decreased fecundity and slower development and are longlived compared to wild type worms (Ewbank et al., 1997; Lakowski and Hekimi, 1996). CoQ is essential for respiration carrying electrons between complex I-II and III and it can act both as ROS scavenger (Genova et al., 2003; Kagan et al., 1990) and prooxidant (Genova et al., 2003; Sohal and Forster, 2007) depending on its reduction state. *clk-1* mutants exhibit a specific decrease in complex I-dependent respiration (Kayser et al., 2004b) and despite elevated ROS production (Lee et al., 2010; Yang et al., 2009), they have reduced mitochondrial oxidative damage (Kayser et al., 2004a) like *gas-1* and *mev-1* mutants. This could be due to enhanced ROS scavenging or fast turnover of damaged proteins. However other studies suggested that ROS was not responsible for lifespan extension, since the effect could not be suppressed by antioxidants in *clk-1* mutants (Yang and Hekimi, 2010a), and recent studies describe a nuclear role for *clk-1* in the assurance of longevity activating a retrograde signaling response to mitochondrial ROS (Monaghan et al., 2015).

Complex III: *isp-1;ctb-1*

The *isp-1* encodes a Rieske iron sulphur protein that is part of the complex III (Figure 1.8.A.). The mutation *isp-1(qm150)* was first identified in a mutagenesis screen and implicated in lifespan extension in 2001 (Feng et al., 2001). All the physiological rates analyzed in this mutant were much lower than the wild type including embryonic and postembryonic development, egg production rate and brood size or oxygen consumption. Despite of these phenotypes the worms appear healthy and show a marked increase in mean and maximum lifespan (Figure 1.8.B.). Interestingly another mutation identified by Feng and colleagues could partially suppress the phenotypes observed in *qm150* mutants without affecting the long lifespan (Figure 1.8.B). This mutation was affecting the cytochrome b (*ctb-*

1) locus. *ctb-1* is the only subunit of the complex III (Figure 1.8.A.) encoded in the mtDNA, and has no phenotype associated with the *ctb-1(qm189)* alone (Feng et al., 2001; Suthammarak et al., 2010). For simplicity, the double mutant *isp-1(qm150);ctb-1(qm189)* will be hereafter referred to only as *isp-1;ctb-1*. The *isp-1* mutation did not only lead to an expected decrease in complex III activity, but also to a decrease in complex I activity and destabilization of I:III2:IV supercomplex, in an apparently allosteric fashion (Suthammarak et al., 2010). *ctb-1,* correspondingly, led to a decrease in complex II activity but improved the complex I and complexes I-III activities and when combined with *isp-1* led to the stabilization the I:III2:IV supercomplex (Suthammarak et al., 2010; Suthammarak et al., 2009).

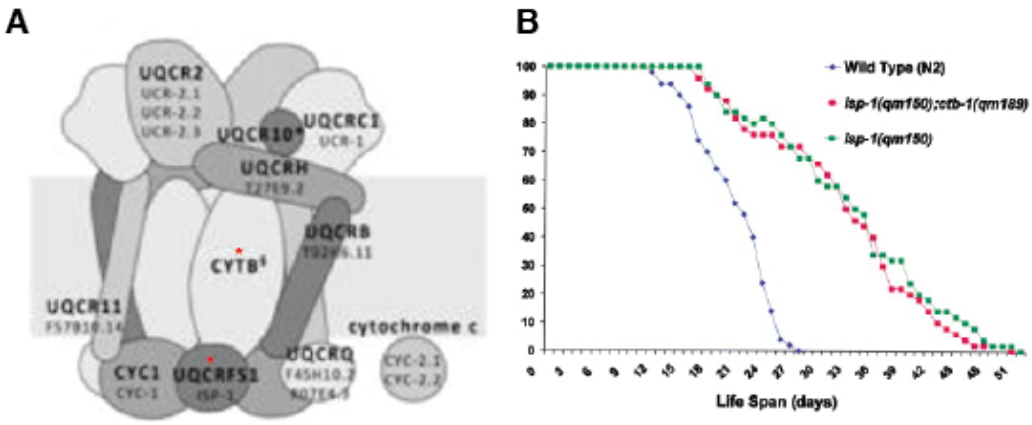

Figure 1.8. *isp-1;ctb-1* mit-mutants are longlived
A) Structure of complex III, mutated subunits in *isp-1(qm150);ctb-1(qm189)* are marked with a red asterisk. Modified from (Munkacsy and Rea, 2014). **B**) Both single mutant *isp-1(qm150)* and double mutant *isp-1(qm150);ctb-1(qm189)* are long lived compared to wild type worms. From (Feng et al., 2001).

In this first publication from Feng and colleagues, it was proposed that *isp-1* induced longevity was achieved due to the low oxygen consumption and decreased sensitivity to ROS, since combination with a *daf-2* mutation, which increases ROS resistance, did not further increase lifespan. Years later in 2010, it was shown that the low respiration of both *clk-1* and *isp-1* mutants was associated a mild increase in oxidative stress that activates HIF-1, which lastly results in altered genes expression and longevity assurance (Lee et al., 2010). Like in the case of *nuo-6* it was shown that both RNAi or *isp-1* mutation lead to lifespan extension trough

different mechanisms (Yang and Hekimi, 2010b): while the RNAi appeared to mediate longevity by inducing autophagy and heat-shock response via HSF-1 (Yang and Hekimi, 2010b), a mitochondrial specific increase in superoxide was the trigger responsible for lifespan extension in the mutant (Yang and Hekimi, 2010a). To date nothing is known about the mechanisms for longevity assurance in the double mutant *isp-1;ctb-1* and precisely this will be the central inquiry guiding our research, focusing on identifying new transcription factors involved in this process and deciphering whether or not ROS plays a role in the longevity assurance of these mutants.

Complex IV:

Knockdown of complex IV subunits has been shown to have opposite effects on lifespan determination (Hamilton et al., 2005; Lee et al., 2003; Suthammarak et al., 2009) and the differences may be due to the differential use of FUdR. Knock down of *cco-1* (ortholog of cytochrome c oxidase-1 subunit Vb) was shown to increase lifespan in previous RNAi screens (Hamilton et al., 2005; Lee et al., 2003) and years later a tissue specific effect of *cco-1* RNAi was proposed for lifespan determination (Durieux et al., 2011). Remarkably, no complex IV mutants have been described to have a longevity phenotype. This observation has been linked to the idea of ROS as a positive modulator of lifespan according to the role of complex IV in OXPHOS, while it is considered rate limiting for the electron flow in the ETC (Arnold, 2012) it has no big impact in ROS formation (Quinlan et al., 2013) and therefore slight changes in complex IV activity may reduce respiration without affecting ROS production and having mild effect on longevity

Complex V: *atp-2*

atp-2 encodes the *C. elegans* ATP5B subunit of the ATP synthase. *atp-2(ua2)* mutants were described to be longlived. However, homozygote mutants arrested at L3 stage and although they were technically longlived, we cannot talk about lifespan extension of the adult lifespan in this case (Tsang et al., 2001). Like for other ETC subunit, knockdown of *atp-2* by RNAi also resulted in an extension of lifespan (Dillin et al., 2002).

1.5. The aim of the study

As above described, the role of mitochondrial (dys)function during aging and lifespan determination is a highly complex process. Our aim is to gain deeper insights regarding the molecular mechanism mediating this phenomenon, and for this purpose we make use of the longlived *C.elegans* mitochondrial mutant *isp-1;ctb-1*. The very first objective of this work was to identify novel mediators of mito-induced longevity with a RNAi feeding screen, which was previously performed in the Lab by Dr. Marija Herholz. The identification of the transcription factor KLF-1 as essential mediator of the lifespan of the mitochondrial mutant gave then rise to the establishment of the following objectives:

- To characterize KLF-1's role in the longevity assurance of *isp-1;ctb-1* mutants by identifying putative target genes.
- To investigate the role of KLF-1 in other longevity models and to define its possible role as conserved mediator of lifespan determination.
- To determine the mechanisms responsible for KLF-1 activation.

2. Material and methods

2.1. *C.elegans* methods

2.1.1. Strains and maintenance

Strains were grown at 20ºC on nematode growth media plates (NGM, see 2.6.2.), spread with *Escherichia coli* OP50, according to standard protocols unless otherwise stated (Brenner, 1974). The following strains were used in this study: Wild type Bristol N2, MQ887 *isp-1(qm150),* MQ989 *isp-1(qm150);ctb-1(qm189),*CB1370 *daf-2(e1370),* CB4037 *glp-1(e2141), klf-1(tm1110),* GR1373 *eri-1(mg366),* ATR2640 *isp-1(qm150);ctb-1(qm189);eri-1(mg366),* TK22 *mev-1(kn1)*, PP752 N2;*geIs10*[*ges-1(long)::skn-1c::gfp;rol-6(su1006)*], ATR4000 *isp-1(qm150);ctb-1(qm189)*;*geIs10,* CL2166 N2;*dvIs19*[*pgst-4::gfp-nls*], ATR1040 *isp-1(qm150);ctb-1(qm189);dvIs19*, *jrIs1* [*prpl-17::HyPer*], ATR1047 *klf-1(tm1110);jrIs1*, ATR1054 *isp-1(qm150);ctb-1(qm189);jrIs1*, ATR1051 *isp-1(qm150);ctb-1(qm189);klf-1(tm1110);jrIs1*, *jrIs2* [*prpl-17::Grx1-rogfp2*], ATR1049 *klf-1(tm1110);jrIs2*, ATR1045 *isp-1(qm150);ctb-1(qm189);jrIs2*, ATR1052 *isp-1(qm150);ctb-1(qm189);klf-1(tm1110);jrIs2*, TJ356 N2;*zIs356* [*pdaf-16::daf-16a/b-gfp;rol-6(su1006)*]. ATR1023 N2;*atEx100*[*pklf-1::gfp;rol-6(su1006)*], was generated by mixing the *pklf-1::gfp* (50ng/µl) and pRF4 (50ng/µl) plasmids and ATR1026 N2;*atEx101*[*pklf-1::klf-1-yfp;prab-3::mcherry;pmyo-3::mcherry*] by using *pklf-1::klf-1-yfp* (40ng/µl), pGH8 (20ng/µl), pCFJ104 (20ng/µl) and adding pBlueScript up to 100ng/µl final concentration. ATR1026 was crossed into *isp-1(qm150);ctb-1(qm189)* genetic background to create ATR1030 strain. ATR4006 N2;*atEx400*[*pklf-1::klf-1-yfp;prab-3::mcherry;rol-6(su1006)*] strain was generated by injecting *pklf-1::klf-1-yfp* (40ng/µl), pGH8 (20ng/µl), and pRF4 (40ng/µl) into N2 strain. This strain was subsequently integrated using UV-irradiation and outcrossed to create ATR4007 N2;*atIs100*[*pklf-1::klf-1-yfp;prab-3::mcherry;rol-6(su1006)*]. The *pklf-1::gfp* plasmid was designed by amplifying a 3kb upstream region of the *klf-1* gene and cloning it into the multiple cloning site of the pPD95.75 vector. *pklf-1::klf-1-yfp* was designed by amplifying *klf-1* cDNA and fusing it C-terminally with yfp, downstream of the *klf-1* promoter. ATR4026 *isp-1(qm150);ctb-*

1(qm189);atEx4026[*pcyp34a8::gfp;prab-3::mCherry*] and ATR4027 *isp-1(qm150);ctb-1(qm189);atEx4026*[*pcyp13a11::gfp;prab-3::mCherry*] were generated by injecting the respective *pcyp::gfp* constructs (50ng/µl), pGH8 (20ng/µl) and pBlueScript (30ng/µl) into *isp-1(qm150);ctb-1(qm189)* worms. ATR4026 and ATR4027 were subsequently backcrossed with N2 to generate ATR4030 N2; *atEx4026*and ATR4031N2; *atEx4027* respectively. KLF-1 mutant strains for phosphorylation sites were obtained by injection of *pklf-1::klf-1-yfp* constructs (40ng/µl) after specific site directed mutagenesis and injection into wt worms with pGH8(20ng/µl) and pRF4(40ng/µl):ATR4008 N2;*atEx4008*[*pklf-1::klf-1(S39A)-yfp;prab-3::mCherry; rol-6(su1006)*], ATR4017 N2;*atEx4017*[*pklf-1::klf-1(S39D)-yfp;prab-3::mCherry; rol-6(su1006)*], ATR4014 N2;*atEx4014*[*pklf-1::klf-1(S280D)-yfp;prab-3::mCherry; rol-6(su1006)*], ATR4021 N2;*atEx4021*[*pklf-1::klf-1(S280A)-yfp;prab-3::mCherry; rol-6(su1006)*]. SD1114 N2;*gaIs237* [*cyp-25A2p::his-24::mCherry + unc-119(+)*], ATR4028 *daf-2(e1370)*; *gaIs237,* ATR4029 *isp-1(qm150);ctb-1(qm189);gaIs237,* CY573 N2;bvIs(pcyp-35b1::gfp;pgcy-7::gfp)

2.1.2. Synchronization of worm populations

Worm populations were synchronized by hypochlorite treatment of gravid adult worms for extraction of the eggs. Worms were washed of the plates with M9 and bleaching solution (see 2.6.2) was added. After 10 minutes incubation with periodic vortexing the samples were spun down by centrifugation (3000 rpm, 1 min) 3 to 5 times adding fresh M9 every time to remove the bleaching solution. Egg suspension was then pipetted on NGM plates on the desired concentrations.

2.1.3. Microinjection and UV integration of transgenic strains

Injection mixes with a final concentration of 100 ng/ µl were prepared as indicated in the *C.elegans strains and maintenance* section, and injected using standard procedures (Mello et al., 1991). Briefly, constructs were injected into the gonad of young adults previously immobilized with mineral oil (Sigma-Aldrich, Seelze, Germany) using Zeiss Observer A.1 microscope equipped with AxioCam ERc5S camera (Zeiss, Oberkochen, Germany), Eppendorf InjectMan NIz injection pump and Eppendorf FemtoJet microinjector (Eppendorf, Hamburg, Germany). After

injection, single worms were transferred to fresh NGM plates and the appearance of transgenic progeny was monitored by following the expression of the coinjection markers (pGH8, pRF4). Strains stably expressing the constructs were used either expressing the constructs as extracromosomal array or further integrated by UV treatment (Evans, 2006; Mariol et al., 2013): transgene expressing L4 stage larvae were exposed to UV 300 to 400 joule/m^2 using a Stratalinker UV Crosslinker (Agilent Technologies, Waldbronn, Germany) and the expression of coinjection markers was followed for three generation until identification of homozygote integrated transgenic animals, which have a 100% transmission. After successful UV integration, strains were backcrossed at least ten times to remove mutation that may result from the UV exposure

2.1.4. RNAi treatment

RNA interference was performed as described previously (Kamath et al., 2003). All genes for RNAi were obtained from the Ahringer RNAi library (Kamath et al., 2003), unless otherwise stated and confirmed by sequencing: few clones not present in the Ahringer library were obtained from the Vidal library (Rual et al., 2004). The clone carrying the empty vector L4440 was used as control. All clones were transformed into the *E. coli* HT115 (DE3) strain. Bacterial cultures were grown in Luria broth media (see 2.6.2) until reaching OD_{595}=0.5, then Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Applichem, Darmstadt, Germany) was added to a concentration of 1mM. The bacteria were then induced for three hours at 37ºC, shaking and seeded on NGM plates containing 100 µg/ml ampicillin (Sigma-Aldrich, Seelze, Germany) and 1mM IPTG. Worms were treated with RNAi from hatching and phenotype was observed on the first or the fifth day of adulthood. In order to obtain five-day old worms, beginning from the first day of adulthood, worms were washed every day with M9 and allowed to settle by gravity, in order to remove eggs and lighter larvae.

- Chromosome III Ahringer library database available online at http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/c-elegans/c-elegans-rnai-library

- *C.elegans* kinase and phosphatases prediction list available at http://www.kinase.com
- Redox enzymes tested see table 2.1

Table 2.1. Redox enzymes RNAi clones

Cells marked in gray refer to clones obtained from the Vidal library.

PeroxiRedoxins PRDX		
prdx-3	R07E5.2	III-2O03
prdx-6	Y38c1AA.11	IV-10F05
ThioRedoxins TRX		
trx-1	B0228.5	II-5F02
trx-2	B0024.9	V-7M01
trx-3	M01H9.1	IV-2E16
trx-4	Y44E3A.3	I-1B03
ThioRedoxin Reducatases TRX*T*		
trxr-1	C06G3.7	IV-9D10
trxr-2	ZK63710	III-8D17
gluthathione peroxidase GPX		
gpx-1	F26E4.12	I-4L14
gpx-2	R05H10.5	11003 B4
gpx-3	C11E4.2	X-4D12
gpx-4	Y94H6A.4	IV-10F14
gpx-5	C11E4.1	X-4D10
gpx-6	T09A12.2	IV-4M01
gpx-7	R03G5.5	X-4E07
gpx-8	F55A3.5	I-5O10
GlutaRedoxin		
glrx-3	D2063.3	V-14K11
glrx-5	Y49E10.2	III-9A07
glrx-10	Y34D9A.6	10070 D7
glrx-22	C07G1.8	IV-9L01

2.1.5. Lifespan analysis

For lifespan analysis, worms were grown at 20ºC, with the exception of the screening (see below). For the experiments with paraquat (Sigma-Aldrich, Seelze, Germany), the drug was added to the plates at a concentration of 0.1mM. First day of adulthood was defined as day 1 of lifespan. Unless otherwise stated, 25 worms were transferred to each plate at day 0, for a total of 100-120 worms per experiment. Worms were examined every second day by prodding with a silver wire. The worms that escaped the plate, or died due to internal hatching or protrusions, were censored. For the switch experiments, worms were grown either on L4440, *klf-1*, *cyc-1*, *atp-5* or *cco-1* RNAi until the L4 larval stage or the third or fifth day of adulthood, as stated. Worms were then transferred to either L4440 or *klf-1* RNAi plates. Fort the experiments with *cyps* and *ugts*, worms were grown on the corresponding RNAi plates from hatching (*cyp-25a1, cyp-13a11, ugt-6* and *ugt-62*). Statistical analysis was done with Graph Pad Prism 5.0 and Log-Rank test (See Appendix A).

2.1.6. Screen for mediators of *isp-1;ctb-1* longevity in chromosome III

RNAi clones from chromosome III of the Ahringer library (Kamath et al., 2003) were inoculated in 384-deep well plates and grown overnight. Bacteria were then seeded on 24-well NGM plates containing 100μg/ml ampicillin (Sigma-Aldrich, Seelze, Germany) and 2mM IPTG (Sigma-Aldrich, Seelze, Germany). Each clone was seeded on quadruplicates induced overnight at room temperature. Afterwards, 15-20 eggs were placed into each well, and the worms were grown at 25ºC to activate the temperature sensitive mutation *eri-1(mg366)* to inhibit the generation of progeny and facilitate the screening process. As a control, *eri-1(mg366)* and *isp-1(qm150);ctb-1(qm189);eri-1(mg366)* strains were grown on a separate 24-well plate, on L4440. Nine to ten days after reaching adulthood, 50% of the *eri-1(mg366)* population was scored as dead. On that day, survival of *isp-1(qm150);ctb-1(qm189);eri-1(mg366)* was assayed and the RNAi clones that gave a similar phenotype as the *eri-1(mg366)* single mutant, were identified. Among all the candidates identified, transcription factors were retested using *eri-1(mg366)*

and *isp-1(qm150);ctb-1(qm189);eri-1(mg366)* strains, at 25ºC. Further KLF-1, the only candidate fulfilling the criteria, was tested at 20ºC in N2 and *isp-1(qm150);ctb-1(qm189)* strains.

2.1.7. KLF-1 and SKN-1 nuclear localization

Worms were grown on control plates (no drug) until first day of adulthood at then transferred to drug containing or control plates and nuclear localization was visualized after 24h: paraquat (Sigma-Aldrich, Seelze, Germany) (16, 18, 4, 1, 0,5 and 0,1 mM PQ) or antimycin A (Sigma-Aldrich, Seelze, Germany) (10, 5, 1, 0,5 and 0,1 µM AA). After the dose-response test all the following experiments where performed using 0,5 µM AA. The level of nuclear localization was divided into four categories attending to the number of intestinal nuclei visible in the worm: 0, <5, 5 to 10, >10. For the screen of factor involved in regulation of KLF-1 translocation into the nucleus the process was the same, growing the worms either on L4440 control or *candidate-gene* RNAi until day one of adulthood and then transferring to corresponding control or AA plates and visualized after 24h.

In the case of SKN-1 localization, the medium localization meant that one or two cells showed nuclear localization and high that there were more than 2 cells with nuclear localization. For the strains used see section 2.1.1.

2.1.8. Post embryonic development

Worms were exposed to RNAi from hatching and scored every 8 hours for vulva formation. Developmental time was defines as the total time passed from hatching until the vulva formation. Alternatively, the developmental stage of worms was assayed four days after hatching and presented as the percentage of total number of worms.

2.2. Molecular biology and biochemistry

2.2.1. Cloning

Cloning of promoter regions and/or cDNA for the generation of transgenic strains was performed using the bellow-listed primers (Table 2.1).

Table 2.2. Primers used for cloning

target	primer
***pklf-1* with PstI site**	Fw 5'-GGGCTGCAGGCCACACCCTTAGACCTTCTAAACCAC-3'
***pklf-1* with KpnI site**	Rv 5'-GGGGGTACCCGTGGATGACGTGGAATTGAATTGTAG-3'
***klf-1* cDNA with KpnI and EcoRI**	Fw 5'-GGGAATTCGGTACCATGACGTCATCATCGATA AAT-3'
***klf-1* cDNA w/o STOP with AgeI and NotI site**	Rv 5'-GGACCGGTTTGCGGCCGCCGAAGTGGCGTTTCATATGGAG-3'
***pcyp-34a8* with PsTI site**	Fw 5'-GGGCTGCAGATTTCATTGTCACCTCGTTT-3'
***pcyp-34a8* with KpnI site**	Rv 5'-GGGGGTACCATTAGTATCAGTAACATTT-3'
***pcyp-13a11* with KpnI site**	Fw 5'-GGGCTGCAGAGAATCTTCGATGTTCAT-3'
***pcyp-13a11* with KpnI site**	Rv 5'-GGGGGTACCAACAGTAGAATAATCATCCT-3'
KLF-1 S39A mutagenesis	Fw 5'-AGATCAGTACATTTG GCA CCGTCTTTT-3'
	Rv 5'-TTGAAAAAAAGACGG TGC CAAATGTAC-3'
KLF-1 S39D mutagenesis	Fw 5'-AGATCAGTACATTTGGATCCGTCTTTT-3'
	Rv 5'-TTGAAAAAAAGACGGATCCAAATGTAC-3'
KLF-1 S280A mutagenesis	Fw 5'-ATGGATAGTCCGACAGCTCCGTGTGTC-3'
	Rv 5'-TCTTTTGACACACGGAGCTGTCGGACT-3'
KLF-1 S280 mutagenesis	Fw 5'-ATGGATAGTCCGACAGATCCGTGTGTC-3'
	Rv 5'-TCTTTTGACACACGGATCTGTCGGACT-3'

Digestions were performed using restriction enzymes from New England Biolabs (Ipswich, USA) and all the inserts were cloned into the plasmid pPD95_75.

Using heat shock transformation of chemical competent *E.coli* DH5 bacteria followed by antibiotic selection, constructs were cloned and amplified. Plasmid extractions were done using StrataPrep Plasmid Miniprep Kit (Agilent Technologies, Waldbronn, Germany) following manufacturers instructions. Constructs were verified by restriction enzyme digestion and sequencing.

2.2.2. Genotyping of mutant strains

To confirm the genotype of new strains generated by crossing single adult worms (previously allowed to lay eggs in individual plated) were picked into PCR tubes containig 10 µl of worm lysis buffer (see 2.6.2) with proteinase K (Applichem, Darmstadt, Germany). After 10 min at -80° C to break the cuticles, samples where exposed to a cycle of 60 °C for one hour for protein degradation followed by 15 minutes at 95 ° C for proteinase K inactivation. 2 µl of the worm lysate were used as template for the PCR. When necessary, after PCR, the amplified products were digested with specific restriction enzymes (Table 2.3) (New England Biolabs Ipswich, USA). The *ctb-1* mutation has to be followed by ensuring that the *ctb-1* parental worm is the hermaphrodite form and not the male since the transmission is mitochondrial

Table 2.3. Primers used for genotyping

allele	primer	restriction
isp-1(qm150)	Fw 5'- CAAATCGCGAACTTTTCTTCA-3'	bsmAI
	Rv 5'-AACGTCGTGCTCTTCCAACT-3'	
daf-2(e1370)	Fw 5'-ATGATTCATCAATGCGTACT-3'	blpI
	Rv 5'-GTCAAACTGTACGGAGTT-3'	
klf-1(tm1110)	Fw 5'-GGG TGTCATTTGAAAGCTCTCAACGTGCTG-3'	
deletion	Rv 5'- GAATCGAATGAATCAGCCGAATTGGGACA -3'	
wt	Rv 5'-AGCACACTGAGAGCTTCAAAATAACACCC-3'	-

2.2.3. Polymerase Chain Reaction (PCR)

All the PCR reactions were performed using the Veriti 96 well Thermal cycler (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). The amplification of DNA fragments with cloning purposes where performed using proofreading Pfu DNA polymerase (Promega, Mannheim, Germany). For standard genotyping PCR DreamTaq DNA polymerase and buffer (Thermo Scientific, Braunschweig, Germany) were used. Amplifications were performed according to manufacturer's instructions in a final volume of 20 µl: 10.7 µl of dH_2, 0,.1 µl of DreamTaq polymerase, 3,2 µl of dNTPs (1.25 mM each), 1 µl of each primer (10 µM), and 2 µl of DreamTaq buffer and 2 µl of sample DNA.

PCR products were then analyzed by agarose (Roth, Karlsruhe, Germany) gel electrophoresis using Gene Ruler DNA Ladder Mix (Fermentas, Thermo Scientific, Braunschweig, Germany) and GelRed gel stain (Biotium, distributed by VWR International GmbH, Darmstadt, Germany). For extraction QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used according to manufacturers instructions.

2.2.4. Microarray analysis

RNA isolation

For microarray experiments, worms were grown on control L4440 plates until fifth day of adulthood. To separate the progeny from the aging worms, they were washed with M9, letting the adults settle by gravity and removing the larvae in the supernatant fraction. For *klf-1* RNAi , *isp-1;ctb-1* worms were grown either on *klf-1* RNAi plates from the eggs and then at the L4 stage switched to control L4440 plates to the fifth day of adulthood, or they were grown on L4440 plates and then switch to *klf-1* RNAi plates at the L4 larval stage. At the fifth day of adulthood, worms were collected from the plates with M9 buffer, and washed subsequently several times to remove bacteria. The pellet was resuspended in 1ml of TRIzol ® reagent (Life Technologies, Darmstadt, Germany). The worms were then transferred to a screw top tube together with 0.7mm zirconia/silica beads (Roth, Karlsruhe, Germany) (to approx. 0.2 ml mark) and beadbeat at maximum setting for 30 seconds with Precellys (Bertin Technologies, Versailles, France). The

homogenate was incubated for 5 minutes at room temperature. Then 200 µl of chloroform (Applichem, Darmstadt, Germany) and 100 µl of 1-bromo-3-chloro-propane (Sigma-Aldrich, Seelze, Germany) were added. The mixture was shaken vigorously for 15 seconds and then incubated on room temperature for 3 minutes. The homogenate was centrifuged at 12000g for 15 minutes at 4 °C. Upon centrifugation, the upper aqueous phase was transferred to a fresh tube and 1 volume of 70% ethanol (Applichem, Darmstadt, Germany) was added. The final steps of purifications were performed using RNAeasy mini spin kit according to manufacturer's recommendations (Qiagen, Hilden, Germany). Subsequently DNAse treatment was performed using DNAfree kit (Invitrogen, Thermo Scientific, Braunschweig, Germany) according to manufacturer's recommendations. For each condition five isolates were made and the quality of the preps was analyzed using Tapestation (Agilent Technologies, Waldbronn, Germany). The samples with 50-100ng/µl, OD260/230>1.5 and RIN>8 were used for microarray analysis, three for each condition.

Microarray analysis

Microarray was performed using GeneArray 1.0 ST (Affymetrix) columns. Three independent replicates were used for each condition. All Affymetrix protocols were performed at the Cologne Center for Genomics (CCG) at the University of Cologne, Germany. Unpaired T-test analysis was performed. The fold change cutoff of 2 and unpaired T-test cutoff of 0.01 was used to compare the WT control sample to *isp-1;ctb-1* control and *isp-1;ctb-1* control to *isp-1;ctb-1* adult switch to *klf-1* RNAi. For functional grouping of the genes which expression changed DAVID Bioinformatics Resources 6.7 (National Institute for Allergies and Infectiuos diseases (NIAID), NIH) was used. For clustering the genes and creating the heatmaps Cluster 3.0 (open source clustering software) was used. See Appendix B and C.

2.2.5. Quantification of gene expression by Real-Time qPCR

Isolation of RNA

Worms were collected from 9 cm plates into RNAse free Eppendorf tubes using at least 5 samples per strain and condition. RNA was isolated with TRIzol ® reagent (Life Technologies, Darmstadt, Germany). DNAse treatment was performed on the samples using Invitrogen DNAse Free Kit (Invitrogen, Thermo Scientific, Braunschweig, Germany). Treated supernatant were finally transferred to fresh RNAse free tubes and RNA concentration was measured using Nanodrop (Nanodrop Technologies, Wilmington, DE, USA).

cDNA synthesis and real-time PCR

Using the RNA extracts mentioned above and the Applied Biosystems high capacity cDNA reverse transcription kit cDNA synthesis was performed (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). Initial RNA concentration was 800ng in 10 µl DEPC water. The following PCR program was used for amplification: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, 4°C until ready for use. Final products were diluted in 30 µl DEPC water and all the samples were synthesized in duplicates. Finally quantitative PCR was done using 2µl of 1:2 diluted cDNA samples and 10 µl of Technologies Brilliant III Ultra Fast SYBR Green (Agilent Technologies, Waldbronn, Germany) qPCR Master Mix containing 0.6 µl forward primer (10 pmol/µl), 0.6 µl reverse primer (10 pmol/µl), 6 µl SYBR-Green, 0.18 µl ROX dye (diluted 1:50) and 2.62 µl DEPC water for each specific primer pair (Table 2.3) with the Applied Biosystems 7900HT Fast Real-Time PCR System 384 well (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany) and SDS 2.3 software (95°C for 3 min and 40 cycles of 95 °C for 5s and 60°C for 15s). Relative quantification was performed against either *act-1* or Y45F10D.4

Table 2.4. Primers used for qPCR

Gene	Primer
sod-1	Fw 5'-GGCGATCTAGGAAATGTGGA-3'
	Rv 5'-CTTCTGCCTTGTCTCCGACT-3'
sod-2	Fw 5'-GGTCTCCAAAGGAAACGTCA-3'
	Rv 5'-GATCCGAAGTCGCTCTTAATTGC-3'
sod-3	Fw 5'-TCGGTTCCCTGGATAACTTG-3'
	Rv 5'-AAAGTGGGACCATTCCTTCC-3'
ctl-1	Fw 5'-CGCAATCCACAGACTCACAT-3'
	Rv 5'-AACCTTGAGCAGGCTTGAAA-3'
ctl-3	Fw 5'-TCCTACACGGACACGCATTA-3'
	Rv 5'-CGGAAACTGTTCGGGAAGTA-3'
cyp-13a7	Fw 5'-AAAAATGGCAATGGGACAAG-3'
	Rv 5'-AATACTTTGAATATCGGTAG-3'
cyp-34a9	Fw 5'-AGCAAGGCAGAAACTTCCAA-3'
	Rv 5'-ACCTGTGCCCAAAAGTGTTC-3'
cyp-14a1	Fw 5'-CCTTTCTTGGGGTCTCATCA-3'
	Rv 5'-AAGTAGCGGCTTGGATTGAA-3'
cyp-14a3	Fw 5'-CAGGCACTGGAGACAAATCA-3'
	Rv 5'-GCAAAAGAGAATGGGGGATT-3'
cyp-13a11	Fw 5'-GCAAATTCTCGCCGTTGTAT-3'
	Rv 5'-TCGTCTCCTGATTCCCATCT-3'
ugt-6	Fw 5'-GGAGCACGAATGGAAATCAT-3'
	Rv 5'-TGGGAAAACCACTTGGCTAC-3'
ugt-62	Fw 5'-CGATGATGAAATGGGCTTCT-3'
	Rv 5'-CAATGTCGGACGAGGAAGAT-3'
C23G10.6	Fw 5'-AGAGGATCATAGTTATGTAC-3'
	Rv 5'-GAAGCAGATGGGAGAAGCAC-3'
F56A4.4	Fw 5'-CCGTGCCACAACTTTACTAC-3'
	Rv 5'-TTTCCTGCCTGTCCACCTTC-3'
Y45G12C.3	Fw 5'-TGCGTCATTTGGGAAGAGTT-3'
	Rv 5'-ACCTTGTCGGCCTTTCTCTT-3'
gst-4	Fw 5'-TGCTCAATGTGCCTTACGAG-3'
	Rv 5'-AGTTTTTCCAGCGAGTCCAA-3'
klf-1	Fw 5'-TAGTCCGACAAGTCCGTGTG-3'
	Rv 5'-TGAAGCCGTCAGTTCGTCTA-3'
skn-1	Fw 5'-ATCCACCAGCATCTCCATTC-3'
	Rv 5'-CGCTACTGTCGATTTCTCTGTC-3'
gcs-1	Fw 5'-AACCGTACCGCACATTGATT-3'

	Rv 5'-TCTTCAGCTTTGCACGACAC-3'
act-1	Fw 5'-TCGTCCTCGACTCTGGAGAT-3'
	Rv 5'-GCCATTTCTTGCTCGAAGTC-3'
Y45F10D.4	Fw 5'-GTCGCTTCAAATCAGTTCAGC-3'
	Rv 5'-GTTCTTGTCAAGTGATCCGACA-3'
klf-2	Fw 5'-AAAATGCCCATCACCAACAT-3'
	Rv 5'-TGTAGTGCTGCTCGATGACC-3'
klf-3	Fw 5'-TTCATTCGATGACTCCACCA-3'
	Rv 5'-AGGGTGAAGAGCGAGTTGAA-3'

2.2.6. Western blotting

For protein sample preparations, worms were collected from three full 9cm plates and wash extensively with M9 buffer, in order to remove the bacteria. The pellets were then frozen in liquid nitrogen. 200μl of lysis buffer (see 2.6.2.) were added to the pellet prior to thawing. Three freeze-thaw cycles were performed in liquid nitrogen, followed by sonication. The debris was spun down for 10 minutes at 12000 rpm/ 4^{o}C and the supernatant was transferred to a fresh tube. Protein concentration was measured with Bradford assay and protein samples were loaded in NuPAGE® Novex® 4-12% Bis-Tris Gels (Life Technologies, Darmstadt, Germany) and runned using MES buffer (see 2.6.2). Membrane transference was performed using iBlot® nitrocellulose Transfer Stack (Novex®) and iBlot® 7-Minute Blotting System (Life Technologies, Darmstadt, Germany). Primary antibodies were blocked with 5% milk (Applichem, Darmstadt, Germany) in TBST (see 2.6.2) incubated over night at 4°C and secondary antibodies at room temperature for 45min. Blot development was done by addition of ECL solution (GE Healthcare, Munich, Amersham) and image acquisition in ImageQuant LAS4000 reader (GE Healthcare) chemiluminescent immunodetection system. Western Blotting was performed using antibodies against MnSOD (Upstate, Millipore, Darmstadt, Germany) and Tubulin (Calbiochem Millipore, Darmstadt, Germany). Detection of carbonylated proteins was performed using Oxyblot Protein Oxidation Detection Kit (Merck Millipore, Darmstadt, Germany) following manufacturer's instruction and the data was normalized to Ponceau S (Sigma-Aldrich, Seelze, Germany) staining of the membrane.

2.2.7. ROS measurements

DCFDA

ROS relative quantification was performed using standard protocols (Arczewska et al., 2013; Lee et al., 2010). Samples of approximately 1000 synchronized adult worms were collected by extensively washing with S-Basal (see 2.6.2). The samples were immediately frozen in liquid nitrogen and the worm pellets were resuspended in a final volume of 200 µl of S-Basal and protein inhibitor cocktail. From this point on, the samples were kept on ice. Samples were sonicated with 50% (vol/vol) zirconia/silica beads (Roth, Karlsruhe, Germany) and bead beaten twice for twenty seconds in with Precellys (Bertin Technologies, Versailles, France). The worm lysates were spun in a microcentrifuge at 4°C at 14000 g. Supernatant were transferred to clean Eppendorf tubes and protein quantification was performed with Bradford (Sigma-Aldrich, Seelze, Germany) assay. ROS measurement was conducted in flat bottom 96-well dark plates with Paradigm TM detection Platform (Beckman Coulter GmbH, Krefeld, Germany) 50 µg nematode extracts incubated in 500 µl of a 5 µM DCFDA (Life Technologies, Darmstadt, Germany) solution in S-Basal plus protease inhibitor cocktail (Roche, Basel, Switzerland). Each sample was read in triplicate (150 µl each) at excitation and emission wavelengths of 485 and 528 nm, respectively. Readings were performed over 2 h every 15 minutes.

Mitochondrial dyes

The mitochondrial stainings were performed in vivo following a protocol adapted from (Zarse et al., 2012). Synchronized populations of adult worms were collected by extensively washing with S-Basal and transferred into Eppendorf tubes and let settle by gravity. 1mM working stocks of all three mitochondrial stains were done by dissolving the dyes with DMSO (Merck, Darmstadt, Germany) according to manufactures' indications (Invitrogen Karlsruhe, Germany). The worms were incubated for 10 minutes with a suspension of previously heat inactivated *E.coli* OP50 with 5 µM concentrations of the mitochondrial dyes with gently shaking (VWR Microplate shaker). After the incubation time the worms were extensively washed with S-Basal and transferred to fresh NGM plates seeded with OP50, to

remove excessive dye, and incubated for at least three hours at 20°C. Worms were finally collected into 50 ml falcon tubes in a volume of approximately 10mL and fluorescence was measured by using BioSorter (Union Biometrica, Geel, Belgium)(See 2.4.).

Genetically encoded reporter proteins

Synchronized population of reporter strains were imaged at day five and/or day one of adulthood using Perkin Elmer Spinning Disk Microscope (see 2.3.) and fluorescence was quantify by using Image J software.

2.3. Microscopy

General handling and observation of nematodes was performed using Olympus KL 1500 compact stereomicroscopes. For analysis of ROS production with transgenic reporter strains animals were immobilized on 2% agarose (Roth, Karlsruhe, Germany) pads in M9 buffer. Paralyzing agents were not used, in order to avoid ROS induction in the worms that may lead to false results. Imaging of *jrIs1 [prpl-17::HyPer]* and *jrIs2 [prpl-17::Grx1-rogfp2]* strains was performed using a Perkin Elmer Spinning Disk Microscope with a 20x objective. Stacks of regions of the anterior part of the worm were taken and compiled to generate maximum intensity projections, using Volocity software. The rest of the imaging (see strains 2.1.1) was performed using an AxioImager Z.1 epifluorescence microscope, equipped with a Hammamatsu camera (OrcaR^2) and AxioVision software 4.8. Images were analyzed using ImageJ (National Institutes of Health)

2.4. Large Particle Flow Cytometry

Quantification of fluorescent intensity in living transgenic reporter strains was done alternatively by large particle flow cytometry with BioSorter (Union Biometrica, Geel, Belgium). Worm samples were collected from the plates with M9 and transferred to 50 mL Falcon tubes, which are place in the BioSorter for analysis. The data obtained from the cytometer, refer to a value of fluorescence (Red, Yellow, Green) and specific size or Time Of Flight of each worm. Samples

from at least 100 to 300 worms were analyzed for each strain and condition. Statistical analysis was done with Graphpad Prism 5.0.

2.5. Software, databases and digital resources

This work was written using OS X Yosemite 10.10.5 in a MacBook Air (Apple Inc.) using standard software for data analysis and text preparation: Microsoft Office 2011 (Microsoft Corp.), EndNote X6 (Thomson Reuters), Adobe Illustrator CS6 (Adobe Systems Inc.), ImageJ (National Institutes of Health), AxioVision (Carl Zeiss), ApE plasmid editor (free software, M.Wayne Davis). All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc). The functional grouping of the genes identified by microarray was done using DAVID Bioinformatics Resources 6.7 (National Institute for Allergies and Infectiuos diseases (NIAID), NIH) (Huang da et al., 2009). For clustering the genes and creating the heatmaps Cluster 3.0 (open source clustering software) was used. Information about *C.elegans* genes, sequences and proteins was obtained from the Wormbase (http://www.wormbase.org/). *C.elegans* kinase and phosphatase predictions are available online at http://www.kinase.com (Salk Institute For Biological Studies). Scientific literature was obtained using the database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Generic and kinase specific phosphorylation sites predictions were obtained using NetPhos2.0 Server and NetPhosk1.0 Server available online (http://www.cbs.dtu.dk) (Center for Biological Sequence Analysis, Technical University of Denmark). Prediction of mitochondrial targeting sequences was obtained by using free software MITOPROT available at (ftp://ftp.biologie.ens.fr/pub/molbio)(Claros and Vincens, 1996). The sequencing of DNA fragments and plasmids was routinely performed by GATC Biotech (https://www.gatc-biotech.com).

2.6. Statistical Analysis

All statistical analysis were done using GraphPad Prism 5.0 (GraphPad Software, Inc). To determine statistical significance, a two-tailed unpaired student's t-test

was generaly used. Fort he analysis of lifespan experiments Log-Rank analysis was performed. The p values below 0.05 were considered significant. Error bars represent standard error of the mean (S.E.M.) *p<0.05; **p<0.01; ***p<0.001 compared to control

2.7. Chemicals, equipment and biological materials

2.7.1. Chemicals and biological material

For PCR reactions, Pfu DNA polymerase (Promega, Mannheim, Germany), and DreamTaq DNA polymerase and buffer (Thermo Scientific, Braunschweig, Germany) were used. Size markers for agarose gel electrophoresis (Gene Ruler DNA Ladder Mix) and for SDS-PAGE (Page Ruler Prestained Protein Ladder Mix) were from (Thermo Scientific, Braunschweig, Germany.) and for DNA stain GelRed (Biotium, distributed by VWR International GmbH, Darmstadt, Germany) was used. Restriction enzymes were from (New England Biolabs, Ipswich, USA).

Following commercially available Kits were used: DNA extraction QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), RNAeasy mini spin kit (Qiagen, Hilden, Germany), DNAfree kit (Invitrogen, Thermo Scientific, Braunschweig, Germany), high capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany), Brilliant III Ultra Fast SYBR Green (Agilent Technologies, Waldbronn, Germany), Oxyblot Protein Oxidation Detection Kit (Merck Millipore, Darmstadt, Germany) and precasted NuPAGE® Novex® 4-12% Bis-Tris Gels (Life Technologies, Darmstadt, Germany). All other chemicals used in this work are listed in table 2.5.

Table 2.5. Chemicals used and their suppliers

Chemical	Supplier
1-bromo-3-chloro-propane	Sigma-Aldrich, Seelze, Germany
2- [N-morpholino] ehtanesulfonic	Roth, Karlsruhe, Germany
Agarose Neo Ultra	Roth, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Seelze, Germany
Antimycin A	Sigma-Aldrich, Seelze, Germany

Bradford Reagent	Sigma-Aldrich, Seelze, Germany
Calcium chloride	Merck, Darmstadt, Germany
Chloroform	Applichem, Darmstadt, Germany
Cholesterol 95%	Sigma-Aldrich, Seelze, Germany
DCFDA	Life Technologies, Darmstadt, Germany
Deoxynucleotides (dNTPs)	Sigma-Aldrich, Seelze, Germany
Dimethylsulfoxide (DMSO)	Merck, Darmstadt, Germany
Disodium phosphate	Sigma-Aldrich, Seelze, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Seelze, Germany
ECL solution	GE Healthcare, Munich, Amersham
EDTA	Sigma-Aldrich, Seelze, Germany
Ethanol	Applichem, Darmstadt, Germany
Gelatin	Sigma-Aldrich, Seelze, Germany
Hydrochloric acid	Applichem, Darmstadt, Germany
IPTG	Applichem, Darmstadt, Germany
Magnesium chloride	Sigma-Aldrich, Seelze, Germany
Magnesium sulfate anhydrous	Merck, Darmstadt, Germany
Mineral oil	Sigma-Aldrich, Seelze, Germany
MitoSox	Invitrogen Karlsruhe, Germany
MitoTracker CMH_2XROS	Invitrogen Karlsruhe, Germany
MitoTracker Deep Red	Invitrogen Karlsruhe, Germany
Monopotassium phosphate	Sigma-Aldrich, Seelze, Germany
Nitrogen (liquid)	Linde, Pullach, Germany
Non-fat Dried Milk Powder	Applichem, Darmstadt, Germany
Nonidet P40 (NP-40)	Sigma-Aldrich, Seelze, Germany
Nystatin Powder	Sigma-Aldrich, Seelze, Germany
Paraquat	Sigma-Aldrich, Seelze, Germany
Pepton	Merck, Darmstadt, Germany
PonceauS	Sigma-Aldrich, Seelze, Germany

Protease Inhibitor Cocktail Tablets	Roche, Basel, Switzerland
Proteinase K	Applichem, Darmstadt, Germany
Sodium chloride	Sigma-Aldrich, Seelze, Germany
Sodium dodecyl sulfate (SDS)	Applichem, Darmstadt, Germany
Sodium hydroxide	Sigma-Aldrich, Seelze, Germany
Sodium hypochlorite 14%	VWR International GmbH, Darmstadt,
Tetracycline Hydrocholride	Applichem, Darmstadt, Germany
TrishydroxymethylaminomethaneT	Applichem, Darmstadt, Germany
Trizma Base	Sigma-Aldrich, Seelze, Germany
Trizol Reagent	Life Technologies, Darmstadt, Germany
Tween-20	VWR International GmbH, Darmstadt,
Yeast extract granulate for	Merck, Darmstadt, Germany

2.7.2. Buffers and solutions

All solutions and buffers used in this work are listed in table 2.5.

Table 2.6. Buffers and solutions

Bleaching Solution		
	Amount	Ingredient
For 3.5 ml Sample	0.356 ml	Sodium hypochlorite, 14% active chlorine
	0.5 ml	5M NaOH
KPI Buffer pH 6.0		
	Amount	Ingredient
	66 ml	K_2HPO_4 (1 M)
	434 ml	KH_2PO_4 (1 M)
LB Medium		
	Amount	Ingredient
	10 g	Pepton
	3 g	Yeast Extract
	5 g	NaCl

	Up to 1 L	H_2O
M9 Buffer		
	Amount	Ingredient
	3 g	K_2HPO_4
	6 g	Na_2HPO_4
	5 g	NaCl
	Up to 1 L	H_2O
After autoclave	1 ml	1M $MgSO_4$ (1 M)
S-Basal		
	Amount	Ingredient
	5.9 g	NaCl
	Up to 1L	H_2O
After autoclave	50 ml	KPI
	1 ml	Cholesterol
MES Running Buffer pH 7.3		
	Amount	Ingredient
	50 mM	MES
	50 mM	Tris Base
	1 Mm	EDTA
	0.10%	SDS
Single Worm Lysis Buffer		
	Amount	Ingredient
	50 mM	KCl
	10 mM	Tris pH 8.3
	2.5 mM	$MgCl_2$
	0.45%	NP-40
	0.45%	Tween-20
	0.01%	Gelatin
Autoclave and store at -20 °C		
Lysis Buffer for western blotting pH 7.4		
	Amount	Ingredient
	25 mM	Tris-HCl
	0.15M	NaCl
	1 mM	EDTA

	1%	NP-40
	0.5%	SDS
	10mM	DTT
1x Protease inhibitor cocktail		
10X TBS Buffer pH 7.6		
	Amount	Ingredient
Part 1	24 g	Tris Base
	88 g	NaCl
	Up to 1L	H_2O
For TBST	100 ml	10X TBS Buffer
	900 ml	H_2O
	500 µl	TWEEN-20
NGM Medium		
	Amount	Ingredient
	3 g	NaCl
	2.5 g	Peptone (Tryptone)
	17 g	Agarose
	Up tp 1L	H_2O
After autoclave	1 ml	$MgSO_4$
	1 ml	$CaCl_2$
	25 ml	KPI
	1 ml	Nystatin
	1 ml	Cholesterol
For RNAi	2 ml	Ampicilin (50mg/ml)
also add:	1 ml	Tetracycline (25mg/ml)
	5 ml	IPTG (0.2 M)

2.7.3. Equipment and consumables

All standard plastic consumables were supplied by Sarstedt (Nümbrecht, Germany) and VWR International GmbH (Darmstadt, Germany). Following equipment was used: Thermo scientific Genesys 10UV scanning spectrophotometer (Darmstadt, Germany), Eppendorf Centrifuges 5424, 5430R and 5810R (Hamburg, Germany), Veriti 96 well Thermal cycler , StepOne Plus real-time PCR system and 7900HT Fast Real-Time PCR System 384 well (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany), Stratalinker UV Crosslinker (Agilent Technologies, Waldbronn, Germany), Precellys (Bertin Technologies, Versailles, France), ImageQuant LAS4000 reader (GE Healthcare, Munich Germany), Nanodrop (Nanodrop Technologies, Wilmington, DE, USA).Paradigm TM detection Platform (Beckman Coulter GmbH, Krefeld, Germany), iBlot® transfer system (Life Technologies, Darmstadt, Germany), Zeiss Observer A.1 microscope equipped with AxioCam ERc5S camera (Zeiss, Oberkochen, Germany), Eppendorf InjectMan NIz injection pump and Eppendorf FemtoJet microinjector (Eppendorf, Hamburg, Germany), Olympus KL 1500 compact stereomicroscopes, AxioImager Z.1 epifluorescence microscope, equipped with a Hammamatsu camera (OrcaR2) and large particle flow cytometer BioSorter (Union Biometrica, Geel, Belgium).

3. Results

3.1. KLF-1 activity exclusively during adulthood is essential for longevity of mitochondrial mutants

In order to uncover the mechanisms responsible for mito-induced longevity, a genome-wide RNAi screen was performed in the lab by Dr. Marija Herholz (unpublished data). The screen was started with the chromosome III of an existing feeding library (Kamath et al., 2003) and using the above described long lived mitochondrial mutant *isp-1;ctb-1.* We looked for genes that, when knocked-down, suppressed the longevity of the mutant without affecting the lifespan of the wild type, to rule out the possibility of identifying genes involved in pathways other than mitochondrial. Transcription factors were in the spotlight of the screen, since they are central players in cellular signaling that gather information and orchestrate specific responses. The only candidate that fulfilled our criteria was the Krüppel-like transcription factor KLF-1 (Figure 3.1), which was recently involved in longevity assurance induced by dieraty restriction (Carrano et al., 2014).

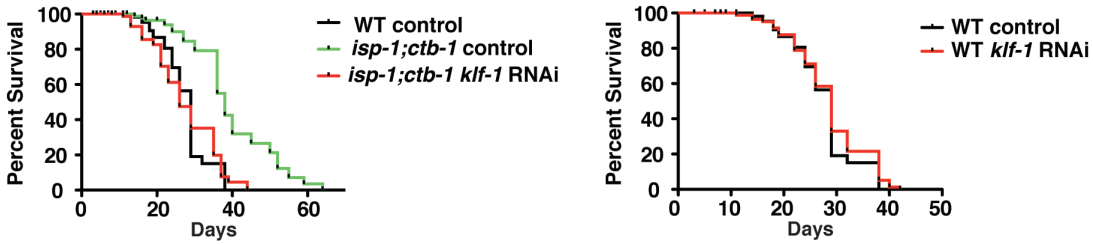

Figure 3.1. KLF-1 is needed for the longevity of *isp-1;ctb-1* in *C.elegans*
Lifespan analysis of wild type worms and *isp-1(qm150);ctb-1(qm189)* mutants treated with RNAi targeting *klf-1* gene. Adopted from Dr. Marija Herholz.

Interestingly, in addition to the suppression of the longevity of the double mit-mutant *isp-1;ctb-1*, we also observed an impact in the longevity of the single mutant *isp-1(qm150)* as well as in the short lived complex II mutant *mev-1(kn1)*

(data not shown). This result is in agreement with our previous observation that both long- and shortlived mutants activate the same longevity-assurance responses (Pujol et al., 2013).

Developmental suppression of subunits of the MRC has been shown to be capable of extending lifespan (Dillin et al., 2002). Dillin and colleagues showed that due to the mild mitochondrial dysfunction caused by RNAi of some MRC subunits, the respiration and ATP production rates were reduced, thus leading to an extension of lifespan in *C.elegans*. They proposed that *C. elegans* possesses a regulatory system that senses, interprets, and remembers the rate of mitochondrial respiration during development that is key for the determination of lifespan (Dillin et al., 2002). Remarkably, the timing and intensity of the mitochondrial dysfunction has to be tightly regulated in order to result in lifespan extension and not being detrimental (Rea et al., 2007).

In order to determine whether KLF-1 plays a role in this process, we downregulated different components of the MRC until the L4 larval stage and then transferred them either to control (L4440) or *klf-1* RNAi plates at day one of adulthood. We selected subunits of different complexes *cyc-1* (complex III), *cco-1*(complex IV) and *atp-5* (complex V), and confirmed that indeed, knocking down *klf-1* during adulthood was sufficient to decreased the longevity induced by the developmental suppression of MRC subunits (Figure 3.2.A) as well as by the *isp-1;ctb-1* mutations (Figure 3.2.B). Altogether these results reinforce the role of KLF-1 in mito-induced longevity. Conversely, developmental suppression of *klf-1* had no impact in longevity of *isp-1;ctb-1* mutants suggesting that KLF-1 is needed during adulthood in order to exert its beneficial effects on longevity (Figure 3.2.C). Additionally and in agreement with KLF-1 being needed during adulthood, we observed a high upregulation of *klf-1* expression in *isp-1;ctb-1* mutants at day one of adulthood (not shown).

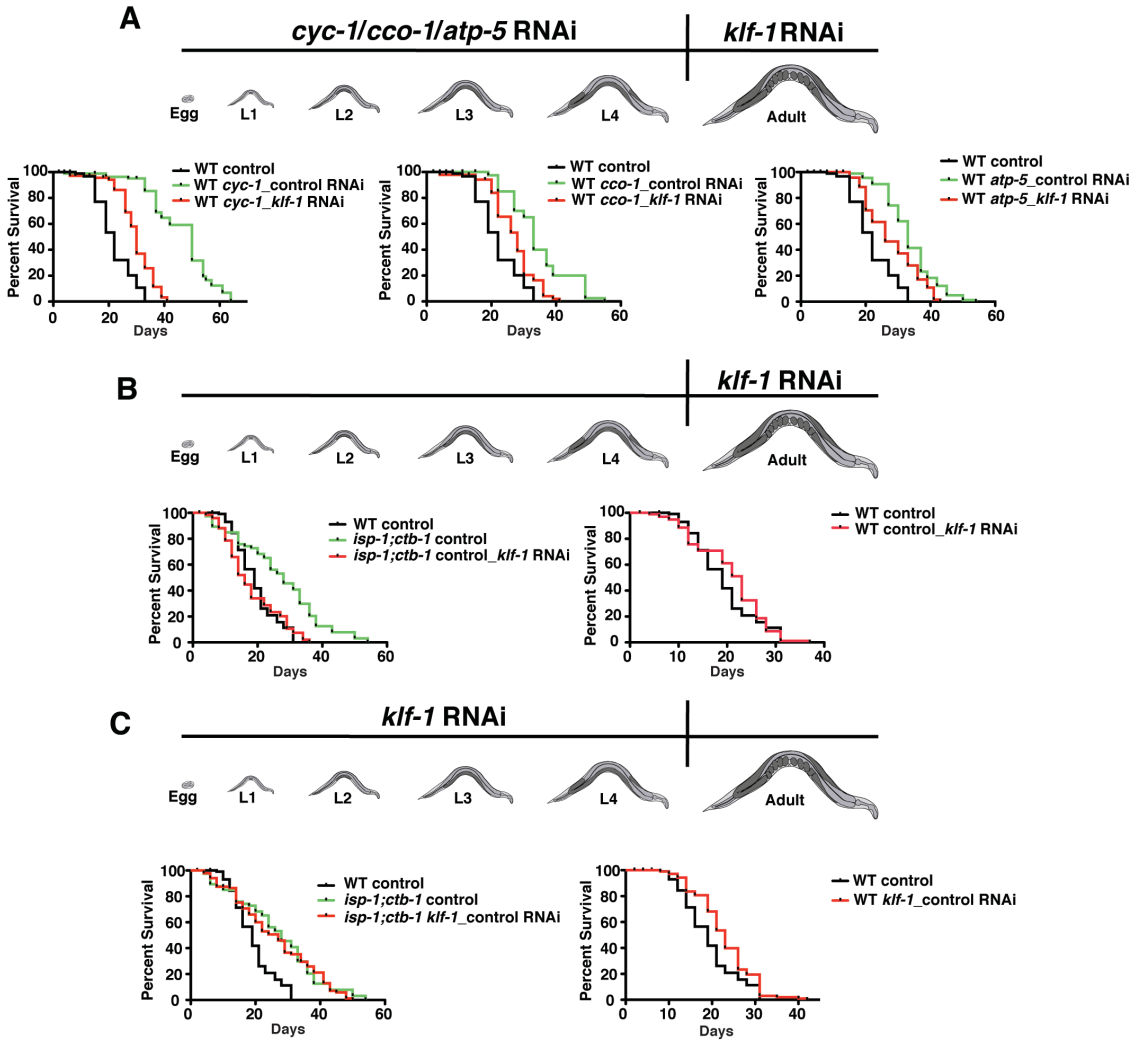

Figure. 3.2 KLF-1 is needed during adulthood to mediate mito-induced longevity
The scheme above the lifespan curves indicates when the different RNAi treatments were performed: development (egg to late larval stage L4) or adulthood. **A)** Lifespan analysis of wild type worms treated with RNAi targeting *cyc-1*, *cco-1*, or *atp-5* genes during development (until late L4 stage). The adult worms were then transferred to control plates containing bacteria expressing the empty vector (L4440) or to *klf-1* RNAi plates. **B** and **C**) lifespan analysis of wild type and *isp-1;ctb-1* worms treated with *klf-1* RNAi exclusively during adulthood (**B**) or development (**C**). Adopted from Marija Herholz.

3.2. KLF-1 is essential for the mitohormetic response to oxidative stress

In 2001 *isp-1;ctb-1* mutants were first described and implicated in lifespan extension. In agreement with the ROS-theory of aging, ROS produces damage, whose accumulation leads to aging phenotypes. Therefore, an increased protection against ROS should increase lifespan: Feng and colleagues proposed this mechanisms as the determinant of *isp-1;ctb-1* longevity. They showed that *isp-1*

and *isp-1;ctb-1* mutants exhibit a low oxygen consumption compared to wild type as well as a decreased sensitivity to ROS. Moreover, they showed that combination with a mutation (*daf-2*), which increases resistance to ROS, did not result in significant further increase in adult life span. Therefore they proposed that both *isp-1* and *daf-2* mutations increase life span by lowering oxidative stress (Feng et al., 2001).

Work done by the same lab years later (Yang and Hekimi, 2010a) postulated an alternative explanation for the *isp-1;ctb-1* longevity: they showed that *isp-1;ctb-1* mutants exhibit a mitochondrial-specific increase in superoxide but not in overall ROS production. They proposed that this increase in superoxide acts as a signal that triggers a beneficial protective response that leads to longevity. Therefore we took a closer look at the oxidative stress response and ROS production in our double mutant *isp-1;ctb-1,* to decipher the role that KLF-1 may play on it.

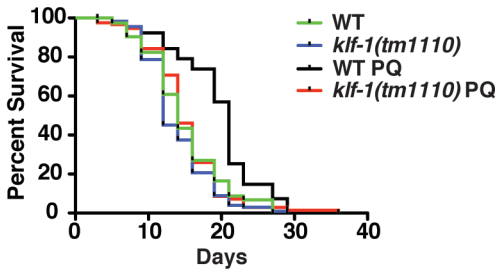

Figure 3.3. KLF-1 mediates oxidative-stress-induced longevity
Lifespan analysis of wild type (WT) and *klf-1(tm1110)* mutant worms grown on control plates or on plates containing 0.1mM PQ. Adopted from Marija Herholz.

Exposure to low concentrations of prooxidants like antimycin A (AA) (Boveris and Cadenas, 1975; Turrens, 1997) or paraquat (PQ) (Cocheme and Murphy, 2008), results in a lifespan extension of the wild type trough the same mechanism proposed for the mitochondrial mutants (Yang and Hekimi, 2010a). We were able to reproduce this result and also showed that lifespan extension trough PQ is completely abolished when performed on a *klf-1(tm1110)* mutant background (Figure 3.3). This result clearly shows that KLF-1 is essential for the oxidative stress-induced longevity. Additionally Yang and Hekimi (2010) demonstrated that the lifespan of the *isp-1;ctb-1* mutant can not be further

increased with exposure to low concentrations of PQ, suggesting that the mechanism responsible for longevity assurance is the same.

The analysis of carbonylated proteins is broadly accepted as an indirect measurement of oxidative stress. However it is worthwhile noting that protein carbonylation is characteristic of an irreversible oxidative damage, and therefore implies that the term oxidative stress refers to an intrinsically deleterious event. Therefore, more precisely, carbonylated proteins can be used as readout for oxidative damage that can occur due to an intense and/or prolonged oxidative stress. We measured the level of these carbonylated proteins using OxyBlot (Merck Millipore, Darmstadt, Germany) and observed that there is an increase of protein carbonilation with age in wild type worms, whereas the *isp-1;ctb-1* mutants exhibit an opposite trend: the levels of oxidative damage are high in one-day-old worms and they decrease with age until almost equaling the levels of young wild type controls (Figure 3.4.A). At day five of adulthood the damage can be further increased upon *klf-1* knockdown in both wild type and *isp-1;ctb-1* mutants (Figure 3.4.A).

Based on the fact that all our results pointed to a role of KLF-1 in responding to oxidative stress, we generated a transgenic reporter strain in order to analyze the subcellular localization of KLF-1 upon exposure to pro-oxidants. Our protein of interest KLF-1, was tagged with yellow fluorescent protein (YFP) in the C' terminus and expressed under the control of its own endogenous promoter (*pklf-1::klf-1-yfp*). The wild type worms were exposed to different concentrations of PQ and AA at day one, and the nuclear localization of KLF-1 was scored approximately 24 hours later. With both drugs the nuclear localization appear to follow a directly proportional trend to the drug concentration (Figure 3.4.B.). The only exception was 16mM PQ, which is a very high concentration frequently used to assess resistance to high oxidative stress. In this case the concentration was too high and the worms looked impaired and exhibited an *egl* phenotype characterized by the congestion of the adult hermaphrodite with an excess of fertilized eggs due to a decrease in the rate of egg-laying. The excess of eggs precludes the observation of the intestinal cells.

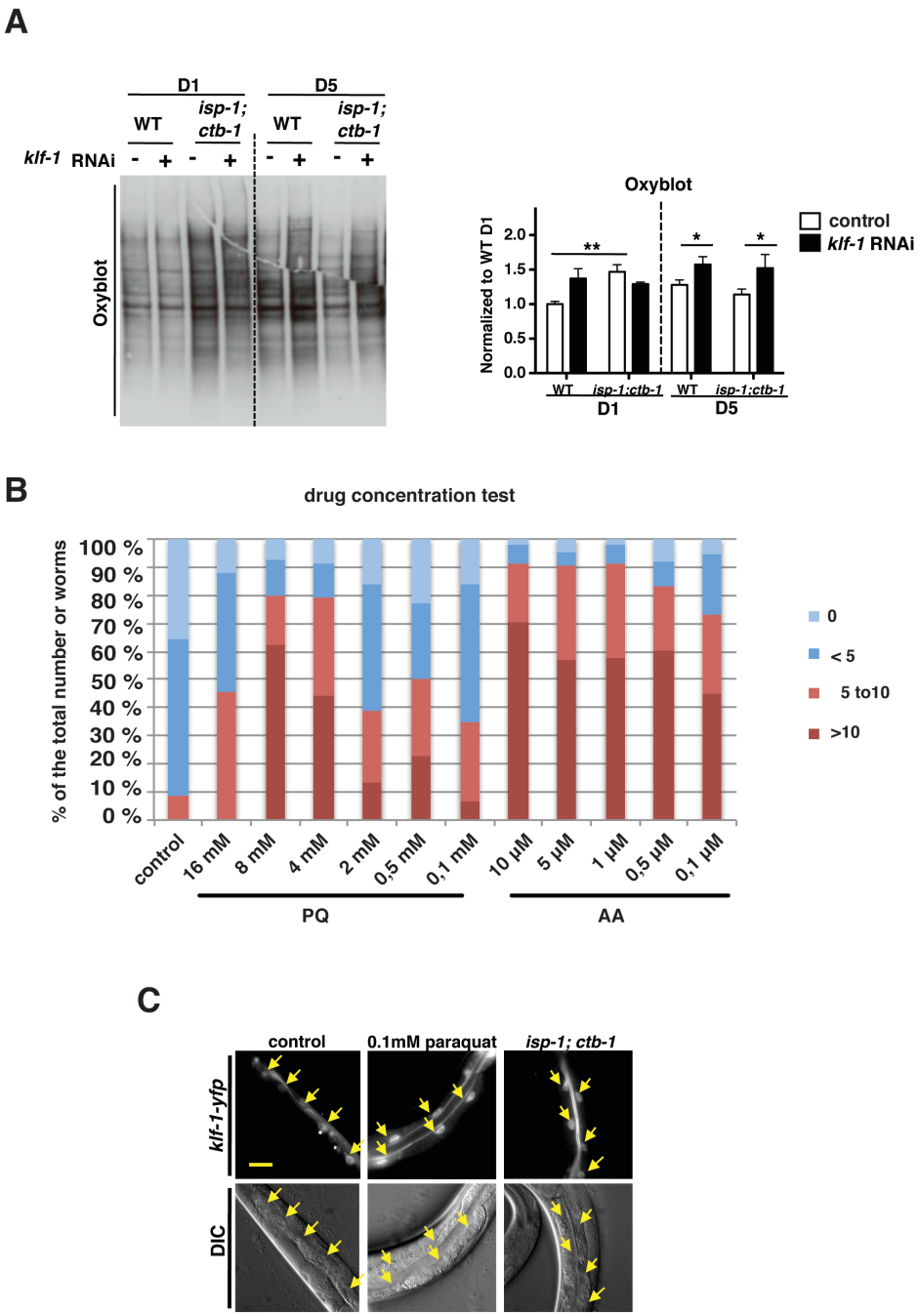

Figure 3.4. KLF-1 responds to oxidative stress
A) Analysis of protein carbonylation leves via OxyBlot in wild type and *isp-1;ctb-1* worms at day one of adulthood (D1) and day five (D5) upon exposure to *klf-1* knockdown (+) or feeding bacteria expressing the empty vector control L4440 (-). On the left the blot and on its left the quantification and statistical analysis. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, Student's T-test) (n=6) Adopted from Marija Herholz **B**) Analysis of the cellular localization of the KLF-1 protein in wild type worms grown on plates containing different concentrations of paraquat (16, 18, 4, 1, 0,5 and 0,1 mM PQ) or antimycin A (10, 5, 1, 0,5 and 0,1 µM AA) expressing the construct *pklf-1::klf-1-yfp*. Scoring indicates the percentage of worms exhibiting different amounts of nuclei visible (none, less than five, five to ten or more than 10 nuclei per worm) **C**) Representative images of KLF-1 subcellular localization in wild type and *isp-1;ctb-1* worms expressing *pklf-1::klf-1-yfp* under control (no drug) or low PQ exposure.

Figure 3.4.C. depicts how under control conditions in the wild type, KLF-1 was mostly localized to the cytosol and upon exposure to mild oxidative conditions, it translocated to the nucleus. In a similar way, and in agreement with the idea that mitochondrial mutants induce longevity through a pathway that overlaps with oxidative stress signaling, we observed nuclear localization of KLF-1 in the *isp-1;ctb-1* mutant without any drug treatment (Figure 3.4.C.).

Taken together, our results (Figures 3.1 to 3.4) strongly suggest that oxidative stress could be the signal that triggers the KLF-1-mediated longevity in the *isp-1;ctb-1* mutants. Moreover these results are consistent with the concept of mitohormesis, where exposure to low levels of a certain stressor like mitochondrial ROS (mtROS), confers an enhanced resistance to the same stressor later in life (Yun and Finkel, 2014).

3.3. Mitochondrial ROS activates an antioxidant response in *isp-1;ctb-1* mutants

After confirming that KLF-1 reacts to pro-oxidants by changing its subcellular localization, and that the same response is observed in *isp-1;ctb-1* mutants, we asked ourselves where this oxidative stress signal is coming from: Is the ROS production increased in *isp-1;ctb-1* mutants? Or is there a decreased capacity to eliminate equal levels of ROS? Maybe a combination of both events?

We proceeded analyzing the expression levels of the major ROS scavengers super oxide dismutases (SODs) and catalases (CTLs). SODs convert superoxide in hydrogen peroxide, and the latter is further degraded to water and oxygen by CTLs. The increase in oxidative stress at day one in *isp-1;ctb-1* mutants, is accompanied by an upregulation tendency (just statistically significant for *sod-3*) of *sod* transcripts that is KLF-1 dependent (Figure 3.5.A.). Remarkably, the protein levels of SOD-2, a major mitochondrial antioxidant enzyme, are upregulated at day one (Figure 3.5.B.). Conversely, *klf-1* does not seem to regulate the expression levels of catalases (Figure 3.5.C.).

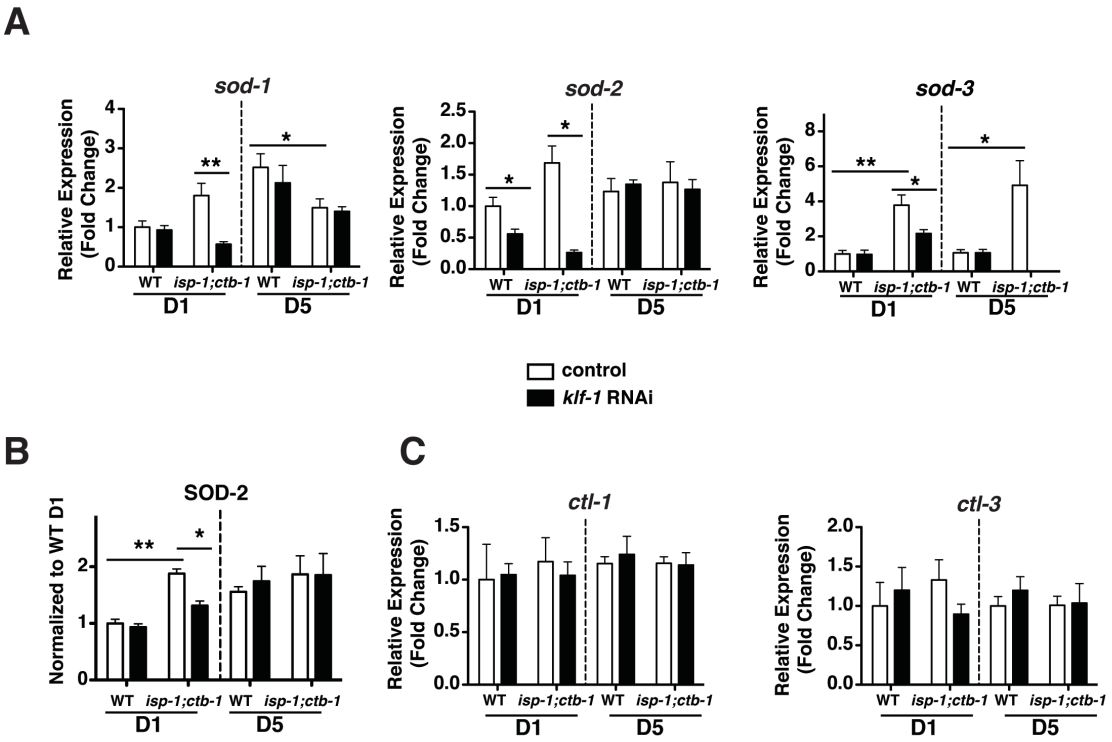

Figure 3.5. *isp-1;ctb-1* mutations induce the overexpression of *sods* but not *ctls*
Expression levels measured by qPCR of the **(A)** *sods* **(C)** *ctls* and quantification of protein levels via Western Blot of **(B)** at day one (D1) and day five (D5) of adulthood of *isp-1;ctb-1* and wild type worms fed bacteria expressing RNAi targeting the *klf-1* gene or the empty vector L4440 (control). Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test) (n=5).

In order to further determine the origin of the oxidative-stress signal we proceeded by analyzing the production of ROS by several methods. Currently, a great selection of fluorescent sensors is available for compartmentalized ROS detection. They can be mostly divided into two groups: small molecule probes and genetically encoded fluorescent proteins.

Starting with the latter group, we measured the levels of H_2O_2 using HyPer-expressing strains. Hyper is a peroxide-specific sensor protein that consists of a circularly permuted yellow fluorescent protein fused to the H_2O_2-sensing domain of *E. coli* OxyR (Back et al., 2012; Belousov et al., 2006). Upon exposure to H_2O_2, HyPer suffers a conformational change that results in a ratiomatric shift. With increasing levels of peroxides the oxidation/reduction ratio increases and can be use as readout for intracellular H_2O_2 production. We observed reduced H_2O_2 levels in the *isp-1;ctb-1* mutants compared to wild type worms at both day one and day five of adulthood, being the latter more pronounced. However, the

oxidized/reduced ratio appears to be KLF-1 independent (Figure 3.6.A). Next we measured the glutathione redox potential (GSSG/2GSH ratio) using the fluorescent reporter Grx1-roGFP2. Glutathione (GSH) is an important antioxidant peptide and its antioxidant protection is associated with multiple GSH-linked antioxidant enzymes. These enzymes catalyze the reduction of H_2O_2, and of lipid hydroperoxides, using GSH as the electron donor. Glutaredoxin one (Grx1) catalyzes glutathione-dependent dithiol reactions, reducing protein disulfides and mixed disulfides between proteins and GSH. Therefore Grx1- roGFP2 is a ratiometric biosensor that specifically detects GSH redox potential (Back et al., 2012; Gutscher et al., 2008). We observed an increased oxidized/reduced Grx1-roGFP2 in *isp-1;ctb-1* mutants compared to wild type at day one of adulthood, and this ratio was significantly reduced by *klf-1(tm1110)* mutation (Figure 3.6.B.). The reduction in cytosolic hydrogen peroxide levels could be due to a previously activated antioxidant response, in agreement with the *sod-2* upregulation. Nevertheless the mit-mutants exhibit a redox imbalance.

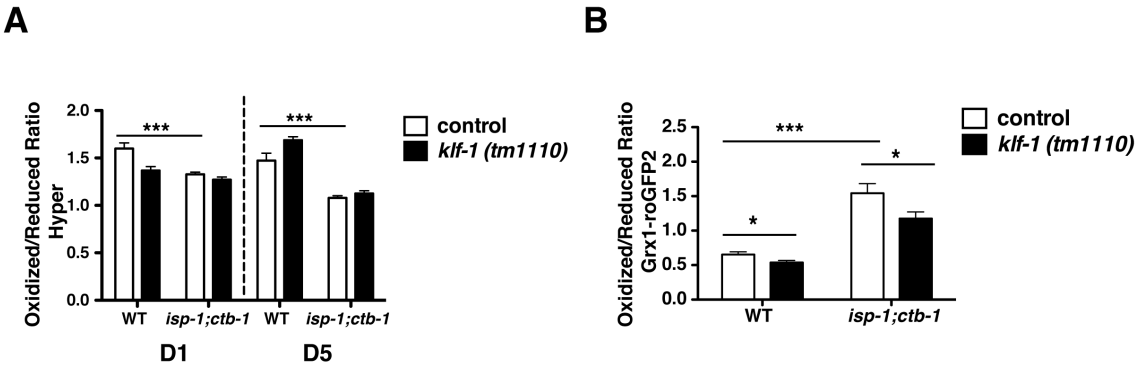

Figure 3.6. *isp-1;ctb-1* mutants exhibit a decrease in cytosolic H_2O_2 but an increased redox imbalance
A) Worms expressing HyPer (wild type, *isp-1;ctb-1*, *klf-1(tm1110)* and triple mutant *isp-1;ctb-1;klf-1*) were imaged at day one (D1) and five (D5) of adulthood and the levels of H2O2 were assessed as the change in fluorescence between oxidized and reduced HyPer. **B**) Glutathione redox potential was measured by Grx1-roGFP2 in wild type, *isp-1;ctb-1*, *klf-1(tm1110)* and triple mutants *isp-1;ctb-1;klf-1*, on the first day of adulthood. Data are presented as mean±SEM. *p<0.05, **p<0.01, ***p<0.001, Student's T-test

Switching to the second group of fluorescent reporters (small molecule probes), we proceeded using the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA), a fluorogenic dye that measures the activity of hydroxyl, peroxyl and other ROS within the cell. First After DCFDA diffuses into the cell and

is deacetylated by cellular esterases to a non-fluorescent compound. This compound is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF) that is highly fluorescent. The signal can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm respectively. Three different conditions were considered for this experiments including wild type, wild type exposed to AA and *isp-1;ctb-1.* At day one of adulthood the samples were collected and the whole-worm extracts analyzed. Interestingly wild type worms showed higher values of fluorescence than both wild type AA and *isp-1;ctb-1*. wild type AA and *isp-1;ctb-1* showed a similar trend, however it was the mutant the one that exhibited the lowest ROS values (Figure 3.7.), which again, could be the result of antioxidant response activated by AA and in the mit-mutants.

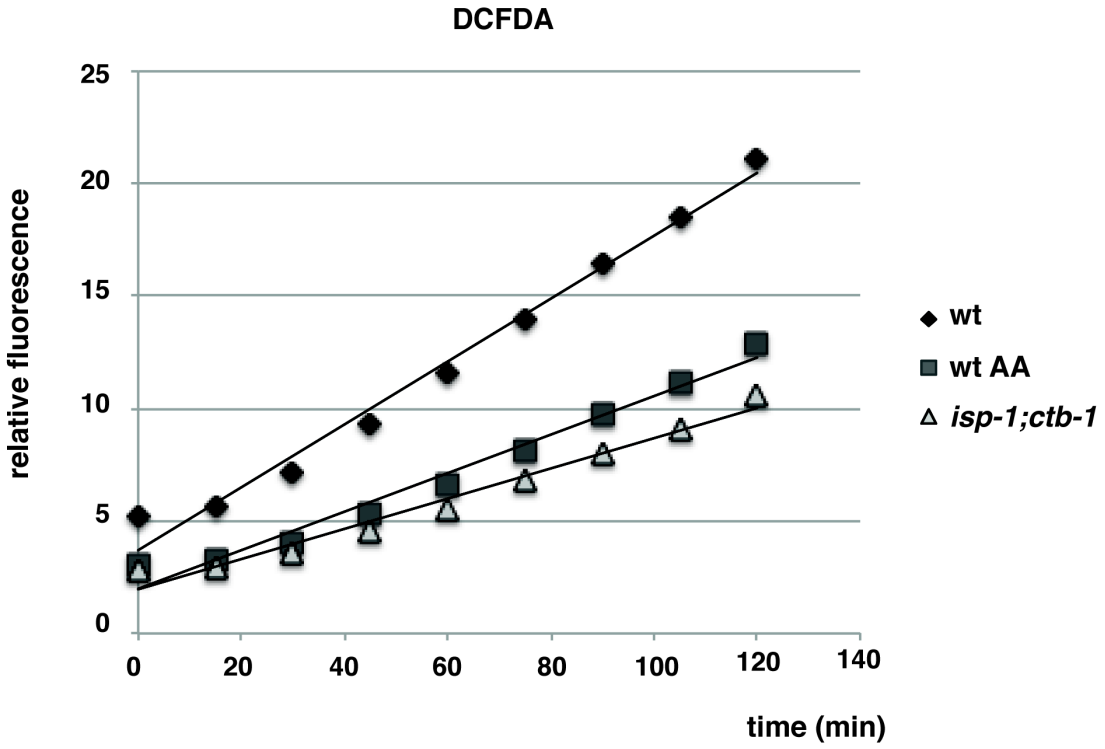

Figure 3.7 ROS levels in whole worm lysates are reduced by *isp-1;ctb-1* mutations and AA treatment
Worms were collected at day one of adulthood (The AA treatment was started at late L4/young adult stage), the relative levels of H_2O_2 were assessed in technical triplicates and the results are shown as the increased signal produced over time (measurements every 15 minutes for two hours).

Next we made use of mitochondrial specific dyes to analyze mitochondrial ROS production. The worms were fed with heat-inactivated *E.coli* mixed with the

corresponding dye and then the fluorescence was analyzed *in vivo* by sorting. MitoTracker ® Red CMH_2XROS (Invitrogen, Karlsruhe, Germany) is a reduced probe that does not fluoresce until it enters living cells, where it is oxidized to the corresponding fluorescent mitochondrion-selective probe and then sequestered in the mitochondria. Therefore we can use this dye as a marker for mtROS production. We observed an increase in total fluorescence (absolute flourescence relative to worm size) in the *isp-1;ctb-1* worms compared to wild type (Figure 3.8.A). To correctly interpret this piece of data we need a value for total mitochondrial mass that we can normalize to.

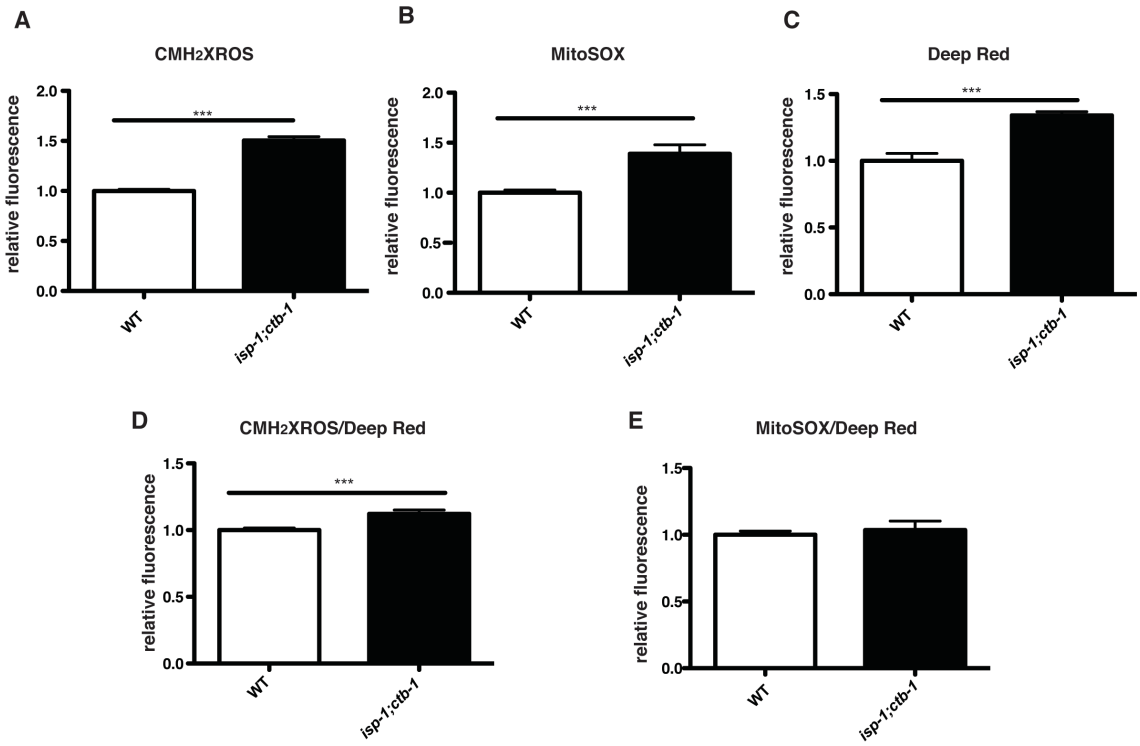

Figure 3.8. *isp-1;ctb-1* mutants exhibit a mitochondrial specific increase in ROS
Mitochondrial ROS (**A**), mitochondrial superoxide (**B**) and mitochondrial mass (**C**) were analyzed in wild type and *isp-1;ctb-1* worms by feeding them with specific mitochondrial dyes and fluorescence was measured by BioSorter(Union Biometrica, Geel, Belgium). Ratio of mtROS per mitochondria (**D**) and mito-Superoxide per mitochondria (**E**). Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, Student's T-test) 100 to 200 worms were used per strain and condition.

Therefore we measured mitochondrial mass with the MitoTracker ® Deep Red (Invitrogen, Karlsruhe, Germany) and observed that *isp-1;ctb-1* worms have increased mitochondrial mass compared to wild type (Figure 3.8.C). Nevertheless, the ratio ROS/mitochondrial mass calculated by CMH_2XROS /Deep Red, still shows a significant increase in the *isp-1;ctb-1* mutant (Figure 3.8.D). Additionally we

utilize MitoSOX ™ (Invitrogen, Karlsruhe, Germany), which stains specifically for mitochondrial superoxide. Here again we observe a higher signal in the *isp-1;ctb-1* mutants compared to wild type (Figure 3.8.B) but this difference is not significant when we normalized it to the mitochondrial mass (Figure 3.8.E).

These data altogether suggest that there is an increase in mtROS in *isp-1;ctb-1* worms, however the observation that overall ROS production (Hyper and DCFDA) shows lower levels in mitochondrial mutants, and that we detect a redox imabalance (Grx1-roGFP), together with the SOD-2 upregulation, could indicate that we are analyzing an already active antioxidant response and that we have missed the major moment of mitochondrial ROS increase, which we postulate will coincide with L3/L4 larval stage and somatic mitochondrial replication.

3.4. KLF-1 regulates the expression of xenobiotic detoxification genes

To further elucidate the function of KLF-1 in the assurance of the longevity of the *isp-1;ctb-1* mutants, we performed microarray analysis. The transcriptome of five-day-old *isp-1;ctb-1* worms was compared to the same mutants after *klf-1* knockdown either during development (long-lived) or during adulthood (suppressed longevity). These two were then compared to wild type worms. We detected 251 genes that had an altered expression in the *isp-1;ctb-1* mutant, which were normalized to wild type levels after *klf-1* knockdown. Making use of the gene enrichment analysis tool DAVID 6.7 (National Institute for Allergies and Infectiuos diseases (NIAID), NIH) (Huang da et al., 2009), the statistical representation of the gene categories was analyzed based on gene ontology: “oxidation-reduction” was identified as the most affected biological process and the “Metabolism of xenobiotics by cytochrome P450” together with the “Drug metabolism” were the two KEGG-pathways that were found to be mostly affected by *klf-1* knockdown during adulthood in the *isp-1;ctb-1* mutant (See Appendix B and C).

The drug-metabolizing enzymes (DMEs) are classified in two main groups according to their functions: Detoxification starts with phase I by oxidative steps exerted by NADPH-cytochrome P450 reductases/cytochrome P450 (CYPs) and is

followed by phase II characterized by conjugative reactions conducted mainly by UDP-glucuronosyltransferases (UGTs), glutathione S transferases (GSTs), sulfotransferases and acetyltransferases.

Our microarray data reveal an overrepresentation of genes involved in xenobiotic detoxification like CYPs, UGTs and GSTs among those that were upregulated under control conditions in the *isp-1;ctb-1* mutants. Great part of these genes were also downregulated by *klf-1* RNAi in the *isp-1;ctb-1* worms in following proportions: 24 out of 86 CYPs as well as 22/72 UGTs and 13/48 GSTs (Figure 3.9). For the purpose of further characterizing some of these putative KLF-1 targets, we analyzed expression levels of some of the newly identified genes by quantitative polymerase chain reaction (qPCR) (Figure 3.9). Members of all three families CYP (Figure 3.9.B), UGT (Figure 3.9.C) and GST (Figure 3.9.C) showed an increased expression at day one of adulthood in the *isp-1;ctb-1* worms compared to wild type. Later in life we observed a general downregulation of expression in the mutants (with the exception of *cyp-13a11* and *gst-4*) always in a KLF-1-dependent manner. In the wild type worms, most of the CYPs, UGTs and GSTs appear to be regulated by KLF-1 both at day one and day five of adulthood. The changes in expression throughout time in the wild type worms are not as obvious as in the mutants, with just a slight tendency to increase later in life being *gst-4* the one gene with the highest upregulation.

Altogether, these results strongly suggest that the xenobiotic detoxification response is upregulated in *isp-1;ctb-1* mutants in a KLF-1 dependent manner.

Remarkably no ROS scavengers like superoxide dismutases or catalases were found to be targets of KLF-1.

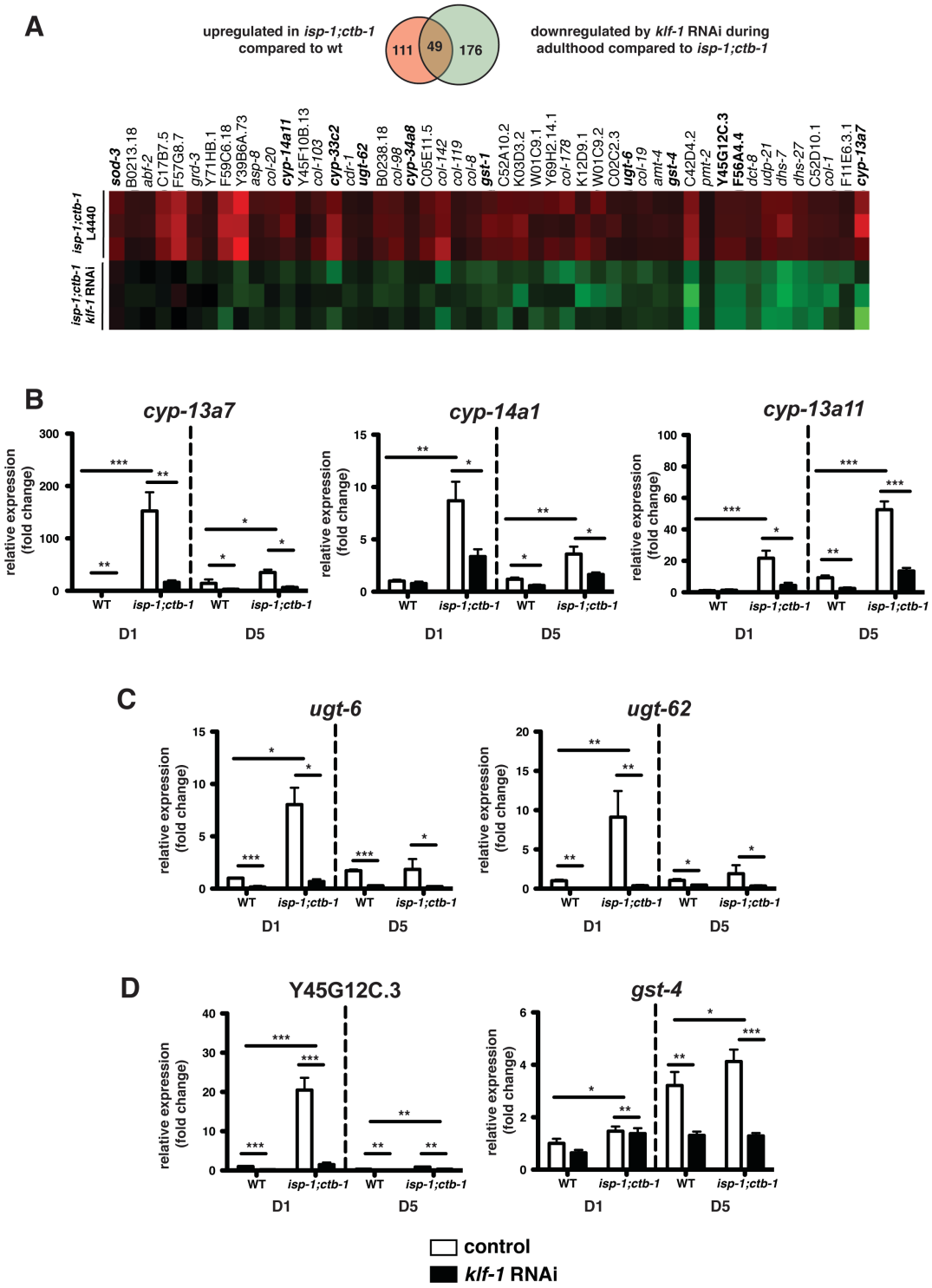

Figure 3.9. *isp-1;ctb-1* strongly upregulates detoxification enzymes in a KLF-1-dependent manner

A) Heat map clustering of the 49 genes simultaneously upregulated in the *isp-1;ctb-1* mutants and downregulated upon *klf-1* knockdown during adulthood (p<0.001). The genes coding for detoxification proteins are in bold. **B**-**D**) Expression levels measured by qPCR of the (**B**) *cyp,* (**C**) *ugt* and (**D**) *gst* genes at day one (D1) and day five (D5) of adulthood of *isp-1;ctb-1* and wild type worms fed bacteria expressing RNAi targeting the *klf-1* gene or the empty vector L4440 (control). Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test) (n=6)

We next examined the expression of some of the candidate targets of KLF-1 *in vivo* utilizing reporter strains: we started exposing the strains to either control conditions or low concentrations of AA. In case of observing a significant change, we subsequently analyzed the impact of the *isp-1;ctb-1* mutation on the reporter's expression.

The strain CY573 obtained from the *Cenorhabditis* Genetic Center (CGC, University of Minnesota) carries the green fluorescent protein (GFP) under the control of *cyp-35b1's* promoter. CY573 showed no significant change in expression when exposed to AA (Figure 3.10) and therefore we didn't further analyze this strain.

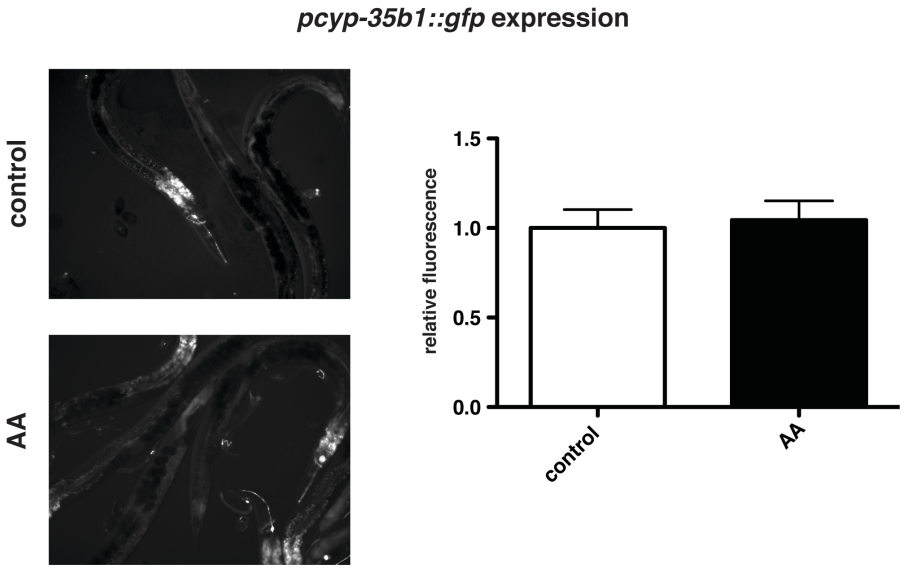

Figure 3.10. *pcyp-35b1::gfp* expression does not change upon exposure to AA
Wild type worms were fed bacteria containing the empty vector L4440 until reach adulthood, then exposed to 0,5 µM AA at day one and imaged at early day two. Representative pictures of both conditions are shown on the left, and the quantification in the right. Bars represent mean ± S.E.M. Student's T-test (n=10)

By contrast, the strain SD1444 (CGC, University of Minnesota) showed a great increase in expression when exposed to pro-oxidant conditions (Figure 3.11.A.). SD1444 carries the red fluorescent protein mCherry downstream of *cyp-25A2's* promoter that is hardly visible under control conditions: a weak red signal can be observed in the head and sometimes tail of wild type worms. Upon exposure to AA there is a massive upregulation of expression and red nuclei can be clearly visible throughout the entire gut of the worms (not shown). In agreement

with this observation, the expression was also significantly increased on the *isp-1;ctb-1* background (Figure 3.11.B) Remarkably upon exposure to pathogens (contamination on the plates) the expression was also very significantly increased (not shown). This reinforces the idea that different signals can trigger DMEs expression.

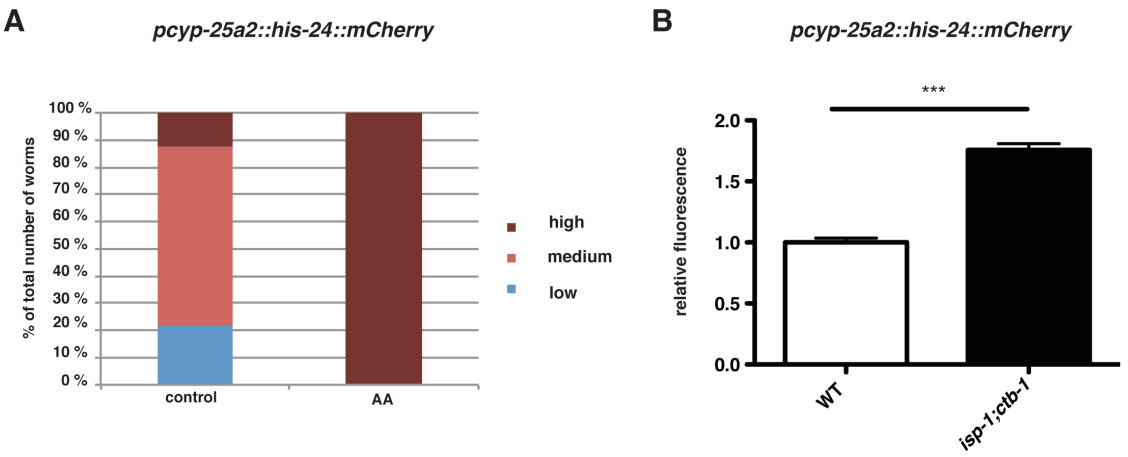

Figure 3.11. *pcyp-25a2::his-24:mCherry* expression is strongly upregulated by AA and *isp-1;ctb-1* mutations
A) Day one wild type adult worms were fed bacteria containing the empty vector L4440 until reaching adulthood, then exposed to 0,5 µM AA and imaged at early day two. The expression was scored as low (mCherry just visible in the head), medium (signal visible in head and tail) and high (mcherry signal throughout the whole body of the worm). At least 30 worms per condition were analyzed. **B**) *pcyp-25A2::his-24:mCherry* expression was measured on one-day-old wild type and *isp-1;ctb-1* worms by BioSorter (Union Biometrica, Geel, Belgium) n>100 Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, Student's T-test)

Additionally, we generated two more reporter constructs expressing GFP under the control of the endogenous promoters of *cyp-34a8* and *cyp-13a11,* respectively. Both constructs where then injected into *isp-1;ctb-1* worms and backcrossed with wild type generating a total of four strains (ATR4030, ATR4031, ATR4026 and ATR4027). The GFP expression pattern showed that both CYPs were mostly expressed in the gut (Figure 3.12.A) and both exhibited an increased GFP signal in the mutant background (Figure .3.12.B).

Taken together, these data reinforce our idea that both mitochondrial dysfunction and oxidative stress act as a trigger of the protective response mediated by detoxifying enzymes trough overlapping mechanisms.

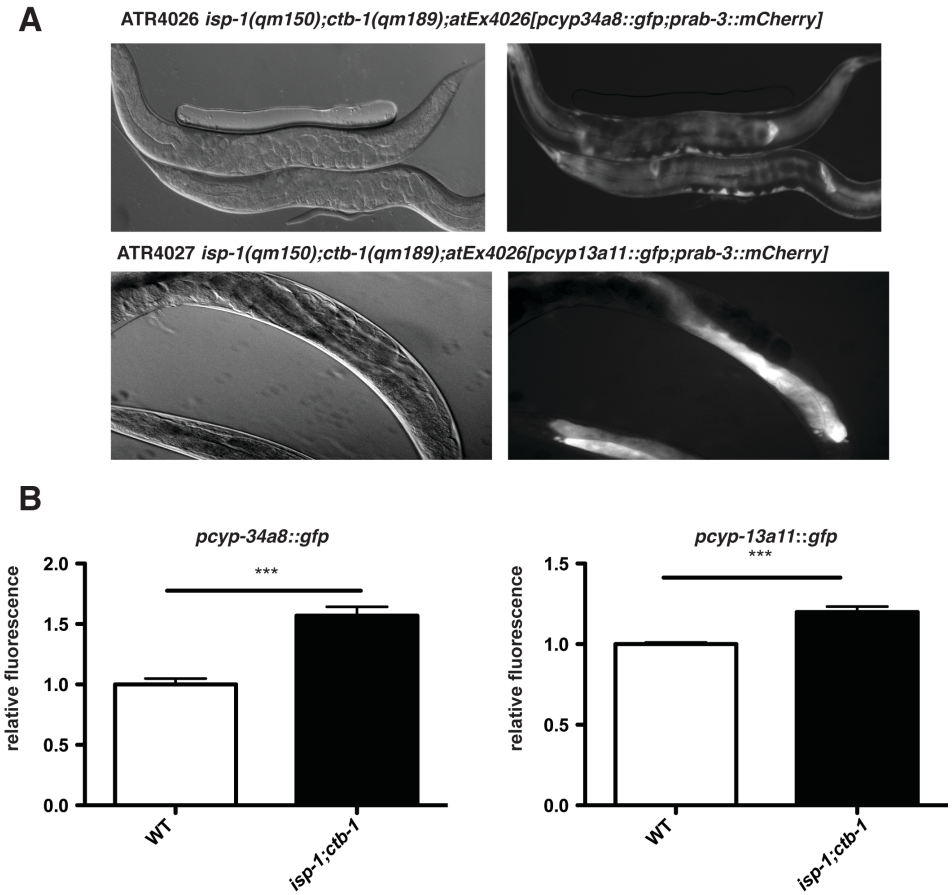

Figure 3.12. *cyp-34a8* and *cyp-13a11* expression is upregulated in *isp-1;ctb-1* worms
A) Expression pattern of *pcyp-13a11::gfp* (hypodermis and gut) and *pcyp-13a11* (gut) in one-day-old *isp-1;ctb-1* worms. **B**) Quantification of expression of both *cyp* reporter constructs on wild type and *isp-1;ctb-1* worms measured by BioSorter (Union Biometrica, Geel, Belgium). At least 100 to 200 worms per strain and condition were scored. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, Student's T-test)

3.5. CYPs are needed for mito-induced longevity and development

Next we wanted to ascertain whether this oxidative-stress-dependent expression of CYPs/DME was in fact responsible for the increased longevity observed in our mitochondrial mutant. For this porpoise we performed lifespan experiments with wild type and *isp-1;ctb-1* worms on either L4440 plates or on CYPs RNAi. The *cyps* were selected for being mostly overexpressed in the *isp-1;ctb-1* and/or mostly down regulated by the suppression of KLF-1. Knockdown of *cyp-25a1* and *cyp-13a11*, strongly suppressed the longevity of *isp-1;ctb-1* mutants, and had little to no impact in the wild type (Figure 3.13.A). Remarkably, not only *cyps*

but also *ugts* (*ugt-6* and *ugt-62*) had an impact in the lifespan of the long-lived *isp-1;ctb-1* (Figure 3.13.B.). Altogether, these data indicate that DMEs play an essential role in the assurance of the longevity of *isp-1;ctb-1*.

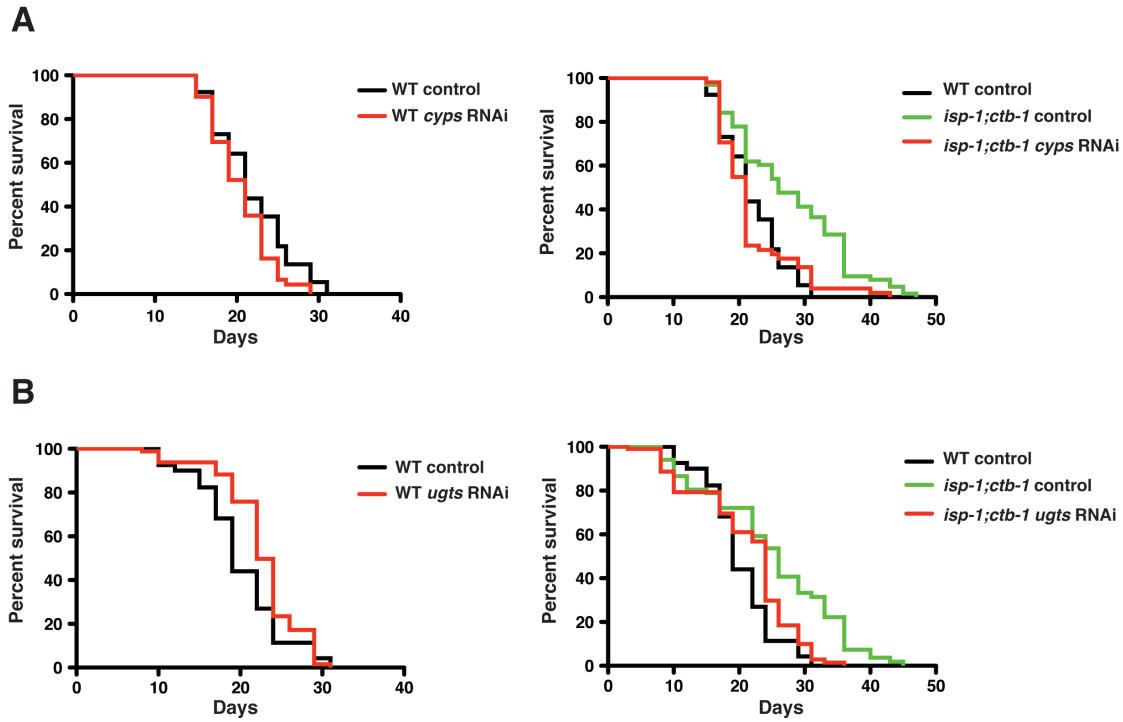

Figure 3.13. *DMEs* mediate the longevity of *isp-1;ctb-1* mutants
Lifespan analysis of wild type and *isp-1;ctb-1* mutants feeding bacteria carrying the empty vector L4440 (control), (**A**) *cyp-25a1* and *cyp-13a11* RNAi (*cyps* RNAi) or (**B**) *ugt-6* and *ugt-62* (*ugts* RNAi).

Interestingly, the knockdown of either of the two *cyps,* normalized the developmental delay of the *isp-1;ctb-1* animals (Figure 3.14.A and B). This effect was not observed after knockdown of *klf-1* (Figure 3.14.C). In this case again, the effect was not exclusive for *cyps* but also exerted by phase II detoxification enzymes *ugts* (not shown). Remarkably the opposite phenomenon was also observed: early exposure of the SD1444 strain to low concentration of AA (0,5 µM) resulted in a great increased in expression of the fluorescent reporter that was accompanied with a marked developmental delay compared to the same strain in control conditions (not shown). The fact that *klf-1* knockdown did not affect development, could be due to different levels of suppression on *cyps* and *ugts* (when performed directly with RNAi targeting them or indirectly through *klf-1)*. Additionaly, KLF-1 could regulate the expression of genes other than DMEs to

counteract this detrimental effect on development exerted by CYPs and UGTs or alternatively not regulate DMEs during development

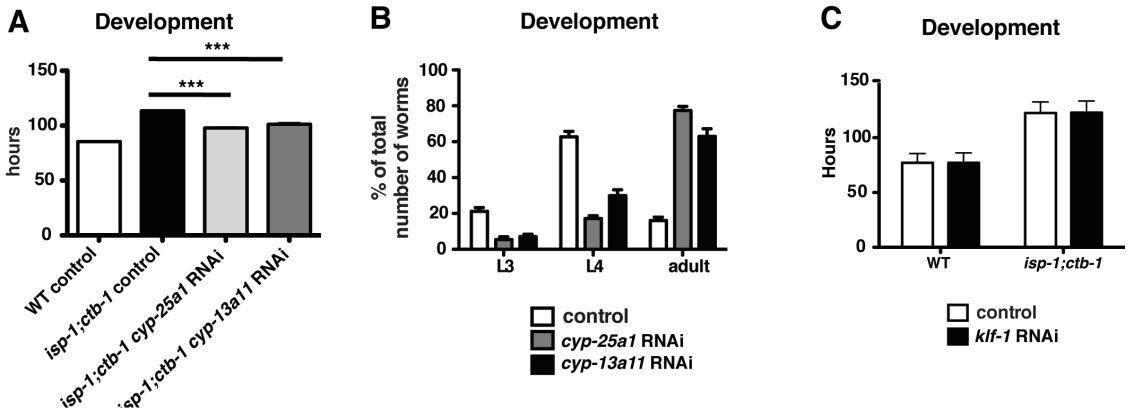

Figure 3.14 Knockdown of *cyp*s partially corrects the developmental delay of *isp-1;ctb-1* mutants
Both wild type worms and *isp-1;ctb-1* mutants were exposed to RNAi targeting either *cyp-25A1* or *cyp-13A11* and the developmental time was assayed either as the time needed for the worms to reach adulthood (**A**) or as the number of worms that reached a particular stage four days after hatching (**B**): larval stage 3 (L3), larval stage 4 (L4) or adulthood (adult). Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test) (n=50). (**C**) Time needed by wild type worms and *isp-1;ctb-1* mutants to reach adulthood. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test) (n=50).

3.6. KLF-2 and KLF-3 do not phenocopy KLF-1

KLF-1 was previously described as mediator of dietary-restriction-induced longevity (Carrano et al., 2014). Carrano and colleagues indicated that KLF-1 is an unspecific mediator of longevity in regard to mitochondrial-dysfunction since its homologs *klf-2* and *klf-3* also had a similar minor impact in the longevity of the *isp-1* mutant. The impact on lifespan exerted by *klf-1* RNAi on *isp-1;ctb-1*mutants that we observe is not minor (Fig 3.1), since it completely brings it back to wild type levels. Nevertheless we wanted to determine whether the homologs of *klf-1* also play a role in regulating its targets. Expression levels measured by qPCR, indicate that the activation of the detoxification enzymes seems to be exclusively regulated by KLF-1 and not KLF-2 or KLF-3 (Figure 3.15.A). KLF-2 does not seem to regulate the expression levels of the genes tested and *klf-3* RNAi led even to an upregulation of *cyp-13a7* and *cyp-13a11*. Moreover the expression of *klf-2* and *klf-3* is not affected in the *isp-1;ctb-1* in contrast to *klf-1* (Figure 3.15.B).

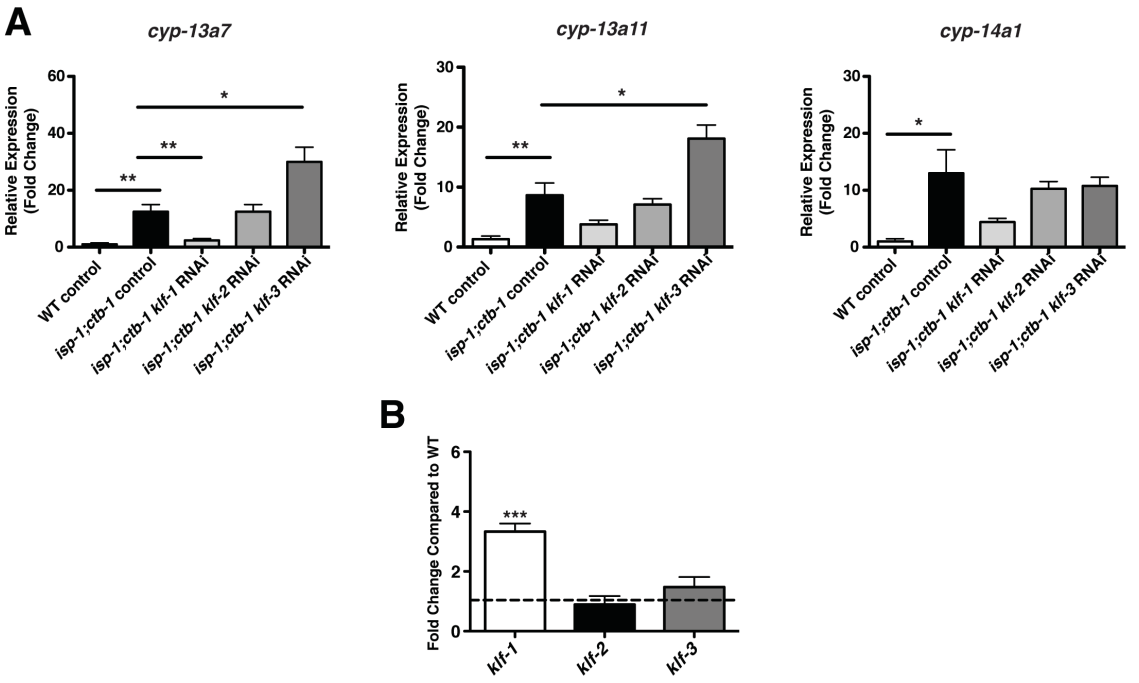

Figure 3.15. KLF-1 specifically regulates CYPs in *isp-1;ctb-1* mutants
A) Expression level of *cyp-13A7*, *cyp-13a11* and *cyp-14a1* measured by qPCR in one-day-old wild type worms and *isp-1;ctb-1* mutants treated with RNAi targeting either *klf-1*, *klf-2* or *klf-3* genes. **B**) Assessment of the expression level of *klf-1*, *klf-2* or *klf-3* by qPCR at the day one of adulthood, in *isp-1;ctb-1* mutants relative to expression in wild type worms. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test), (n=5).

3.7. KLF-1 in part regulates *daf-2*-induced longevity

There is a very rapidly increasing body of scientific literature about genes and pathways involved in lifespan determination in *C.elegans*. Despite the great advances done, little number of studies have tried to globally analyze these results and study aging as a whole interconnected network instead of isolated pathways. Transcriptional comparison of different lifespan models is a great way to identify potential "universal" mediators of longevity. Shore and Ruvkun review the evidence for the involvement of cytoprotective functions in lifespan determination. Cytoprotection includes several different processes like detoxification, innate immunity, oxidative stress response, proteostasis, and others. They present evidence that these processes are evolutionarily, genetically, and functionally integrated and suggest that understanding the variation in those pathways might help understanding the aging process itself (Shore and Ruvkun, 2013). The fact

that KLF-1 was recently reported as mediator of dietary-restriction-induce longevity (Carrano et al., 2014), together with the implication of the detoxification system in the longevity of dauer larvae and long lived *daf-2* mutants (McElwee et al., 2004) gave rise to the question whether the KLF-1-dependet regulation of DMEs is a conserved mechanisms of longevity in other models different than mitochondrial.

To shed more light on the role of KLF-1 on longevity we performed lifespan experiments on the *daf-2 (e1370)* mutant. We observed a clear impact of *klf-1* RNAi on the longevity of the insulin signaling mutant (Figure 3.16.A), although the lifespan was not completely normalized to the wild type values as it was on the *isp-1;ctb-1.* A variance in the effect of *klf-1* RNAi on the lifespan of *daf-2* animals could be a consequence of different classes of mutants used in previous studies (Carrano et al., 2014; Gems et al., 1998). Additionally, we also observed a slight upregulation of *klf-1* expression in *daf-2(e1370)* mutants at day one of adulthood (Figure 3.16.B).

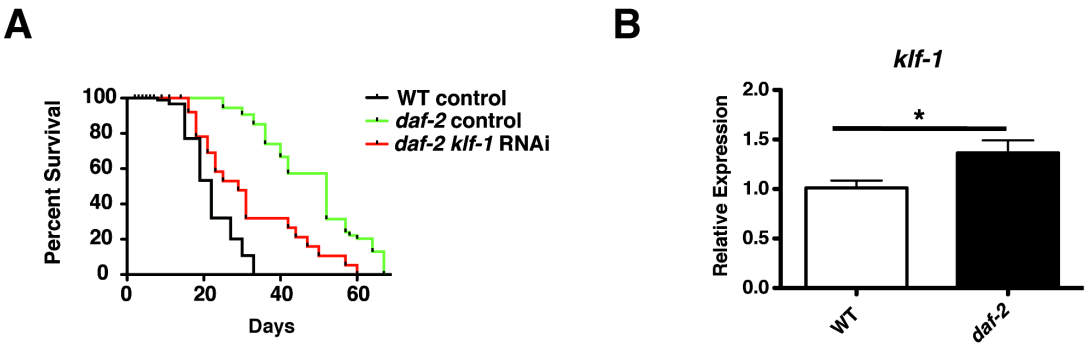

Figure 3.16. KLF-1 partially regulates *daf-2(e1370)* longevity
A) Lifespan assay of *daf-2(e1370)* mutants and wild type worms fed with either control bacteria carrying the empty vector L4440 or targeting *klf-1* gene. **B**) *klf-1* expression levels was assessed by qPCR at day one of adulthood in wild type and *daf-2(e1370)* worms. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test)

daf-16 encodes the sole forkhead box O (FOXO) homolog , and functions as the major transcription factor involved in the insulin/IGF-1-mediated signaling (IIS) pathway. DAF-16 function has been extensively studied and is notable for being the primary transcription factor essential for the prominent lifespan extension observed upon mutation of the insulin-like receptor *daf-2*(Kenyon et al., 1993; Ogg

et al., 1997). Therefore we decided to analyze whether *klf-1* RNAi is having an impact in longevity because is affecting DAF-16. We did not observe any obvious changes in expression in the *daf-16* flourescent reporter strain (*pdaf-16::daf-16a/b-gfp)*(Figure 3.17.A.) upon *klf-1* RNAi, however, there is a clear age-dependent upregulation. Moreover no changes in expression of *klf-1* mRNA were detected when exposed to *daf-16* RNAi. (Figure 3.17.B). These results suggest that the KLF-1 has a partial impact on the lifespan of *daf-2(e1370)* mutant trough a pathway independent of DAF-16. Interestingly no nuclear localization of KLF-1 was observed in this mutant background (not shown). Nevertheless we cannot exclude the possibility that low concentrations of the protein may be active in the nucleus and enough to activate gene expression.

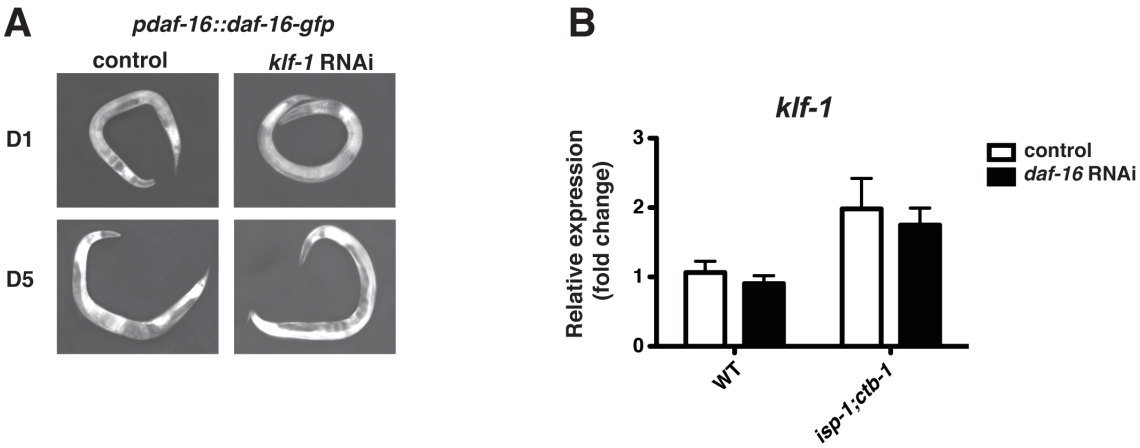

Figure 3.17 KLF-1 effect on IIS pathways is independent of DAF-16
A) The expression levels of *daf-16* were analyzed by imaging wild type worms (expressing *pdaf-16::daf-16-gfp* reporters) at day one (D1) and day five (D5) of adulthood after exposure to bacteria targeting *klf-1* or control L4440 empty vector. Representative pictures are shown. **B**) The expression of *klf-1* gene was assessed by qPCR in wild type and *isp-1;ctb-1* worms at day one of adulthood after feeding with bacteria carrying the empty vector L4440 or targeting *daf-16* gene. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test)

In agreement with the impact on lifespan and the transcriptional upregulation of *klf-1* in the *daf-2(e1370)* mutant (Figure 3.16.B), we also observed an upregulation of KLF-1 targets including *cyps*, *ugts* and *gsts* in this mutant (Figure 3.18.A). Moreover this increase in expression was suppressed by *klf-1* RNAi (Figure 3.18.A). Additionally we crossed the above described CYP reporter strain SD1114 with a *daf-2(e1370)* mutant, in order to analyze the impact of this mutation on *cyp-25a2*::his-24::mCherry expression *in vivo*. Sorting analysis of one-day-old adult

worms did not reveal any significant difference on the fluorescent signal of *daf-2 vs.* wild type worms (Figure 3.18.B), indicating that this insulin/IGF-1 signaling mutation does not upregulate the expression of *cyp-25a2* under the conditions tested.

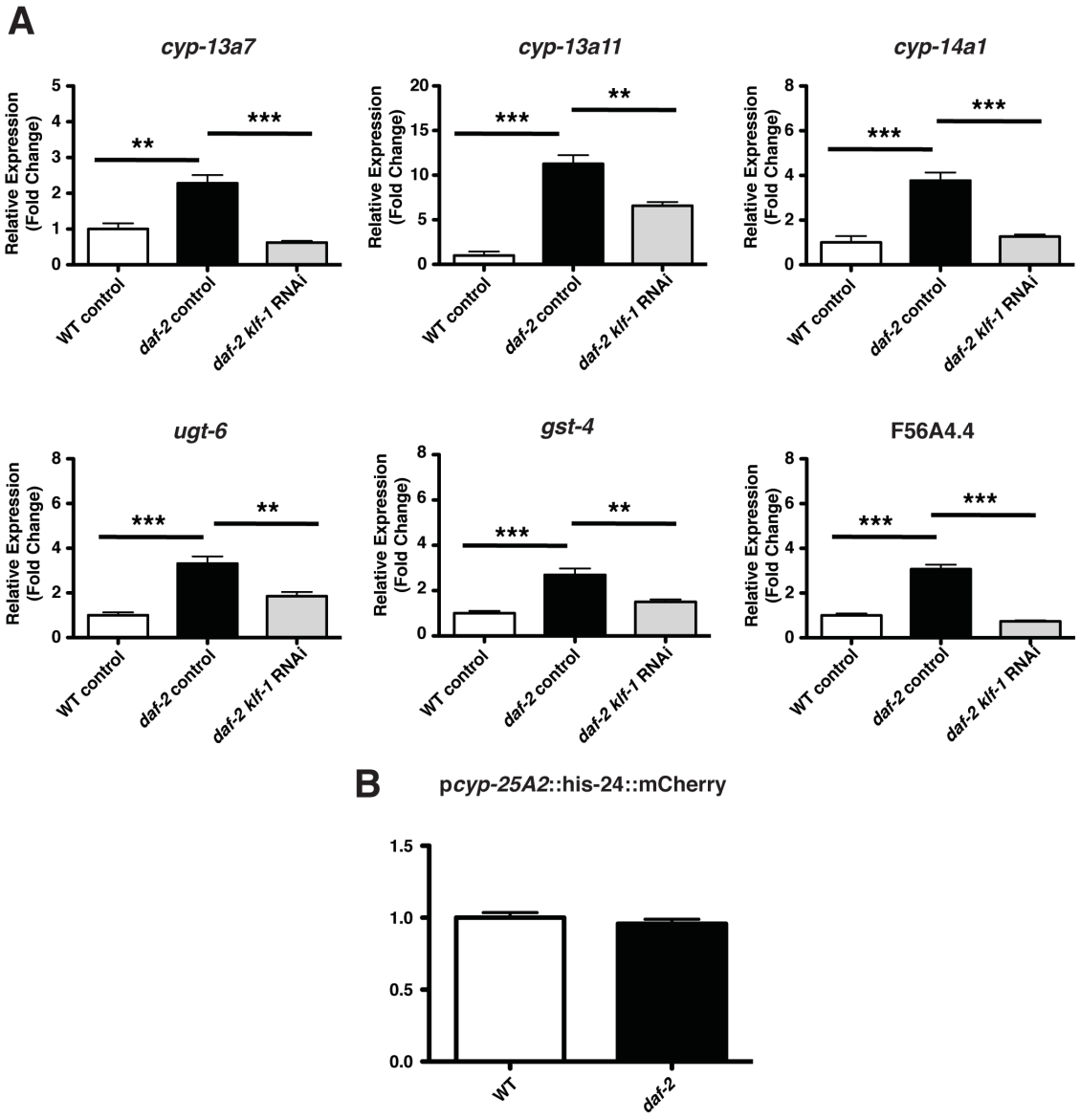

Figure 3.18. KLF-1 is needed for the upregulation of detoxification genes in long-lived *daf-2(e1370)* mutants

A) Expression levels of the detoxifying genes *cyp-13a7, cyp-13a11, cyp-14a1, ugt-6, gst-4* and F56A4.4 assed by qPCR in wild type worms and *daf-2(e1370)* long-lived mutants fed with bacteria carrying the empty vector L4440 or targeting *klf-1*. n=5 **B**) Quantification of expression of *pcyp-25a2::his-24::mCherry* with BioSorter(Union Biometrica, Geel, Belgium) in wild type and *daf-2(e1370)* worms at day one of adulthood. 100 to 200 worms per strain. **A** and **B)** Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test)

The fact that *klf-1* RNAi partially suppresses the longevity of *daf-2(e1370)* mutant together with the KLF-1-dependent upregulation of DMEs observed in this mutant, strongly suggested that these enzymes might have a conserved role on lifespan determination. Strikingly, knockdown of *cyps* by RNAi did not affect the longevity of *daf-2(e1370)* (Figure 3.19.). This fact has two probable explanations: first, other targets of KLF-1 different than DMEs are responsible for the suppression of longevity exerted by KLF-1 in the *daf-2(e1370)* mutant. Second, we cannot exclude the possibility that other subset of KLF-1 dependent DMEs could impact insulin/IGF-1 signaling. Despite of the partial overlap in regulation of the detoxification response in different mutants it seems that at least these specific subset of genes studied, have actually different impact on longevity depending on the genetic background that they are analyzed on. Additional analysis of more genes involved in the detoxification response in different long-lived models would be necessary in order to further elucidate the role that DMEs play in longevity.

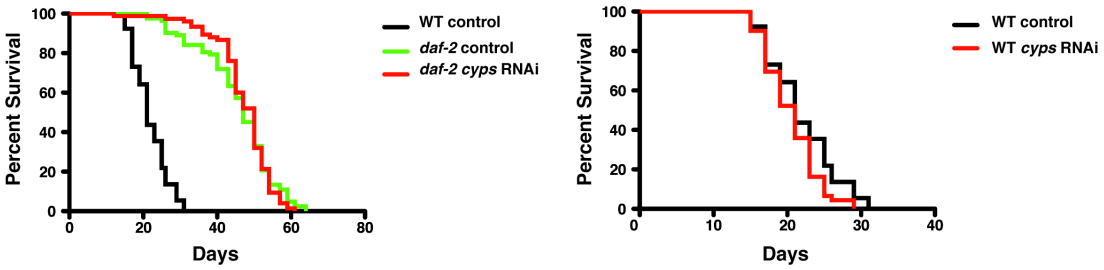

Figure 3.19. *daf-2(e1370)* longevity is not affected by *cyps* RNAi
Lifespan analysis of wild type and *daf-2(e1370)* long-lived mutants feeding bacteria with the empty vector L4440 or targeting *cyp-25a1* and *cyp-13a11.*

3.8. KLF-1 may be needed indirectly for SKN-1-mediated Phase II activation

The Nrf2 (NF-E2-related factor) transcription factor is a well established regulator of the phase II DMEs in mammals (reviewed in (Giudice et al., 2010), and similarly SKN-1 has also been reported to regulate the same set of genes in *C.elegans* (Oliveira et al., 2009). SKN-1 plays an essential role in mesodermal development but it has been also extensively studied for its role in oxidative stress response(An and Blackwell, 2003). Upon exposure to exogenous stressors (Oliveira et al., 2009) or in response to intracellular oxidative stress, SKN-1 translocates into the nucleus and regulates the expression of phase II

detoxification genes like *gcs-1* or *gst-4* (An and Blackwell, 2003; Inoue et al., 2005). Given the similarities in responding to oxidative stress of both KLF-1 and SKN-1, and the overlap in the putative targets of KLF-1 with those already known to be under control of SKN-1, our interest in understanding the relationship between these two transcription factors was clear.

Lifespan experiments revealed an impact of *skn-1* RNAi on the longevity of both wild type and *isp-1;ctb-1*, suggesting that the effect exerted by SKN-1 is at least partially independent of the mitochondrial dysfunction (Figure 3.20.A) and probably related to the role of SKN-1 in development (An and Blackwell, 2003; Bowerman et al., 1992).

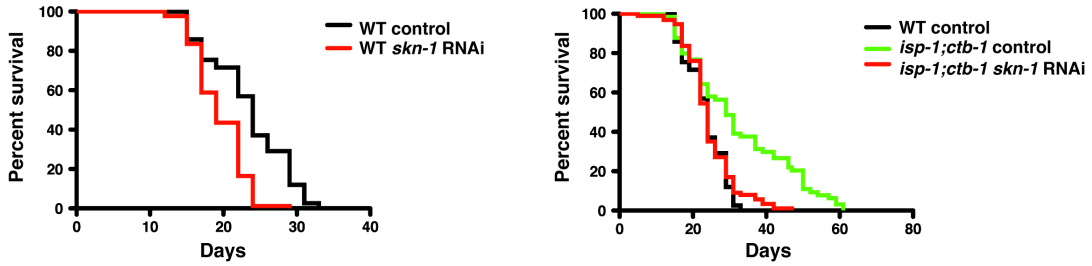

Figure 3.20. SKN-1 impacts longevity of *isp-1;ctb-1* and wild type worms
Lifespan assay of wild type and *isp-1;ctb-1* mutants fed with bacteria expressing the empty vector L4440 (control) or targeting *skn-1* gene.

The transcript levels of *skn-1* do not show any significant changes between *isp-1;ctb-1* and wild type worms at day one of adulthood and only a slight decrease in the *skn-1* expression with increased age in the mutants. Additionally the expression is independent of KLF-1 (Figure 3.21.A). In agreement with this observation, we detect no difference in expression in some SKN-1 targets like *ctl-1* and *ctl-3* (An and Blackwell, 2003) neither induced by the *isp-1;ctb-1* mutations nor by *klf-1* RNAi (Figure 3.5.B.). Other targets like *gcs-1* show differential expression in the *isp-1;ctb-1* mutants compared to wild type but in a KLF-1-independent manner (Figure 3.21.B). These data suggest that KLF-1 does not simply affect overall SKN-1 activation. Noteworthy, the expression of the bona fide SKN-1 target *gst-4*, that encodes a major phase II enzyme involved in both xenobiotic detoxification and ROS responses (An and Blackwell, 2003), was increased in the *isp-1;ctb-1* mutants and this transcriptional activation was dependent on KLF-1 (Figure 3.9.D). Additionally, and corresponding to the *klf-1*

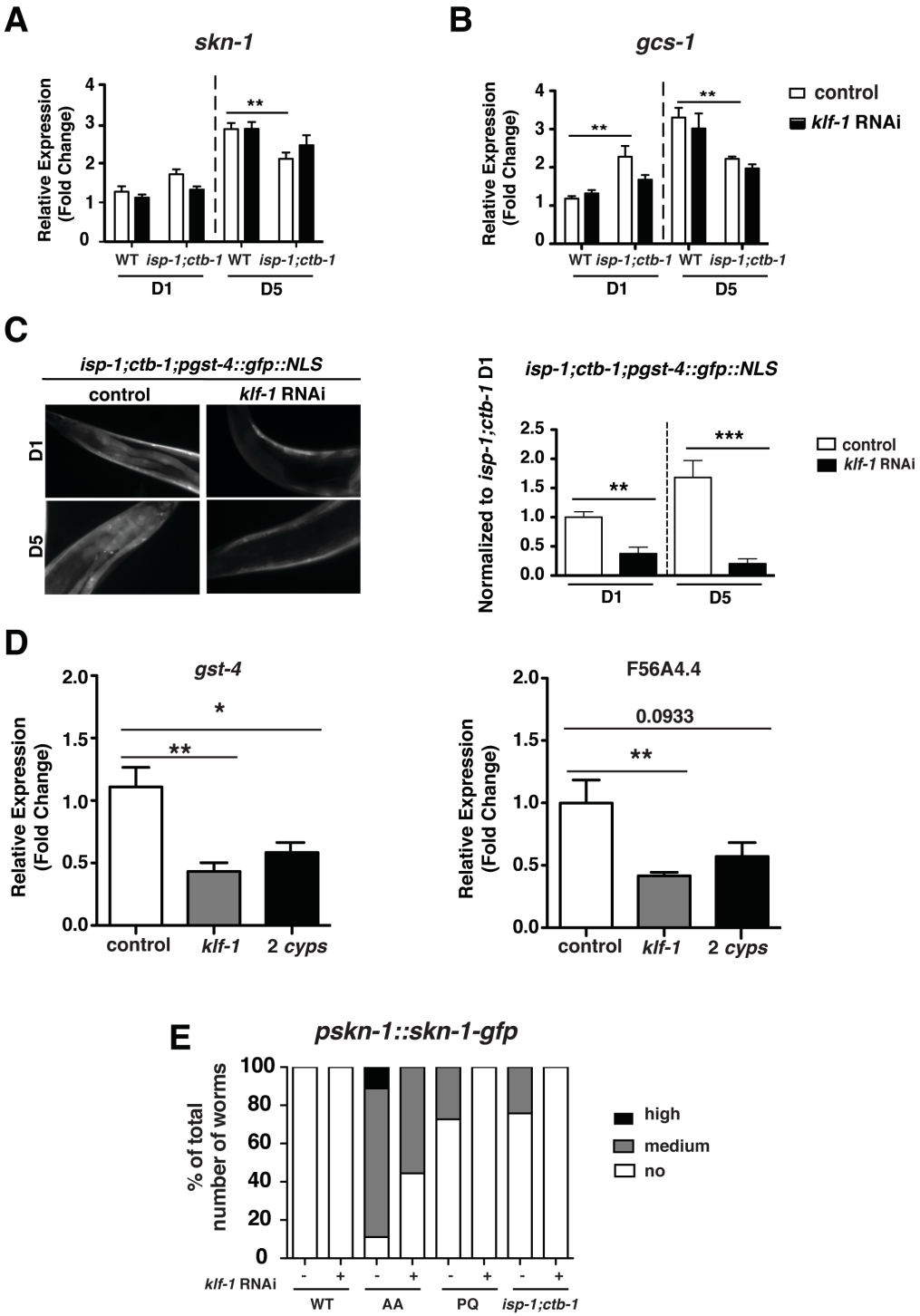

Figure 3.21. KLF-1 enhances the SKN-1-dependent phase II activation
Expression levels of *skn-1* (**A**), and its *gcs-1*(**B**) measured by qPCR in wild type and *isp-1;ctb-1* worms at day one (D1) and day five (D5) of adulthood feeding on bacteria carrying the empty vector L4440 or targeting *klf-1* gene. **C**) The expression of the *pgst-4::gfp::NLS* reporter construct was analyzed at day one (D1) and (D5) of adulthood by imaging *isp-1;ctb-1* mutant worms fed with bacteria carrying the empty vector L4440 or targeting *klf-1*. On the right representative images are shown, on the left quantification **D**) Expression levels of the genes *gst-4* and F56A4.4 upon control conditions (L4440 empty vector), *klf-1* or *cyps* (*cyp-25a1, cyp-13a11)* RNAi. Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test).

expression pattern, *klf-1* knockdown suppressed the activation of *gst-4* expression in the gut of *isp-1;ctb-1* animals (Figure 3.21.C).

As introduced above, the detoxification phase I exerted mainly by CYPs is often required for the modification of the toxic compounds so that these can be further processed and eliminated by the phase II machinery (Iyanagi, 2007). In order to test whether this process is happening in our model we analyzed the impact of *klf-1* RNAi as well as RNAi of KLF-1 targets and phase I DMEs, *cyp-25a1 and cyp-13a11*, on the activation of phase II enzymes *gst-4* and F56A4.4 (a *gst-10* homologue). Both GSTs were strongly downregulated by *cyp*s RNAi, and this downregulation was mimicking the effect exerted by *klf-1* knockdown (Figure 3.21.C). Furthermore, the nuclear localization of SKN-1 induced by exposure to prooxidants like PQ or AA or *isp-1;ctb-1*, was hindered by *klf-1* RNAi (Figure 3.21.D.). Altogether these data suggest that KLF-1 and its phase I targets, CYPs, are needed to stimulate the activation of the phase II regulated by SKN-1.

3.9. KLF-1 nuclear localization is regulated by a conserved MAPK signaling cascade

To gain deeper insights regarding the function of KLF-1 in the assurance of the mito-induced longevity we proceeded analyzing the steps previous to the nuclear localization by screening for putative upstream regulators.

The screen was started with kinases and phosphatases: Phosphorylation, as well as many other post-translational modifications, is essential for a great number of cellular signaling events, and therefore an interesting starting point for the screen. Making use of an available *C.elegans* kinase and phosphatase database (kinase.com, Salk Institute For Biological Studies) that predicts over 600 kinases and phosphatases, we selected these specific subset of genes of our interest out of the existing Aringer RNAi feeding library to study their impact on the nuclear localization of KLF-1. The worms were fed with the RNAi against the different candidates and at day one of adulthood they were exposed to low concentrations of AA and then scored for nuclear localization, annotating those genes that reduced

this oxidative stress-mediated mobilization into the nucleus. Out of the many genes tested, several candidates were annotated (Table 3.1).

Table 3.1. Annotated kinases regulating KLF-1 nuclear localization

gene name	sequence	description
hpo-11	H37N21.1	is an ortholog of human NRBP2 (nuclear receptor binding protein 2) and NRBP1 (nuclear receptor binding protein 1)
cdk-9	H25P06.2	encodes an ortholog of the metazoan transcription elongation factor kinase
gcy-21	F22E5.3	encodes a receptor-type, transmembrane guanylyl cyclase
nsy-1	F59A6.1	also called *ask-1* and *esp-8*; encodes a MAP3K orthologous to the mammalian apoptosis signal-regulating kinase (ASK) family of protein kinases
ttbk-2	F36H12.8	ortholog of human TTBK2 (tau tubulin kinase 2) and TTBK1 (tau tubulin kinase 1)
elo-3	D2024.3	encodes a paralog of *elo-1* and *elo-2*, each of which encodes a polyunsaturated fatty acid (PUFA) elongase
pmk-3	F42G8.4	homolog of p38 MAPK, activated in vitro by MEK6, indicating that it does respond like p38 to physiological stress
pkg-2	C09G4.2	ortholog of human PRKG2 and PRKG1 (protein kinase, cGMP-dependent, typeII and I)
wnk-1	C46C2.1	orthologous to the human gene PROTEIN KINASE, LYSINE-DEFICIENT 1 which when mutated leads to pseudohypoaldosteronism type II
kin-5	T13H10.1	predicted protein TK closely related to the non-receptor TKs Fes/Fps and Fer that contain an SH2 domain and a TK domain (OMIM:190030)
nekl-3	F19H6.1	ortholog of human NEK7 and NEK6 (NIMA-related kinase); predicted to have protein kinase activity and ATP binding activity
odr-1	R01E6.1	putative guanylyl cyclase required for normal responses to all AWC-sensed odorants; ODR-1 is expressed in chemosensory neurons
kin-19	C03C10.1	S/T kinase orthologous to Casein Kinase:involved in endoderm formation, spindle orientation, asymmetric division and fate specification in epidermal seam cells
pdhk-2	ZK370.5	ortholog of human PDKs (pyruvate dehydrogenase kinase, isozyme 4,2,3 and 1); is localized to the perinuclear region of cytoplasm

Among all the genes, we detected an overrepresentation of kinases belonging to the mitogen-activated protein kinase (MAPK) family. The strongest and most consistent reduction of nuclear localization was corresponding to *pmk-3* (Figure 3.22.A). *pmk*s enconde *C.elegans* homologs of mammalian p38: *pmk-1* (B0218.3), *pmk-2* (F42G8.3), *pmk-3* (F42G8.4). MAPKs are serine/threonine protein kinases that can phosphorylate both cytoplasmic and nuclear targets.

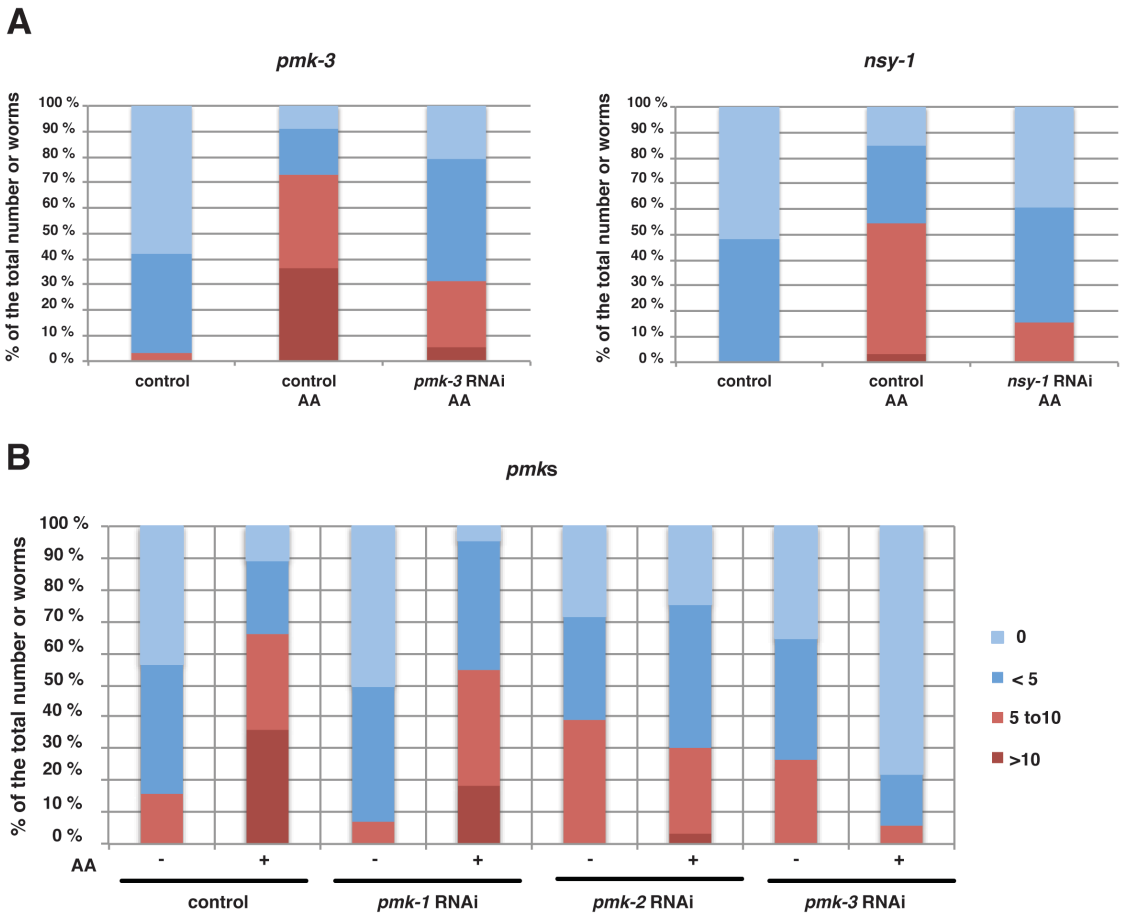

Figure 3.22. p38/PMK-3 and ASK1/NSY-1 mediate the nuclear localization of KLF-1
A and **B**) Nuclear localization of *pklf-1::klf-1-yfp* in wild type worms feeding bacteria carrying the L4440 empty vector (control) or targeting specific kinases (*pmk-1, pmk-2, pmk-3, nsy-1*) without prooxidant treatment or exposed to 0,5 µM AA. The AA treatment was started at day one of adulthood and the scoring was performed at day two indicating the percentage of worms exhibiting different amounts of nuclei visible (none, less than five, five to ten or more than 10 nuclei per worm).

The activation of p38 MAPKs has been associated traditionally with the stress responses and apoptosis. However, p38 MAPKs play additionally an important role in cell proliferation, differentiation, survival and immune response (Nebreda and Porras, 2000). Interestingly we also observed a reduction in nuclear localization of

KLF-1 by *nsy-1* RNAi (Figure 3.22.A). *nsy-1* is the only homolog of the apoptosis signal-regulating kinase (ASK) in *C.elegans,* and is also known as *ask-1*. Its functions are well conserved with those of mammalian ASK1: NSY-1 was first described as regulator of neuronal cell fate (Sagasti et al., 2001). It was later shown that NSY-1 (MAP3K)/SEK-1(MAP2K)/PMK-1(MAPK) signaling pathway is indispensable for the immune response to various bacterial infections (Kim et al., 2002) as well as for oxidative stress response (Inoue et al., 2005; Kondo et al., 2005).

In order to analyze whether the effect on nuclear localization exerted by PMK-3 was specific we analyzed the other two p38 homologs PMK-1 and PMK-2: *pmk-1* RNAi AA did not reduce the nuclear localization of KLF-1compared to control AA while *pmk-2* RNAi AA slightly although the effect seemed to be AA independent (Figure 3.22.B). Finally *pmk-3* RNAi AA showed a clear reduction in KLF-1 nuclear localization (Figure 3.22.B).

In addition to the effect observed in the nuclear localization, *pmk-3* resulted a promising candidate for KLF-1 regulation, due to the fact that there are three predicted phosphorylation sites for this kinase on KLF-1 (Figure 3.23.A)((NetPhos 2.0, (Blom et al., 1999)). We therefore proceeded by mutagenizing the predicted *pmk-3* phosphorylation sites. We started with S29 and S280 because of their high prediction scores. We specifically mutated each of the serines to either aspartic acid (D), to mimic a constitutively active form, or to alanine (A), to inhibit phosphorylation and therefore resulting in a inactivable form. These mutated versions of KLF-1 were then fused to YFP and expressed under the control of its endogenous promoter. All four constructs were then individually microinjected into wild type worms to generate for reporter strains of KLF-1 nuclear localization.

Remarkably, none of the four strains showed any nuclear localization (Figure 3.23.B.). In the case of the constitutively active forms, where one could expect enhanced nuclear localization, a feasible explanation for the lack of nuclear localization is that the switch in the amino acidic sequence results in a conformational change and therefore an altered function. In the case of the inactivable forms, the absence of nuclear localization is in agreement with these

two phosphorylation sites being essential for KLF-1 nuclear localization, although we cannot exclude the possibility that conformational changes have happened in this case too. Therefore in vitro studies will be performed to overcome this limitation.

A

position	context	Phosphorylation score	p38MAPK specific phosphorylation score
*S*39	SVHLSPSFF	0.886	0.52
*S*280	DSPTSPCVK	0.991	0.59
*T*178	SGFNTPTTA	0.696	0.52

B

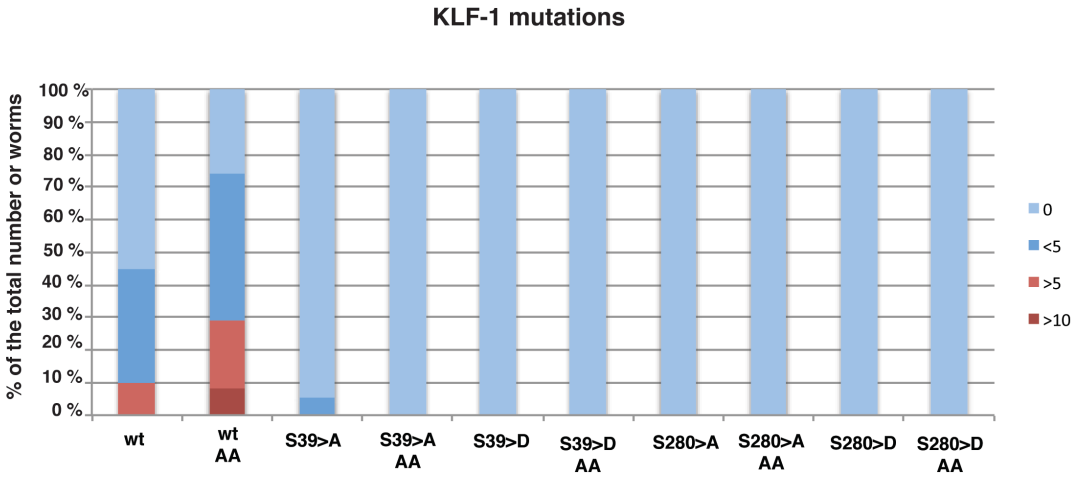

Figure 3.23. Mutation of predicted phosphorylation sites abolishes nuclear localization of KLF-1
A) List of the three predicted p38 MAPK phosphorylation sites with their sequence context and prediction scores **B**) Nuclear localization of the KLF-1 mutations at serines 39 and 280 to alanine (A) or aspartic acid (D) in the presence (AA) or absence of AA.

Although the nuclear localization assays strongly implicated *pmk-3* in the activation of KLF-1, the variability in such an observational study is quite high. Therefore we decided to study the impact of *pmk-3* on the activation of KLF-1 predicted targets (Figure 3.24.) Knockdown of *pmk-3* clearly reduced the mito-induced upregulation of *cyp*s expression in the *isp-1;ctb-1* mutants.

By contrast, the effect of *pmk-3* in wild type worms was not consistent, having a different impact on each gene tested. The effect of *pmk-1* and *pmk-2* RNAi in *isp-1;ctb-1* was either not significant or downregulated the expression of *cyp*s but not

to the same extend as *pmk-3*. In the wild type background again, the knockdown of both *pmk-1* and *pmk-2* had different effects on each *cyp* tested (Figure 3.24.A). Therefore, altogether these results suggest that *pmk-3* regulates *cyp* expression in *isp-1;ctb-1* mutants. In order to analyze whether this effect was similar to the one exerted by *klf-1*, we measured the levels of the same three enzymes in *isp-1;ctb-1* worms after *klf-1* or *pmk-3* RNA. We observed that in all three cases *pmk-3* has the same effect, or even stronger than *klf-1*(Figure 3.24.B), reinforcing the idea that both PMK-3 and KLF-1 act together in the same pathway regulating the expression of DMEs.

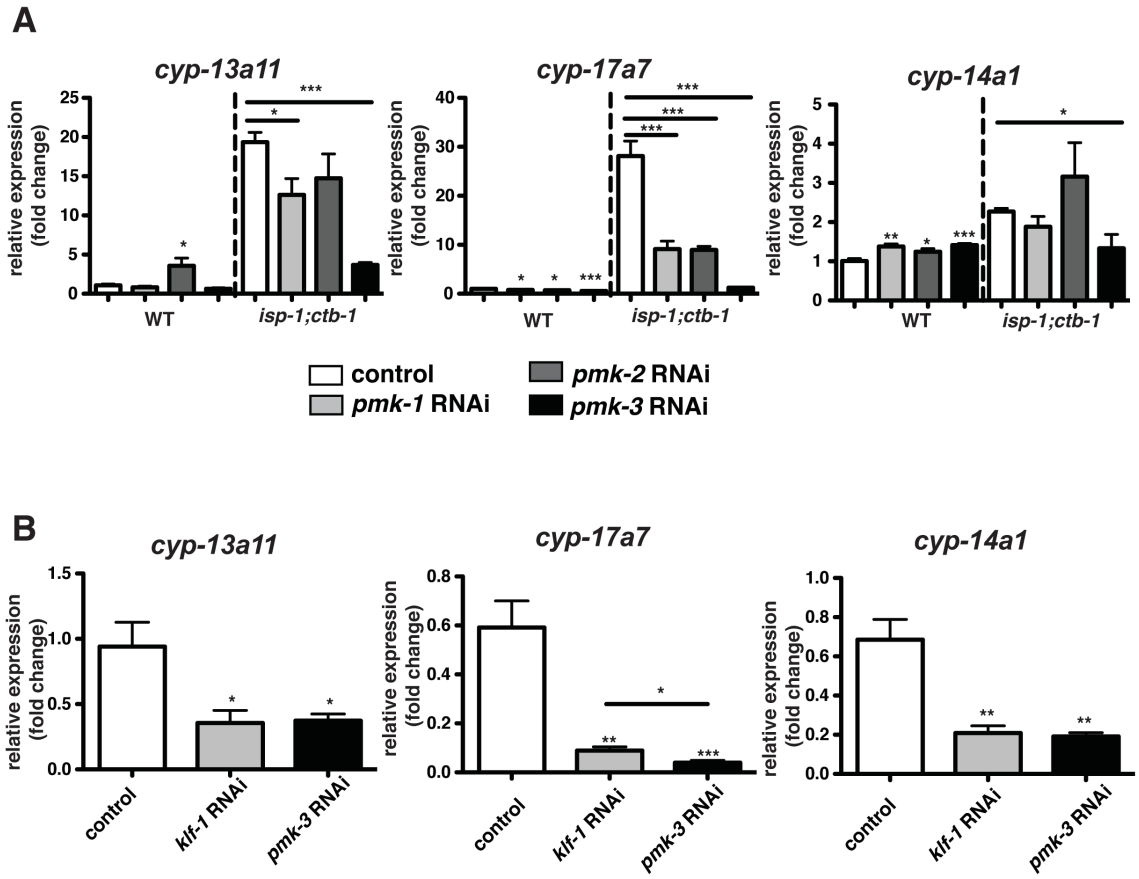

Figure 3.24. The expression of *cyp*s in *isp-1;ctb-1* mutants is specifically regulated by *pmk-3* resembling *klf-1* RNAi
Expression levels measured by qPCR of the genes *cyp-13a11*, *cyp 17a7* and *cyp-14a1* at day one of adulthood in wild type worms and *isp-1;ctb-1* mutants feeding bacteria carrying the L4440 empty vector or targeting *pmk-1*, *pmk-2*, *pmk-3* (**A**) and exclusively in the mutants feeding L4440, *pmk-3* or *klf-1* RNAi (**B**). Bars represent mean ± S.E.M. (*p<0.05, **p<0.01, ***p<0.001, Student's T-test.

The fact that we pinpointed two kinases previously described to be in a common pathway, directed us to identify the other intermediate players involved in it. Since we already had a MAPK and MAPK3 we were interested in identifying a MAPK2 to complete the characteristic MAPK-cascade structure(Seger and Krebs, 1995). Therefore and based on bibliographic references we selected the genes fulfilling our criteria and tested their impact on KLF-1 nuclear localization. *sek-1*, a MAPK2 previously described to be downstream of *nsy-1* and upstream of *pmk-1* in SKN-1 (Hoeven et al., 2011) activation among other processes (Tanaka-Hino et al., 2002), and *mkk4* involved in presynaptic development upstream of *pmk-3* (Nakata et al., 2005) both reduced nuclear localization of KLF-1, being the impact of *sek-1* more prominent (Figure 3.25.A).

Despite of the evidence collected involving oxidative stress as a trigger and MAPK signaling as mediator of KLF-1 activation, a direct link between these two was still missing in our model. Therefore we focused on studying the role that redox enzymes may play in this process since they act as electrochemical biosensors that can sense and oxidative signal and transduce it into a signaling cascade (Thevenot et al., 2001) and they have been previously described in signaling pathways like *prdx-2*-involvement in stress resistance and longevity upstream of SKN-1 (De Haes et al., 2014; Olahova and Veal, 2015). We performed a directed feeding screen of redox enzymes available in our library (See Table 2.1) and analyzed the impact of their knockdown on KLF-1 nuclear localization. This approach showed great variability among repetitions and therefore we could not conclude with certainty about the involvement of these genes in KLF-1 activation. As an example of this great variability we show *trx-1* (Figure 3.25.B1 and B2). The rest of the genes showed similar variation (not shown). The further upstream we go in the signaling cascade, the higher variation we observe, which is understandable since the interference with the pathway is minor, since alternative mechanisms can be involved in activating the cascade and little expression levels of the proteins may be enough to activate the nuclear localization. A different approach is needed for studying the role of redox enzymes in the KLF-1 mediated longevity, for example using specific mutants for the genes of interest.

Nevertheless, what mostly called our attention during this experiment was the fact that RNAi of *trx-4* combined with AA treatment was sufficient to increase KLF-1 nuclear localization to levels even higher than AA control (Figure 3.25.C). This result could be in agreement with previous reports of TRXs acting as negative modulator of signaling cascades: Trx1 binds kinase ASK1 and the ROS-mediated oxidation of Trx1 dissociates the Trx1-ASK1 complex and liberate the first kinase of the signaling cascade (Saitoh et al., 1998) (Figure 3.25.D). We did not observed any significant changes in expression of *trx-4* based on our microarray data but detected a +1,3 fold increase of *trx-5* in *isp-1;ctb-1* mutants and a -1,6 and -2,1 fold decrease of *gpx-6* and *gpx-7* respectively after *klf-1* knockdown during adulthood (See Appendix C). We have just collected preliminary results about the involvement of redox enzymes in the activation of KLF-1-mediated longevity of *isp-1;ctb-1* mutants, but we consider that they are promising candidates and further studies are necessary to reveal their potential function in this process.

To sum up, here we describe the role of the transcription factor KLF-1 as the major mediator of the longevity of the *isp-1;ctb-1* mitochondrial mutants. An oxidative stress signal triggers KLF-1 translocation into the nucleus where it activates, among others, the transcription of genes involved in detoxification. We provide evidence that DMEs, especially CYPs and UGTs are essential for the longevity assurance of *isp-1;ctb-1* mutant and its developmental delay. We postulate that KLF-1 and its main targets CYPs can foster SKN-1-dependent activation of phase II genes, that probably also influence *isp-1;ctb-1* longevity. Despite the impact of KLF-1 observed in *daf-2(e1370)* longevity, we can not confirm the mechanisms responsible for the effect observed since the *cyp*s tested failed to affect lifespan of these IIS mutants. Probably a different subset of genes regulated by KLF-1 is responsible for the observed reduction in the longevity of *daf-2(e1370)* and therefore further studies need to be done. Finally, we show that the p38 homolog *pmk-3,* regulates KLF-1 nuclear localization and propose a model where redox enzymes and MAPKs constitute the activation-signaling cascade of KLF-1 (Figure 3.25.D).

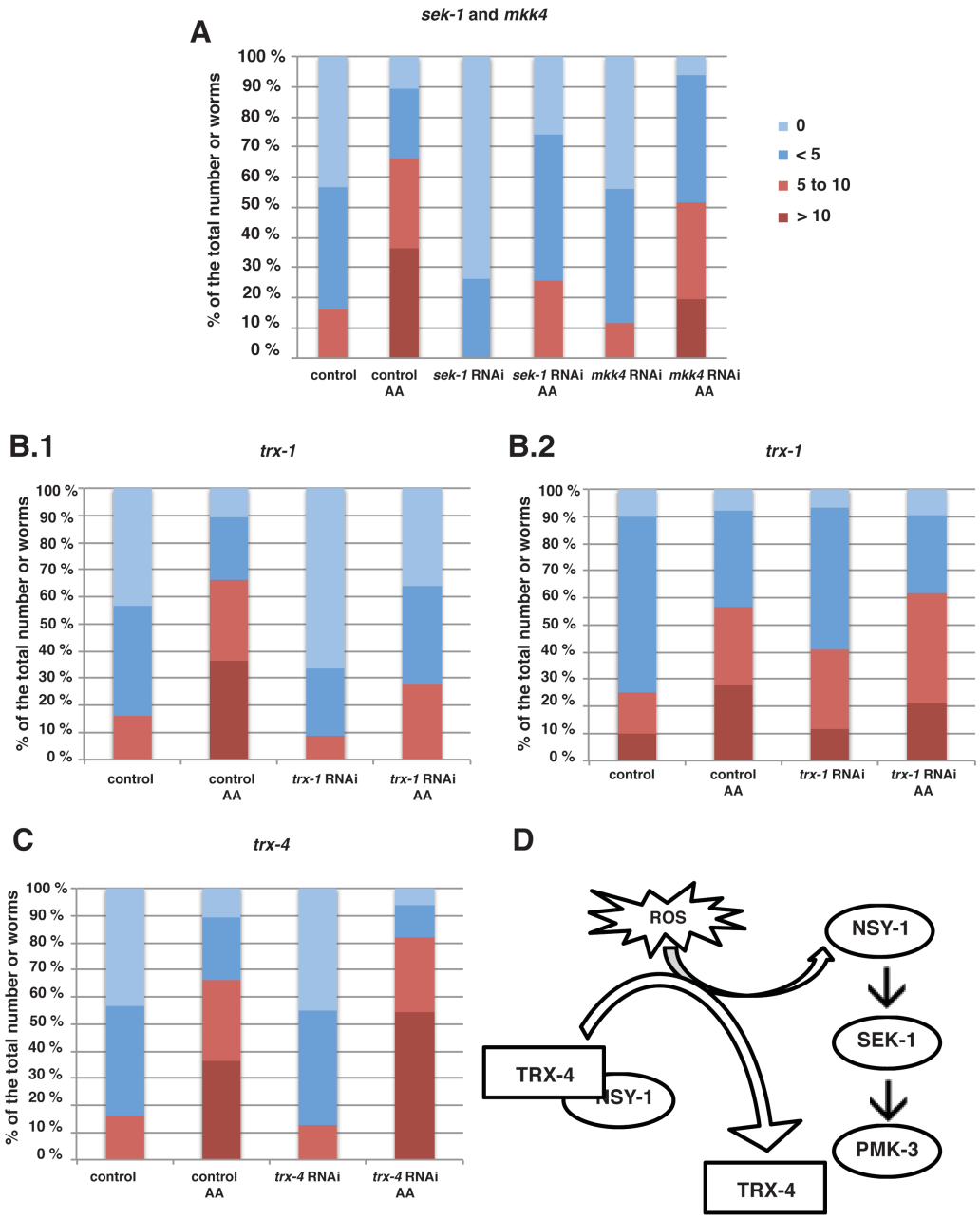

Figure 3.25. KLF-1 may be regulated by a redox-sensitive enzyme upstream of a MAPK signaling cascade

Nuclear localization of *pklf-1::klf-1-yfp* in wild type worms feeding bacteria carrying the L4440 empty vector (control) or targeting specific kinases (*sek-1, mkk4*)(**A**) or redox enzymes like *trx-1*(**B1** and **B2**) or *trx-4* (**C**) without prooxidant treatment or exposed to 0,5 µM AA. The AA treatment was started at day one of adulthood and the scoring was performed at day two indicating the percentage of worms exhibiting different amounts of nuclei visible (none, less than five, five to ten or more than 10 nuclei per worm). **D**) Model proposed for KLF-1 activation.

4. Discussion and outlook

For the past decades, great advances have been done in the discovery of genes involved in lifespan determination from yeast to mammals. Striking progress has been done in the roundworm *C.elegans:* researchers have discovered mutations where lost of function increases lifespan, and assumed that those genes would regulate normal life in wild type, and further elucidated the mechanisms acting upstream and downstream of those genes, resulting in a very powerful tool for identification of aging mechanisms. Some of the first genes identified fitted in three main categories like dauer formation, food intake or genes affecting physiological rates, but the list of "longevity-genes" keeps growing (reviewed in (Kenyon, 2010)). Increasing evidence has also shown that mitochondrial ETC has a great impact in lifespan determination (reviewed in (Dancy et al., 2014)), and the mechanisms responsible for this consequence remain being studied and discussed. The complexity of the aging research field rises, as increasing information is collected and isolated longevity pathways start being correlated. This leads to the establishment of a complex longevity network where different combinations of transcription factors activate a certain transcriptional response that varies depending on the prolongevity signaling-trigger, altogether helping us understand the mechanisms responsible for lifespan regulation in wild type conditions.

Intrigued by understanding the mechanism responsible for the longevity assurance in mitochondrial mutants, we identify the transcription factor KLF-1 as a novel mediator of *isp-1;ctb-1* longevity. We propose a model where mtROS activates a redox dependent signaling cascade that results in KLF-1 activation and nuclear translocation where it initiates a mitohormetic stress response regulating the expression of, among others, detoxification genes, which are essential for the lifespan extension of these mit-mutants.

4.1. Identification of KLF-1 as mediator of mito-induced longevity

The Krüppel-like factor (KLF) family includes transcription factors involved in several biological processes like development, growth, differentiation, proliferation and survival as well as response to external stressors (reviewed in (McConnell and Yang, 2010)). KLFs share structural features: first the conserved carboxy terminus (C') with three Krüppel-like zinc fingers for binding to GC-rich region of DNA and CACCC elements, trough which they mediate transcriptional activation or repression. In the proximity of this zinc-finger motifs they include nuclear localization sequences and finally the amino terminus (N'), which is much more variable, confers the specificity by mediating the interaction with coactivators/repressors and modifiers. KLFs are very conserved among mammals from mouse to human but are also present in lower organisms like worms (*C.elegans*), zebrafish (*Danio rerio*), frog (*Xenopus laevis*) or chicken (*Gallus gallus*). In contrast to humans, where the KLF family is represented by 17 members, the genome of *C.elegans* contains only three predicted homologs: *klf-1* (F56F11.3), *klf-2* (F53F8.1), and *klf-3* (also known as *mua-1*, F54H5.4). *klf-3* has been implicated in cell matrix attachment and more extensively in fat metabolism (Plenefisch et al., 2000; Zhang et al., 2011; Zhang et al., 2013a; Zhang et al., 2009) On the other hand, *klf-1* has also been shown to play a role in lipid metabolism as well as cell death and phagocytosis (Brey et al., 2009; Hashmi et al., 2008) and remarkably, it has been recently described as a target of the E3 ligase WWP-1 and a mediator on dietary-restriction-induced longevity (Carrano et al., 2014).

KLF-1 is mostly expressed in the intestine but also detectable in few hypodermal and neuronal cells. *klf-1* transcript levels progressively increase during development in wild type worms with a maximum level at L4 larval stage followed by a decrease at day one of adulthood (Hashmi et al., 2008). Here we show that *klf-1* is necessary during adulthood to mediate the longevity of *isp-1;ctb-1* mutants as well as the longevity induced by RNAi of different MRC subunits during development, suggesting that KLF-1 acts as a "memory molecule" that responds to a developmental signal to ensure longevity. This is in agreement with

previous observations that suggest that mitochondrial function during development is key for lifespan determination (Dillin et al., 2002).

4.2. KLF-1 is essential for the mitohormetic response to oxidative stress

The use of pro-oxidant drugs that interfere with the ETC and promote the generation of ROS is a broadly accepted intervention to study oxidative stress. Exposure to low concentration of paraquat, which favors the generation of ROS at complex I (Cocheme and Murphy, 2008) can extend the lifespan of wild type worms. A similar mechanism of lifespan extension was proposed for mitochondrial mutants, in view of the fact that exposure to PQ could not further extend the lifespan of the long lived mit-mutants *isp-1* and *nuo-6* but it had an additive effect on other longevity models like *eat-2*, *sod-2*, *daf-2* and *clk-1*(Yang and Hekimi, 2010a). Our result confirms their observation and implicates *klf-1* directly in this oxidative stress-mediated lifespan extension, considering that the pro-longevity effect of PQ is completely abolished in *klf-1*(tm1110) mutants.

Since KLF-1 is a transcription factor, we hypothesized that upon activation it should translocate in to the nucleus to activate its transcriptional program. We confirmed our conception by making use of two different pro-oxidants: not only PQ, but also AA, which acts at the level of complex III (Boveris and Cadenas, 1975; Quinlan et al., 2011), promote the translocation of KLF-1 into the nucleus at different concentrations. Remarkably, this drug treatment phenocopied the nuclear localization of KLF-1 in *isp-1;ctb-1* mutants without any drug treatment, convincingly suggesting that the nuclear localization of KLF-1 on both, drug treated and mit-mutants, is due to oxidative stress. After our dose-response experiment with PQ and AA to determine the impact on nuclear localization, we chose to further work with the lowest AA concentration that showed the highest effect, 0,5 µM. We opt for AA due to its specific effect on complex III.

The electron transfer in complex III operates by a Q cycle mechanism where two electrons are bifurcated down different paths: the first trough the high potential chain from the Rieske Fe-S (*isp-1*) center trough CYT-c1 to CYT-C and

complex IV, and the second down the low potential chain from cytochrome b (*ctb-1*). Thus two turns of the Q cycle result in the oxidation of two quinols at Qo and reduction of quinone at Qi. Antimycin A binds at Qi inhibiting the oxidation of CYT-b hemes, therefore limiting the oxidation of semiquinone at Qo, which is highly reactive to interact with molecular oxygen and generate superoxide (Quinlan et al., 2011) and mimicking the *isp-1;ctb-1* mutations.

4.3. The oxidative stress response in *isp-1;ctb-1* mutants: ROS and ROS scavengers

The redox imbalance and oxidative damage observed in *isp-1;ctb-1* mutants can be attributed to different causes: a higher rate of ROS production, a lower rate of ROS scavenging or a combination of both events. Interestingly, *isp-1;ctb-1* mit-mutants were previously shown to exhibit a mitochondrial specific increase in ROS production (Yang and Hekimi, 2010a).

The hydrogen peroxide scavengers *ctl-1* and *ctl-3* did not show a different expression in the mutant, nor compared to the wild type, neither in response to *klf-1* RNAi. On the other hand the superoxide dismutases *sod-1*, *sod-2* and *sod-3* showed a tendency towards increased expression at day one in the mutants compared to wild type but not always statistically significant. However, more interestingly, the upregulation was *klf-1* dependent, suggesting that ROS scavengers are active already at this point in mit-mutants, whereas in wild type it is common to observe an age dependent increase of expression. Nevertheless the expression pattern of *sod*s can not explain the oxidative damage observation, which is reduced with age in *isp-1;ctb-1* and increased in wild type in a KLF-1-dependent manner, implying that a more complex response has to be activated to fight oxidative stress.

When oxidative stress persists it can generate oxidative damage as we observed is the case in one day-old mit-mutants and old wild type worms. The carbonylation of proteins is an irreversible damage, and therefore it is not necessarily correlated with the expression of ROS scavengers, which could prevent the damage but not remove it once it is done. Moreover, protein carbonylation can

result in an amplification of ROS (Frohnert and Bernlohr, 2013) and be involved in signaling processes (Wong et al., 2008). Therefore the measurement of protein carbonilation via OxyBlot captures a transcient state of a dynamic process dependent on the ROS production/scavenging rate and the clearance of the damaged proteins.

The mechanisms regulating this clearance of carbonylated proteins and their impact in cellular homeostasis remain unclear, however it is known that both autophagy (Campesi et al., 2013; Inoue et al., 2015; Navarro-Yepes et al., 2015) and proteasome (Grune et al., 1997; Pajares et al., 2015; Shringarpure et al., 2001) participate in their degradation. Yang and Hekimi reported that there is no increase in autophagy in *isp-1* mutants while this process is enhanced after *isp-1* RNAi suggesting two alternative pathways for mitochondrial longevity (Yang and Hekimi, 2010b). However this process has to our knowledge never been studied in *isp-1;ctb-1* double mutants and it would be interesting to see if the second mutation *ctb-1* has any impact on autophagy regulation.

Proteosomal activity has been shown to be enhanced in germline-deficient longlived worms, being this process essential for their longevity assurance (Vilchez et al., 2012). The mitochondrial dysfunction induced by *cco-1* RNAi analyzed in that study did not result in an increase in proteosomal activity. But mitochondrial dysfunction has not only been shown to not increase proteosomal activity but to be capable of actually hindering it (Livnat-Levanon et al., 2014; Segref et al., 2014): mitochondrial oxidative stress induced by RNAi of different mitochondrial ETC subunits including *isp-1*, has been shown to result in the accumulation of a short-lived ubiquitin-fusion protein (Segref et al., 2014) extensively used to monitor the activity of the ubiquitin/proteasome system (Johnson et al., 1995). Hence, mitochondrial dysfunction is able to reversibly disassemble the 26S proteasome (Livnat-Levanon et al., 2014) resulting in transient proteosomal dysfunction, which can be restored after antioxidant treatment (Segref et al., 2014). This implies that proteosomal activity is sensitive to redox homeostasis and therefore a defective clearance of damaged proteins could account for the increased levels detected in the *isp-1;ctb-1* mutants at day one, which after activation of the mitohormetic response is restored at day five.

One of the major difficulties in understanding the role of ROS in *C.elegans* aging and its involvement in oxidative stress signaling, is the widely recognized methodological limitation to specifically measure ROS production *in vivo* (Wardman, 2007). Therefore the results produced by using such controversial methods have to be carefully interpreted. Our data obtained from using mitochondrial specific fluorescent dyes suggests that the *isp-1;ctb-1* mutant have an increased mitochondrial mass, which could be a compensatory mechanism to their mitochondrial dysfunction. Due to the increase in mito-mass we observe an increased net superoxide production but not an increase in the superoxide rate relative to mitochondria. Hence the mitochondrial specific ROS trigger could be a different form of ROS other than superoxide, since we detect a mito-specific increase with MitoTracker CMH_2XRos. Due to its short half-life superoxide is not believed to act as signaling molecule but rather H_2O_2, which is more stable and can diffuse out of mitochondria (Andreyev et al., 2005).

The specificity of these dyes is however conflicting (Kaludercic et al., 2014): MitoSOX Red is a derivate of hydroethidine (HE), which upon reaction with superoxide generates a highly specific fluorescent product 2-hydroxyethidum, which has led to the wide use of MitoSOX as specific superoxide formation (Robinson et al., 2006). However another red fluorescent product can result from reaction to other oxidants in living cells (Zhao et al., 2005), therefore limiting the specificity of the measurement. On the other hand reduced dyes like MitoTracker CMH_2XRos (Poot et al., 1996) which is a derivate of dihydro-X-rosamine, become fluorescent and positively charged upon oxidation in living cells and accumulate in mitochondria but are not specific for a single oxidant species.

DCFDA is a popular fluorescent probe used for ROS assessment *in vivo* and *in vitro* but probably one of the most conflicting methods due to the variation in the protocols and to the complexity of its intracellular redox chemistry (Halliwell and Whiteman, 2004; Kalyanaraman et al., 2012; Wardman, 2007). Briefly, when applied on biological probes, DCFDA is first deacetylated by endogenous esterases to dichlorofluorescein (DCFH). DCFH can then react with different ROS species forming the fluorophore DCF, which can be detected. DCFDA has been routinely used measure intracellular generation of H_2O_2 and other ROS species or monitor

redox signaling changes in cells in response oxidative stimuli (Karlsson et al., 2010; Tampo et al., 2003). However there are some key limitations and artifacts associated with the DCF assay for intracellular H_2O_2 measurement being the most important that H_2O_2 itself cannot directly react with DCFH to generate DCF (reviewed in (Kalyanaraman et al., 2012)), indicating that DCFDA can be exclusively used as an indirect measurement of H_2O_2. Redox active transition metals have been shown to be highly reactive and even required for DCF fluorescence, likewise intracellular glutathione levels are also determinant for this signal (Karlsson et al., 2010; Tampo et al., 2003).

Regarding the methodology, DCFDA is often used in worm lysates (Arczewska et al., 2013; Lee et al., 2010) there is big controversy about the correctness of this method precisely because of the limitations named above (Fong et al., 2010). Worm lysate preparation trough freezing and thawing or sonication, disrupts the cuticle as well as the internal membranes. The intraorganelle content is released, including transition metals that can amplify the redox signal by Fenton reactions, therefore being the levels measured not comparable to the physiological ones. Additionally this measurement does not ensure the mitochondrial original of the signal, and therefore some researchers defend its application just to whole worms (Fong et al., 2010). Here we use DCFDA to compare relative levels of ROS production between different conditions and are not seeking for a quantitative result. If we consider that the release of intraorganelle content would result in an amplification of the signal, this would *a priori* not explain how the *isp-1;ctb-1* mutants and more specifically the pro-oxidant AA treatment , result in such low levels of DCFDA signal.

Nevertheless, using DCF signal as a readout of metal-dependent ROS generation, this could be related with the increased ferritin levels observed in the microarray data in *isp-1;ctb-1* old mutants, which could quench free iron therefore resulting in a reduced amplification of the signal. At the moment we lack more data regarding ferritin levels early in life, but we are very interested in further understanding the impact of this process in lifespan determination and some additional remarks regarding the role of ferritin will be discussed later. An alternative explanation for the low levels of ROS detected in both mutant and AA

treated worms, could be that the sick and fragile mitochondria present in the mutants or AA-treated worms are more severely affected and easily broken than wild type mitochondria during lysate preparations, thus resulting in fewer respiring mitochondria in the samples, which are major ROS producers. This measurement should be therefore repeated in living worms.

The previous report of superoxide specific increase in *isp-1* worms was performed in isolated mitochondria (Yang and Hekimi, 2010a), which on the one hand is useful to ensure the origin of the ROS production, but on the other hand the isolation steps can damage or break mitochondria. Additionally the obtention of enough mitochondrial sample in *C.elegans* is a limiting methodological issue.

Many of these technical limitations were overcome by the use of genetically encoded fluorescent proteins, which are great tools for the measurement of ROS *in vivo* and its compartmentalization (Gutscher et al., 2008; Ostergaard et al., 2001). These probes have enabled the successful quantification of H_2O_2 and establishment the glutathione redox state in *C.elegans* (Back et al., 2012) and *Drosophila* (Albrecht et al., 2011). Both constructs used for our measurements are expressed under the control of the *rpl-17* promoter, a cytosolic ribosomal protein, and therefore our data are limited to this subcellular compartment. The HyPer results show that the *isp-1;ctb-1* mutants have decreased levels of hydrogen peroxide at both day one, and even lower at day five of adulthood. These results also correlate with the lower levels obtained with the DCFDA. The reduction in ROS levels observed, combined with the upregulation of *sods* at day one, strongly suggests that an antioxidant response is already active at this point in life. Nonetheless a KLF-1-dependent redox imbalance is detectable with Grx1-roGFP2. Altogether this suggests that we may have missed the moment of ROS upregulation and are looking at an already activated oxidative stress response. We hypothesize that the prolongevity signal arises from a transient ROS pulse produced by the dysfunctional mitochondria of *isp-1;ctb-1* mutant coinciding with the increase on somatic mitochondrial biogenesis during L3/L4 larval stages (Bratic et al., 2009; Dillin et al., 2002). Therefore it will be interesting to measure ROS levels throughout all the different larval stages as well as later in life and additionally

using the genetically-encoded protein-reporters expressed specifically in different cellular compartments.

4.4. KLF-1 regulates xenobiotic detoxification genes

Our microarray data identified "oxidation-reduction" as the most affected biological process together with the two KEGG-pathways "Metabolism of xenobiotics by cytochrome P450" and "Drug metabolism". The drug-metabolizing enzymes (DMEs) are classified in two main groups according to their functions: oxidative and conjugative. The first group or phase I enzymes is formed by NADPH-cytochrome P450 reductases/cytochrome P450 (CYPs): they are oxidative enzymes that mainly hydroxylate endogenous metabolites like steroids, prostaglandins and fatty acids and xenobiotic substrates such as environmental pollutants, agrochemicals or toxins (Iyanagi, 2007). The second group of DMEs that conducts the phase II includes UDP-glucuronosyltransferases (UGTs), glutathione S transferases (GSTs), sulfotransferases and acetyltransferases. UGTs are conjugative enzymes that add side groups to toxic compounds that in most of the cases have been previously activated by the phase I, in order to increase their solubility and therefore expedite their excretion (Iyanagi, 2007). GSTs mainly catalyze the conjugation of electrophilic substrates to glutathione (GSH), but have also other functions like peroxidase and isomerase activities, protection against H_2O_2-induced cell death and non-catalytically binding to a wide range of endogenous and exogenous ligands (Sheehan et al., 2001).

Cytoprotective mechanisms including detoxification, innate immunity, proteostasis, and oxidative stress response have been increasingly described as potential mediators of longevity in *C.elegans* and across species. So far, studies of xenobiotic response have focused on the identification of the effectors of detoxification rather than on the mechanisms by which these effectors are regulated (Shore and Ruvkun, 2013). For this reason, the upstream signaling and regulatory factors are still poorly understood.

The upregulation DMEs appears to be an increasingly recognized transcriptional signature of many long-lived mutants across species (Liu et al., 2014; McElwee et

al., 2004; Shore et al., 2012; Shore and Ruvkun, 2013). Moreover, previous studies have detected increased expression of some *cyp, ugt,* and *gst* genes in long-lived mitochondrial mutants (Cristina et al., 2009; Liu et al., 2014). This phenomenon has been explained as an evolutionary advantage of the soil nematode since many pathogens of *C.elegans* habitat specifically target mitochondria and therefore the coupling of both mitochondrial dysfunction and xenobiotic response would be clearly beneficial (Liu et al., 2014). Strikingly, other longevity promoting pathways, such as decreased insulin/IGF1 signaling or dietary restriction, also activate the expression of detoxification genes in a wide array of organisms ranging from worms (McElwee et al., 2004; Murphy et al., 2003) to mammals (Steinbaugh et al., 2012) and therefore probably the coupling of longevity and detoxification different has arisen from different mechanisms.

The transcriptional regulation of CYPs in mammals is known to be a complex process dependent on nuclear transcription factors, different cofactors, and an elaborated signaling cascades (Tralau and Luch, 2013). Additionally, posttranscriptional regulation at the level or mRNA stability (Lin, 2006), splicing (Corcos et al., 2012), protein degradation (Pabarcus et al., 2009) or epigenetic (Tokizane et al., 2005) has also been reported. Interestingly, some mammalian KLFs have been shown to bind the basic transcription element (BTE) in different *cyp* promoters, and therefore, potentially regulate their expression, although the physiological relevance of this remains unknown (Yanagida et al., 1990; Zhang et al., 1998). In contrast to mammals, little is known about the regulation of the xenobiotic detoxification genes in *C.elegans*. A similar model of regulation with nuclear receptors and the aryl hydrocarbon receptor is proposed for *C.elegans* (Lindblom and Dodd, 2006) although little evidence has been yet collected supporting this model (Butler et al., 2001; Jones et al., 2013; Lindblom et al., 2001; Powell-Coffman et al., 1998) probably due to the massive expansion of the nuclear hormone receptor (NHR) family in *C.elegans* (Maglich et al., 2001; Sluder et al., 1999).

Most of the progress has been done by screening for mediators of resistance after exposure to specific xenobiotics. This has led to widening the list of genes involved in xenobiotic responses in *C.elegans*: *hif-1* (Budde and Roth, 2011; Miller

et al., 2011), *mdt-15* (Taubert et al., 2008), *nhr-8* (Lindblom et al., 2001), *skn-1* (An and Blackwell, 2003; Hasegawa et al., 2010; Miller et al., 2011). In addition to these xenobiotic-response studies, the regulation of DMEs has gained attention, as it has been shown to be upregulated in different longevity model (as above introduced) but without exposure to drugs, implying that alternative regulatory mechanisms may exist. Since we observed a very similar expression pattern for members of both the *cyp* and *ugt* gene families as well as some *gst*s in the *isp-1;ctb-1 mutants*, with very high upregulation in early adulthood and subsequent downregulation later in life, we propose KLF-1 as a novel key regulator of DMEs in *C.elegans* and are intrigued by better understanding this process in the context of different longevity pathways and identifying possible co-regulators.

Additionally, here we provide evidence that these detoxification genes are essential for the longevity assurance of the longlived *isp-1;ctb-1* mit-mutants. Interestingly, knockdown of both *cyps* and *ugts* results in a partial rescue of the developmental delay characteristic of the *isp-1;ctb-1* mutants, suggesting that these genes could negatively affect developmental rates. This is an example of time-sequential antagonistic pleiotropy in which the same genes have beneficial effects at one certain stage in life and detrimental in others (Williams, 1957). Such a negative impact on development has been recently reported for the upregulation of *cyp-35a* family genes by caffeine (Min et al., 2015). Remarkably we do not observe any impact on development after *klf-1* RNAi. There are several possible explanations for this fact. On the one hand, we known that the level of suppression exerted by *klf-1* RNAi on *cyp* and *ugt* levels is not the same as the one exerted by direct inhibition of the target genes. Another possibility is that *klf-1* RNAi results simultaneously in the transcriptional modulation of other genes that counteract the negative impact of increased DMEs expression during development. Alternatively and in even more likely, it is possible that the developmental regulation of *cyps* and *ugts* contrary to the regulation during adulthood is independent of KLF-1, in agreement with our observation of the specific requirement of KLF-1 during adulthood for longevity assurance. Nevertheless we lack enough information about both DMEs and KLF-1 function during development to further explain this observation. Therefore we consider that it would be

interesting to measure the expression levels of DMEs during development in the presence or absence of KLF-1 to better understand this process.

The specific role of *klf-1* in lifespan determination of mitochondrial mutants has been previously dismissed based on the observation that knockdown of *klf-1* and its homologs *klf-2* and *klf-3* resulted in minor changes in longevity of *isp-1* mutants (Carrano et al., 2014). Contrary to this report, we detect a complete normalization of both *isp-1;ctb-1* and *isp-1* lifespans compared to control upon *klf-1* RNAi and we attribute this effect entirely to *klf-1* and not its homologs due to the specific upregultaion of *klf-1* in *isp-1;ctb-1* mutants and the specific impact in the expression of DMEs. This discrepancy could be due to experimental differences in lifespan experiments for example by the use of FUdR ant it should be carefully analyzed.

In *C.elegans* little is known about the function and specificity of individual CYPs in contrast to mammals, therefore we can just speculate about how these enzymes result in a lifespan extension. The idea of enhanced cytoprotection is increasingly accepted as a beneficial feature determining lifespan (Shore and Ruvkun, 2013). One can expect that enhanced DMEs expression results in a better detoxification of metabolic toxic byproducts and enhanced resistance to pathogens. Specific examples of the protective role of CYPs have also been reported in mice (Lingappan et al., 2014).

However DMEs are not exclusively dedicated to detoxification of xenobiotics, and lipids have been described as endogenous targets of CYPs consequently involving them in lipid metabolism in both worms and mammals (Jiang et al., 2005; Westphal et al., 2015; Zhang et al., 2013b). Hence it is interesting to speculate about the idea that CYPs specifically modulate metabolism thus leading to lifespan extension, also in agreement with our microarray data that identifies lipid metabolism as the second most changed pathway in *isp-1;ctb-1* mutants and after *klf-1* RNAi. In mammals most of the CYPs are found in the endoplasmic reticulum and specific targets are known for specific genes (Terfloth et al., 2007; Wishart et al., 2008; Wolf et al., 2000). Interestingly a subset of CYPS, those targeted to mitochondria, have been previously involved in specific cellular metabolic

processes like biosynthesis of hormones and bile acids from cholesterol, metabolic conversion of vitamin D3 and generation of lipid mediator derived from polyunsaturated fatty acids (Omura, 2006; Tyurina et al., 2014). Targeting of CYPs to mitochondria can be done trough two different mechanisms: mito-translocation of microsomal CYPs after processing of the NH2-terminal region involving an unusual TOM20/TOM22 bypass mechanism (Anandatheerthavarada et al., 2009) or direct targeting of inherent CYPs with canonical signals in their sequence (Ahn and Yun, 2010).

Intrigued by this idea, analysis of the predicted mitochondrial targeting sequences with (Mitoprot, (Claros and Vincens, 1996)) of the mostly upregulated *cyps* in *isp-1;ctb-1* reveals high statistical scores : the *cyp-13a7*, which shows a massive upregulation in *isp-1;ctb-1* mutants of approximately 150 fold, shows a 0'9657 probability of being mitochondrial based on sequence prediction. Further, the *cyp-33e2*, which is one of the few functionally characterized CYPs in *C.elegans*, is involved in the metabolism of the predominant polyunsaturated fatty acid in this worms, eicosapentaenoic acid (EPA) (Kosel et al., 2011; Kulas et al., 2008). Our microarray data reveal an upregulation in *isp-1;ctb-1* worms and a KLF-1-dependent downregulation of +1,5 and -2,2 fold respectively for *cyp-33e2* transcripts. CYP-33E2 shares high amino acid sequence homology with mammalian CYP2J2, which is also involved in lipid metabolism (Jiang et al., 2005).

This facts, together with the appearance of other lipid metabolizing enzymes in the microarray data like *acs-2, elo-6, fat-6* being upregulated in *isp-1;ctb-1* and/or downregulated by *klf-1* RNAi, strongly points towards a change in metabolism as a factor contributing to lifespan determination is the *isp-1;ctb-1* mutants. Additionally this would be in agreement with previous reports involving *C.elegans klf-1* and *klf-3* (Hashmi et al., 2008; Zhang et al., 2009) in lipid metabolism, which is also the case of many mammalian KLFs (reviewed in(Brey et al., 2009; Wu and Wang, 2013)). A change in lipid composition has been reported to be key determinant of susceptibility towards lipid peroxidation (Pamplona et al., 1998), interestingly this observation has been confirmed in longlived IIS *C.elegans* mutants, which show a marked decrease in susceptibility to oxidation determined by fatty-acid chain length (Shmookler Reis et al., 2011). It would be highly

interesting to analyze whether this phenomenon is observed in mitochondrial mutants too and if it is mediated by *klf-1.* We have just very preliminary data regarding *klf-1* and lipids in lifespan determination but think that it is a very promising field and will proceed investigating it.

4.5. Alternative pathways that may contribute to the *isp-1;ctb-1* longevity

KLF-1 is not the first transcription factor involved in mediating the longevity of mitochondrial mutants (reviewed in (Chang et al., 2015)) and therefore we are interested in understanding whether all these already described transcription factors are somehow related to each other and with KLF-1. For this purpose we focus on transcription factors that have been previously related with *isp-1* mutation. The hypoxia-inducible factor HIF-1 is a conserved transcription factor that has been shown to activate genes that promote survival during hypoxia (Shen and Powell-Coffman, 2003; Webb et al., 2009) and after inhibition of respiration in *clk-1* and *isp-1* mutants (Lee et al., 2010). In this study RNAi of mitochondrial ETC subunits and mitochondrial mutation lead to enhanced expression of a reporter of HIF-1 activation (Shen et al., 2005) and a transcriptional upregulation of the HIF-1 targets *nhr-57* and *egl-9* is observed in *clk-1* and *isp-1* mutants, however the stage at which these measurements are done its not specified (Lee et al., 2010).

In our hands, the upregulation of *nhr-57* at day one in *isp-1;ctb-1* mutants, although statistically significant, it is minimal and therefore we doubt about its biological significance and no change in *egl-9* expression (not shown). We cannot conclude whether this difference arises from the analysis of single *vs*. double mitochondrial mutants or if there are other reasons for this discrepancy. What is very interesting is the fact that at day five of adulthood we observe a clear downregulation of both *nhr-57* and *egl-9* transcripts and this is *klf-1* dependent (not shown), which could suggest a KLF-1 dependent inhibition of the HIF-1 response.

The possible relationship between HIF-1 and KLF-1 brings us to take up the matter of ferritin in lifespan determination: A screen looking for regulators of iron homeostasis trough regulation of ferritin (in *C.elegans ftn-1* and *ftn-2*), identified *hif-1* and *klf-3* as negative and *klf-1* as positive regulator of *ftn-1* expression (Ackerman and Gems, 2012; Romney et al., 2011). Our microarray data shows a +1,3 upregulation of *ftn-1* expression in *isp-1;ctb-1* mutants and -2,9 downregulation upon *klf-1* RNAi in five-day-old worms. *A priory* contradictory reports regarding the role of *ftn-1* in lifespan determination have been published: on the one hand suppression of *ftn-1* by HIF-1 (Hwang et al., 2014) has been proposed to be necessary for lifespan determination of *isp-1* mutants while downregulation by CEP-1/p53 has been shown to mediate longevity of *isp-1* by positively regulating *ftn-1* (Baruah et al., 2014). However, altogether these results would fit a model where the transcription factors act sequentially during life to determine longevity with an early upregulation of the HIF-1 response and suppression of *ftn-1* (Ackerman and Gems, 2012; Hwang et al., 2014; Lee et al., 2010; Romney et al., 2011), which would result in the amplification of the ROS signal and a reduction later in life regulated by CEP-1 and KLF-1 ((Ackerman and Gems, 2012; Baruah et al., 2014) and this work). A possible synergistic activity of both KLF-1 and CEP-1 results very appealing since there are reports of human KLF4 modulating target selectivity of p53 (Brandt et al., 2012).

The role of ferritin in lifespan determination remains controversial but seems highly promising and further analyses have to be done to determine the expression patterns and regulation during life. Another interesting observation is the fact that *klf-1* and *klf-3* where identified as opposing regulators of *ftn-1* expression (Ackerman and Gems, 2012), we observe opposite impact on *cyp*s expression after RNAi and both have been implicated in lipid metabolism (reviewed in (Hashmi et al., 2011)) suggesting that they may work antagonistically.

Another process that is frequently related to longevity assurance in *C.elegans*, is the activation of the mitochondrial unfolded protein response (mtUPR)(Durieux et al., 2011). Although it is true that mtUPR is active in many longlived models, the requirement of this process for lifespan extension remains

controversial. Initially mtUPR was linked to longevity by showing that RNAi of components of the mtUPR could suppress longevity of *isp-1* or *clk-1* mutants. However many of these interventions resulted in lifespan shortening of wild type animals and other longlived models, with the exception of *ubl-5* (Benedetti et al., 2006) which appear to be specific (Durieux et al., 2011). In contrast to these observations, deletion of *atfs-1*, which is a central player in mtUPR (Nargund et al., 2012), failed to prevent lifespan extension induced by *cco-1* RNAi or *isp-1* mutation. Moreover, constitutive activation of mtUPR by *atfs-1* gain of function failed to extend lifespan in the absence of mitochondrial dysfunction (Bennett et al., 2014; Ren et al., 2015). We do not observe any impact of *klf-1* RNAi on the activation of mtUPR (not shown), and although we cannot exclude the possibility that the constitutively active mtUPR exhibited by *isp-1;ctb-1* mutants contributes to their longevity phenotype, we consider that mtUPR is not a major determinant of longevity assurance in these mit-mutants.

4.6. The role of KLF-1 in IIS-dependent longevity

Interested in the fact that xenobiotic detoxification response is also a known transcriptional signature of Insulin/IGF-1 Signaling (IIS) (McElwee et al., 2003; McElwee et al., 2004; Murphy et al., 2003), we wanted to elucidate whether KLF-1 is a transcription factor that can ensure longevity in different longevity models, including *daf-2* mutants.

We observe a partial reduction in longevity of *daf-2(e1370)* mutants after *klf-1* RNAi contrary to previous observations that attribute no role to KLF-1 in the lifespan determination of *daf-2(e1368)* mutants (Carrano et al., 2014). The *daf-2(e1370)* mutant is the most broadly allele used in IIS studies. Interestingly there are some reports that show a different impact of dietary or caloric restriction on these two *daf-2* mutants. On one hand the lifespan of the *daf-2(e1370)* mutant can be further extended by CR (Houthoofd et al., 2003; Iser and Wolkow, 2007) suggesting that they affect lifespan by different mechanisms and have additive effects. On the other hand, the lifespan of the *daf-2(e1368)* mutants can not be further extended by CR, suggesting that there may be overlapping and not additive mechanisms determining the lifespan of these IIS mutants. Remarkably, the

lifespan of *daf-2(e1368)* was not affected by *klf-1* RNAi, despite the proposed CR-specific effect of KLF-1 in lifespan determination (Carrano et al., 2014). Although we consider that this discrepancy is probably due to the use of different mutant alleles, the different experimental procedure could also contribute to the results observed (FUdR). In order to determine whether or not KLF-1 plays a role in lifespan determination in the IIS pathway, further alleles should be tested.

It is well known that the Fork head transcription factor DAF-16 is the major contributor to lifespan determination of *daf-2* mutants (Kenyon et al., 1993; Ogg et al., 1997) and as above described microarray analysis also point towards detoxification, among many other genes, as targets, whose expression changes in the presence or absence of *daf-16* (McElwee et al., 2003; Murphy et al., 2003). Therefore this raises the question whether these transcriptional signatures are similarly regulated by both DAF-16 and KLF-1 and whether they overlap in different longevity models. Interestingly some prolongevity genes described to be upregulated in *daf-2* mutants and downregulated by *daf-16* (Murphy et al., 2003) also appear in our microarray data: for example *cyp-35b1* and *cyp-34a9* are downregulated by *klf-1* RNAi in *isp-1;ctb-1* mutants and *cyp-13a7* is both upregulated in *isp-1;ctb-1* worms and downregulated by *klf-1* knockdown. Our data reveal no apparent change in the transcriptional levels of *daf-16* upon *klf-1* and *vice versa*, suggesting that the phenotypes observed are KLF-1-specific. However we lack results regarding other levels of regulation like protein abundance, posttranslational modifications or subcellular localization, which are essential for the activity of transcription factors.

Nevertheless, we demonstrate that the upregulation of *cyp*s observed in *daf-2(e1370)* mutants is highly dependent of *klf-1*. Interestingly knockdown of the two *cyp*s that impacted *isp-1;ctb-1* longevity, did not show any effect on the lifespan of the longlived *daf-2(e1370)* mutants. Although this clearly shows that this specific subset of CYPs is not determining for longevity assurance of *daf-2(e1370)* mutants, it does not exclude the possibility that other *cyps* will: therefore it would be interesting to analyze the impact on longevity of the genes mostly affected in this mutants. Alternatively different targets of KLF-1 other than DMEs could be responsible for the impact in longevity of *daf-2(e1370)* mutants. Further

experiments are necessary for understanding the role of KLF-1 in longevity assurance of *daf-2* mutants. In our hands KLF-1 appears to be a conserved regulator of DMEs expression, and increasing evidence (this work and (Carrano et al., 2014) points towards a conserved role in lifespan determination in different longevity pathways: insulin/IGF-1 signaling, dietary restriction, mitochondrial dysfunction. However the mechanism through which *klf-1* exerts its prolongevity effect as well as the magnitude of the latter might vary from mutant to mutant.

4.7. KLF-1 and SKN-1 interplay

The *skn-1* gene encodes the only *C.elegans* homolog of mammalian Nrfs (Nuclear factor-erythroid related factor/CNC family). It is important to note that, despite the nomenclature similarity, the Nrf/CNC proteins are unrelated to the nuclear respiratory factors (NRF), which regulate nuclear encoded mitochondrial genes. Nrf2 is a well characterized regulator of the antioxidant and xenobiotic response in mammals (Itoh et al., 1997; Kensler et al., 2007; Osburn and Kensler, 2008; Ramos-Gomez et al., 2001; Taguchi et al., 2011). *skn-1* was initially described as mediator of mesodermal development during embryogenesis (Bowerman et al., 1992; Maduro et al., 2001) but the description of postembryonic functions of *skn-1* did not take long to be reported: Despite the divergence in the DNA-binding-mechanism of SKN-1 compared to Nrfs (Blackwell et al., 1994; Carroll et al., 1997), SKN-1 is essential for normal lifespan and has been shown to play similar roles in oxidative stress response and detoxification as its mammalian counterpart (An and Blackwell, 2003; Blackwell et al., 2015).

To date, SKN-1 its known to function in a p38 MAPK pathway regulating the oxidative stress response and lifespan in parallel to DAF-16/FOXO in the IIS signaling pathway (Tullet et al., 2008). Likewise to PQ-induced longevity, arsenite-dependent ROS production can extend lifespan, and this lifespan extension has been shown to be SKN-1 dependent (Schmeisser et al., 2013b). Similarly, chemical inhibition of complex I results in a ROS-dependent lifespan extension that requires the activation of neuronal SKN-1 (Schmeisser et al., 2013a), suggesting that SKN-1 could be involved in lifespan determination of mitochondrial mutants. However this conception remains controversial due to the collection of inconsistent results

obtained in different studies. On one side SKN-1 has been reported to not be involved in lifespan extension induced by RNAi of ETC subunits (Rea et al., 2007; Tullet et al., 2008), while on the other it has been involved in lifespan determination of *clk-1* mutants (Park et al., 2010). Additionally, association to mitochondria of a pool of SKN-1, has been shown to be a regulatory mechanism for SKN-1 activation in response to starvation (Paek et al., 2012), suggesting that mitochondrial (dys)function and SKN-1 may be connected. Given all these implication of SKN-1 in lifespan determination and activation of detoxification genes in response to oxidative stress, our interest in deciphering whether KLF-1 and SKN-1 function together to regulate these responses was clear. *skn-1* RNAi impacts the lifespan of both *isp-1;ctb-1* and wild type worms, and therefore our results supports the idea of SKN-1 being involved in lifespan determination of mit- mutants. However, since the lifespan of wild type worms is also affected it implies that the effect of SKN-1 on lifespan is not specific or exclusive to mitochondrial dysfunction and it could be related to developmental functions of SKN-1.

SKN-1 response to oxidative stress is known to activate ROS scavenger like catalases (*ctl-1*) and superoxide dismutases and they exhibit predicted SKN-1 binding sites (An and Blackwell, 2003; Zarse et al., 2012). We see that catalases are unchanged in *isp-1;ctb-1* mutants and we observe an upregulation at day one of *sod*s that seems to be KLF-1-dependent. Additionally some phase II SKN-1 targets also exhibit changes in the expression in *isp-1;ctb-1* mutants and upon *klf-1* RNAi. It is *a priory* not surprising to think that more than one transcription factor can regulate the same subset of genes probably reacting to different activation signals or time points. However we don't know if the transcriptional changes that we observe are the result of direct effect of KLF-1 or if KLF-1 could be somehow modulating SKN-1 activity. Therefore CHIP-seq experiments will be essential to clearly dissect these two pathways.

When we analyze the expression levels of SKN-1 phase II targets like *gst-4* or the *gst-10* homolog F56A4.4 (Kahn et al., 2008; Oliveira et al., 2009; Tullet et al., 2008) we see that they are dependent on *klf-1* RNAi and more interestingly on *cyps* RNAi. This dependence on phase I genes is in agreement with the sequentiality characteristic of the detoxification process, where phase I enzymes modify toxic

products so that they can be further processed by the phase II machinery and finally secreted. Additionally KLF-1 seems to favor the nuclear translocation of SKN-1. Altogether it seems that KLF-1 does not simply result in the activation of the SKN-1-oxidative stress response, but rather that the activation of phase I genes by KLF-1 results in priming of the phase II activation regulated by SKN-1. This would be in agreement with previous observations, where upon stress conditions a complex transcriptional response is orchestrated by SKN-1 but also other unidentified factors (Oliveira et al., 2009). We propose that KLF-1 could be one of these factors, since a specific subset of genes upregulated by stress in a SKN-1-independent manner included namely CYPs and lipid metabolism (Oliveira et al., 2009).

4.8. KLF-1 upstream regulation

In order to better understand the regulation of KLF-1 activation in response to oxidative stress we performed a targeted RNAi feeding screen looking for mediators of KLF-1 translocation into the nucleus upon exposure to AA. We started by testing kinases, since phosphorylation is a common post translational modification broadly involved in cell signaling and sensitive to cellular redox state (reviewed in (Rhee et al., 2000)). In our model of the mitohormetic response orchestrated by KLF-1, a mitochondrial ROS pulse acts as trigger of the signaling cascade. As previously introduced, H_2O_2 fulfills the criteria for being an intracellular messenger since it is a small, diffusible, and ubiquitous molecule that can be synthesized and destroyed rapidly.

So far H_2O_2 has been implicated in cell signaling events in numerous occasions and its essential role as messenger is clear (Greene et al., 2000; Lo and Cruz, 1995; Sundaresan et al., 1995; Ushio-Fukai et al., 1999). However the mechanisms by which H_2O_2 exerts its messenger features remain being studied, and modulation of protein phosphorylation has already been reported as one of those mechanisms (Bae et al., 1997; Bae et al., 2000; Cunnick et al., 1998; Yoshizumi et al., 2000). Hence, our kinase screen let to the identification of the p38 homolog *pmk-3* as responsible for KLF-1 translocation, since its knowdown resulted in significant reduction of the nuclear localization exerted by AA treatment. Remarkably, this

effect appears to be specific for *pmk-3* and not the other *C.elegans* homologs of p38 *pmk-1* and *pmk-2*. Interestingly, *pmk-1* is known to regulate the activation of SKN-1 (Inoue et al., 2005) and moreover the p38 signaling pathway has also been reported to be upstream of some mammalian KLFs (He et al., 2015; Liu et al., 2010).

In silico predictions of KLF-1 phosphorylation sites identified p38 specific sites with high statistical scores, which led us to generate specific mutated reporter strains. The site directed mutagenesis of the serines 39 and 280 to both aspartic acid (activation) and alanine (inactivation) led to a suppression of the nuclear localization of KLF-1. Although that was the result that we predicted exclusively for the inactive forms, this observation has to be carefully considered since the alteration of the amino acidic sequence could result in the modification of the proteins structure and therefore an inhibition of the proteins functionality in all four cases. To overcome this limitation, in vitro kinase assay will be performed in order to determine whether *pmk-3* specifically phosphorylates KLF-1 in the predicted sites. Additionally, we don't know if phosphorylation of both sites simultaneously is necessary for nuclear translocation since we just studied single mutations and therefore this possibility will be studied as well. Alternatively, other posttranslational modifications of KLF-1 will be analyzed via mass spectrometry after pull down of KLF-1, since KLF-1 has already been shown to be subjected to ubiquitination via WWP-1 (Carrano et al., 2014).

wwp-1 encodes a E3 ubiquitin ligase orthologus to human WWPs (Huang et al., 2000a), and it has been shown to be a positive regulator of longevity upon dietary restriction acting upstream of KLF-1 by mediating its poly-monoubiquitination (Carrano et al., 2014; Carrano et al., 2009). The effect of this modification on KLF-1 remains unclear but it has been suggested to lead to a modification of its subcellular localization (nuclear translocation) and therefore activation of KLF-1 activity (Carrano et al., 2014). We have collected preliminary results of the function of WWP-1 in regulating subcellular localization of KLF-1 and we observe a massive upregulation of KLF-1 nuclear localization upon *wwp-1* RNAi suggesting that WWP-1 is rather a negative regulator of KLF-1 (not shown). Further analyses need to be done before we can conclude about the role of WWP-1 in the

modulation of KLF-1 localization and activity. Conflicting results regarding the impact of *wwp-1* on longevity have been collected: while some reports defend that *wwp-1* exclusively mediates longevity upon dietary restriction (Carrano et al., 2009), others place *wwp-1* in lifespan determination of IIS mutants in parallel to *daf-16* (Chen et al., 2010; Samuelson et al., 2007). Again this discrepancy could be due to the use of different *daf-2* alleles (*daf-2(e1368) vs. daf-2(e1370)*). However, what results more puzzling, is the discrepancy in the role of *wwp-1* on lifespan determination of wild type worms: Carrano and colleagues exclusively observed an effect on lifespan of wild type worms exerted by *wwp-1* RNAi or mutation at 25 °C and not at 20 °C in agreement with the role of *wwp-1* in responding to stress (Carrano et al., 2009). Other studies however, show a clear impact of *wwp-1* mutation on wild type lifespan in control conditions (temperature is not specify) and upon exposure to pore forming toxins or pathogens (Chen et al., 2010).

Interestingly, ubiquitination appears to be a conserved mechanism regulating stress related transcription factors like VHL-1/HIF-1 in *C.elegans* (Mehta et al., 2009) and pVHL/HIF1 α in mammals (Kallio et al., 1999; Ohh et al., 2000), or SKN-1 that can also be regulated by ubiquitination by the adaptor protein WDR-23 (Choe et al., 2009) like Nrf2/KEAP1 (Itoh et al., 1999). Therefore further understanding of the role of WWP-1 in KLF-1 (Carrano et al., 2014) regulation results highly interesting.

Further analysis of the impact of PMK-3 in KLF-1 activation was performed by looking at KLF-1 targets. We observe a clear and specific effect of *pmk-3* RNAi, and not *pmk-2* or *pmk-1* (Berman et al., 2001), on the expression of KLF-1 targets. Moreover the effect exerted by *pmk-3* RNAi mimics the one exerted by *klf-1* RNAi in *isp-1;ctb-1* mutants, strongly suggesting that both PMK-3 and KLF-1 work together to assure longevity in these mitochondrial mutants. Cell signaling events trough MAPK cascades function through a multi-tired mechanism characterized by the subsequent phosphorylation of the regulatory enzymes involved in it (MAPK3, MAPK2, MAPK), which amplify and confer specificity to the signaling events (Plotnikov et al., 2011; Seger and Krebs, 1995). After the identification of the MAPK3 *nsy-1* and the MAPK *pmk-3* in our first kinase screen, we performed a targeted analysis based on previous reports to identify the missing MAPK2

(Hoeven et al., 2011; Kim et al., 2002; Nakata et al., 2005; Tanaka-Hino et al., 2002). This lead to the identification of a conserved MAPK pathway, which has been previously described in both worms (with *pmk-1*) and mammals and is involved in many cellular processes like immune response or apoptosis and even detoxification response upstream of SKN-1 (Crook-McMahon et al., 2014; Kim et al., 2002; Tobiume et al., 2001; Wang et al., 2009).

The capacity from H_2O_2 to directly modify cysteine residues on phosphatases and kinases can seem *a priory* quite unspecific. However, there is great evidence of H_2O_2 specifically regulating protein tyrosine phosphatases (PTPs), which relies on their structure singularity that allows the formation of reversible disulfide bonds (Denu and Tanner, 1998; Michalek et al., 2007; Seth and Rudolph, 2006; Sohn and Rudolph, 2003). But not all kinases and phosphatases have this specific structure and therefore could only by activated by H_2O_2 as result of the oxidation of cysteine residues of upstream regulators. We postulate that a redox enzyme will be responsible for triggering the oxidative-stress-induced response, since redox enzymes act as electrochemical biosensors that are dedicated to sense and transduce an oxidative signal into a signaling cascade (Thevenot et al., 2001). The ability of these enzymes to act as redox switches relies, like for PTPs, on the presence of specific sequences (Cys-X-X-Cys) with cysteine residues in their active or regulatory sites. The thiol side chain on these residues participates in redox reactions serving as nucleophile, its susceptible to oxidation and give rise to disulfide derivates that define protein structure and function (Cross and Templeton, 2006; Klomsiri et al., 2011; Ortiz de Orue Lucana et al., 2012). To this group belong, among others, enzymes like peroxiredoxins, thioredoxins, thioredoxin reductases, glutathione peroxidases, glutaredoxins or glutathione disulfide reductases. All these types of enzymes are also present in *C.elegans* (Johnston and Ebert, 2012) and work in close interconnection to ensure global cellular redox homeostasis in response to local redox changes (Klomsiri et al., 2011).

To test our hypothesis, we performed a targeted feeding RNAi screen with available clones encoding *C.elegans* redox enzymes. Due to the high variability of our results regarding nuclear localization upon knock down of redox enzymes, we

cannot clearly determine which gene is involved in this process. For these reason further analyses need to be done: for example by using specific mutants of the genes of interest instead of RNAi. The further upstream we go in the signaling pathway for KLF-1 activation, the more variability we confront, since minimal expression of the target gene can lead to activation of the whole signaling cascade and therefore misinterpretation of the results. However, being aware of the limitation of our approach, the fact that *trx-4* RNAi combined with antimycin A led to an increased nuclear localization of KLF-1, leads us to the proposal of a model where an enzyme like TRX-4 (to be confirmed) sequesters NSY-1 under control conditions and upon the appearance of an oxidative signal this will lead to the conformational change of TRX-4 and the consequently releasing of NSY-1 activating the MAPK cascade. This is in agreement with the mechanism previously reported for TRX1/ASK-1 in apoptosis regulation (Jin et al., 2015; Liu et al., 2000). It would be interesting for example to test whether specific *trx* mutations combined with *isp-1;ctb-1* lead to normalization of lifespan or if these mutations can abolish the PQ induce longevity observed in wild type worms. Again this model of regulation highly resembles the mechanisms controlling SKN-1 activation with *prdx-2* (De Haes et al., 2014; Olahova and Veal, 2015) as initial signal transducer followed by activation of a MAPK cascade and p38/*pmk-1* dependent phosphorylation (Inoue et al., 2005).

4.9. Summary

Taken together, in this work we identify KLF-1 as an essential regulator of the longevity of the mit-mutant *isp-1;ctb-1* by activating phase I DMEs and altering lipid metabolism. We propose that the enhanced cytoprotective capacity combined with the metabolic change leads to lifespan extension. KLF-1 appears to be a conserved regulator of this response in different longevity models; however, the impact of the transcriptional activation on lifespan determination may differ in every case. We place KLF-1 in a central position of the increasingly complex map of lifespan determination of *C.elegans* and postulate that it can be activated by different prolongevity cues, which in case of *isp-1;ctb-1* is a developmental mitochondrial ROS signal (Figure 4.1.).

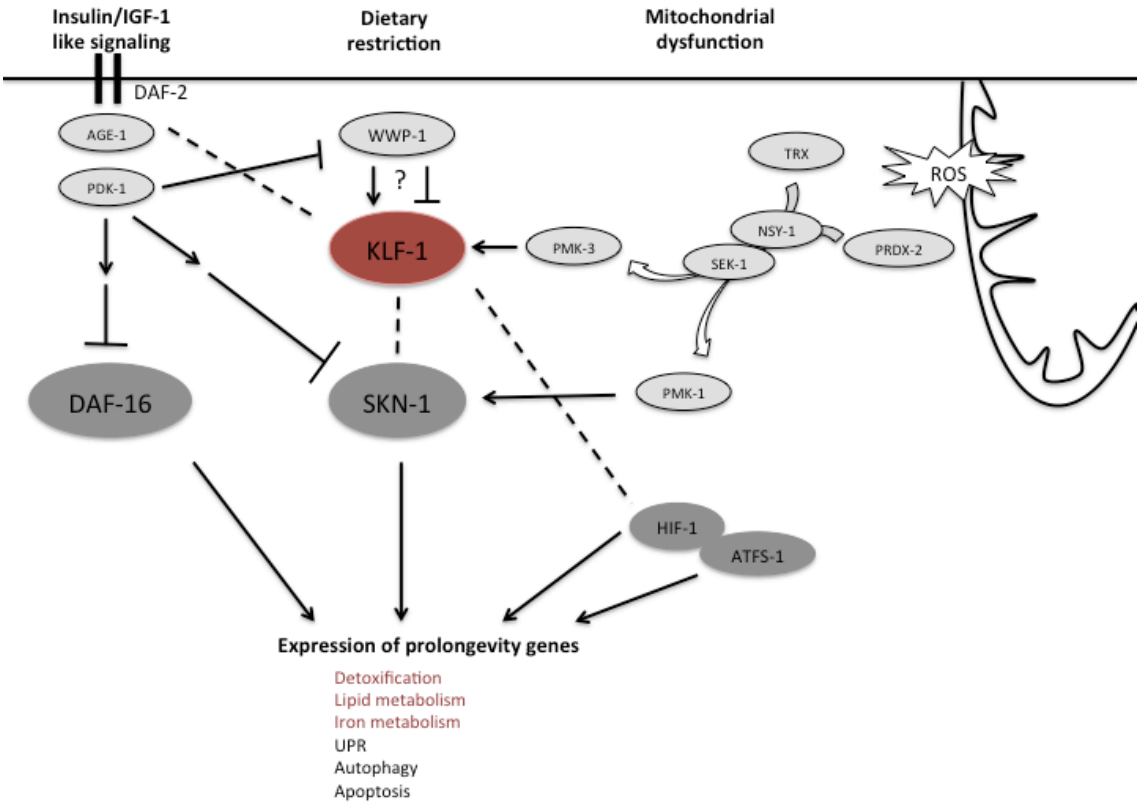

Figure 4.1. The longevity map of *C.elegans*
The figure represents a simplified version of the complex interconnected network of pathways identified to be essential for lifespan determination of different longevity models in *C.elegans,* based on our results and the results of others above discussed. In light gray upstream regulators of transcription factor activation, which are represented in dark gray with the exception of KLF-1 in red. The dashed lines indicate an indirect or still unclear interaction between pathways. Altogether these prolongevity pathways result in the modulation of metabolic and cytoprotective genes: In red we highlight the processes that we show to be regulated by KLF-1.

The search for one gene or pathway responsible for "universal" lifespan determination is an obsolete approach that the aging research community is slowly overcoming. We cannot forget that the final purpose of this research is not to find a "magic key" for lifespan extension but to understand the molecular mechanisms responsible for lifespan determination to ensure a healthier aging. Therefore just a comprehensive analysis of all these discoveries made during the past decades will lead to a correct understanding and application of the results. This work is our modest contribution to the expansion of the complex longevity map and we hope that our work will also help others deciphering further mechanisms involved in it. Future effort should also focus on establishing whether

our findings extend to mammals and lead to the development of medical interventions and therapeutic strategies to combat age-associated diseases.

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Appendix

A. Lifespan data and statistical analysis

genotype	condition	mean±SE	median survival	p value (Log-rank test)	Number of animals died/total
N2	L4440	27.47±0.6084	29		100/116
N2	*klf-1*	28.46±0.7885	29	ns 0.1725[a]	79/91
isp-1;ctb-1	L4440	38.6±1.460	38	<0.0001[a]	62/109
isp-1;ctb-1	*klf-1*	27.41±1.056	26	ns 0.9459[a] <0.0001[b]	66/104
isp-1	L4440	38.60±1.250	36	<0.0001[a]	62/110
isp-1	*klf-1*	22.71±0.7182	22	<0.0001[a] <0.0001[a]	88/109
N2	L4440	21.98±0.6800	22		85/100
N2	*cco-1*_L4440	34.53±1.478	33	<0.0001[a]	40/102
N2	*cco-1_klf-1*	26.4±1.067	28	<0.0001[a] <0.0001[c]	50/104
N2	*cyc-1*_L4440	46.03±1.372	50	<0.0001[a]	73/88
N2	*cyc-1_klf-1*	29.52±0.8660	30	<0.0001[a] <0.0001[d]	85/100
N2	*atp-5*_L4440	33.66±0.8498	33	<0.0001[a]	83/99
N2	*atp-5_klf-1*	27.91±1.010	26	<0.0001[a] <0.0001[e]	66/100
N2	L4440	19.29±0.5817	19		115/121
N2	*klf-1*_L4440 L4	22.72±0.6457	23	0.0003[a]	102/115
N2	L4440_*klf-1 L4*	20.22±0.8090	21	ns 0.1751[a]	83/99
isp-1;ctb-1	L4440	26.47±1.623	28	< 0.0001[a]	66/83
isp-1;ctb-1	*klf-1*_L4440 L4	25.07±1.387	23	< 0.0001[a] ns 0.6424[b]	90/140
isp-1;ctb-1	L4440_*klf-1* L4	17.76±0.8692	16	ns 0.6859[a] < 0.0001[b]	96/127
N2	L4440	14.73±0.5125	14		107/120
N2	PQ L4440	19.42±0.5917	21	< 0.0001[a]	96/120
klf-1	L4440	13.81±0.4482	12	ns 0.1243[a]	104/120
klf-1	PQ L4440	14.26±0.6381	14	ns 0.8815[a] ns 0.1162[f]	74/120

N2	L4440	23,37±0,6249	24		76/103
N2	*skn-1*	19,42±0,3723	19	<0.0001[a]	85/95
isp-1;ctb-1	L4440	32,20±1,781	29	<0.0001[a]	64/101
isp-1;ctb-1	*skn-1*	24,31±0,7310	24	<0.0001[a] ns 0,3143[b]	89/99
N2	L4440	23.37±0.6249	24		76/103
N2	*cyps*	19.18±0.4726	17	<0.0001[a]	78/100
isp-1;ctb-1	L4440	32.20±1.781	29	<0.0001[a]	64/101
isp-1;ctb-1	*cyps*	25.20±0.8207	25	ns 0.0501[a] <0.0001[b]	80/101
N2	L4440	21.98± 0.6800	22		85/100
daf-2	L4440	48.5±1.727	52	< 0.0001[a]	54/100
daf-2	*klf-1*	31.05±3.141	27	0.0007[a] <0.0001[g]	20/100
N2	L4440	22,76±0,5156	22		89/100
N2	*cyps*	22,26±0,5736	22	ns 0,7233[a]	90/101
daf-2	L4440	42,48±1,413	46	< 0,0001[a]	82/94
daf-2	*cyps*	38,58±1,794	46	< 0.0001[a] ns 0,4467[g]	79/95
N2	L4440	19,86±0,6145	19		73/100
N2	*ugts*	22,34±0,5772	22	0,0064[a]	71/100
isp-1;ctb-1	L4440	24,37±1,362	26	< 0,0001[a]	57/100
isp-1;ctb-1	*ugt*	20,81±0,9154	24	0,0239[a] 0,0005[b]	72/100

[a] Log-Rank test compared to N2 L4440
[b] Log-Rank test compared to *isp-1;ctb-1* L4440
[c] Log-Rank test comapred to N2 *cco-1*_L4440
[d] Log-Rank test compared to N2 *cyc-1*_L4440
[e] Log-Rank test compared to N2 *atp-5*_L4440
[f] Log-Rank test compared to *klf-1* L4440
[g] Log-Rank test comapred to *daf-2* L4440

B. GO- and KEGG-pathway analysis of the microarray data

GO Pathway

GO-ID	Pathway	P-value
55114	oxidation reduction	6,60E-04
30258	lipid modification	1,80E-02
6635	fatty acid bet- oxidation	8,80E-02
9062	fatty acid catabolic processes	8,80E-02
19395	fatty acid oxidation	9,70E-02
34440	lipid oxidation	9,70E-02

KEGG Pathway

Pathway	P-value
Metabolism of xenobiotics by cytochrome P450	1,70E-03
Drug metabolism	2,40E-03
Fatty acid metabolism	1,50E-02
Glycolysis / Gluconeogenesis	4,60E-02

C. Microarray data

The microarray results are available at the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo/) under the Geo Accession number **GSE61771**

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Aleksandra Trifunovic betreut worden.

Köln, den 09.11.2015

Curriculum Vitae

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Professional and educational training

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05/2012-07/2012	**Research Project in Aleksandra Trifunovic's Lab** at the Institute for Genetics oft he University of Cologne: „Who is R144.3? A first approximation to R144.3 gene's function in the aging context“
10/2011-07/2012	**Exange** year at the University of Cologne with european ERASMUS scholarship.
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Conferences

27-30/09/2014	Mito Retreat, Stockholm Sweden. Short talk „KLF-1 regulates mitohormesis through activation of the xenobiotic response“
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Languages

Spanish: native **German:** fluent **English**: fluent