The Genetics and Biochemistry of Longevity in Axenically Cultured *Caenorhabditis elegans*

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Natascha

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Summary

Aging is a complex phenomenon which encompasses the progressive structural and functional decline over time, starting at reproductive age, and inevitably results in death. Since human aging imposes a considerable social and economical impact, much effort has been invested to elucidate the mechanism underlying the aging process.

One of the major theories proposed is the Free Radical Theory of Aging which suggests that extensive damage to cellular components by reactive oxygen species (ROS) underlies the aging process. As primary ROS production sites, the mitochondria are assigned a crucial role in this process.

The nematode model system *Caenorhabditis elegans* provides an ideal tool for aging research. Its short generation time and lifespan allow fast and repeated screening for lifespan-affecting mutations and conditions. Furthermore, the *C. elegans* genome is fully sequenced and annotated and multiple long-lived mutants are available.

Dietary restriction (DR), or the restriction of nutrients without malnutrition, is the only environmental intervention shown to induce consistent lifespan extension in a wide variety of species, ranging from yeast, worms and flies to rodents. Since dietary restriction retards the aging process, it has been the subject of intensive research. In *C. elegans*, multiple DR methods can be applied. One way is to culture the worms in the absence of bacteria, in axenic medium (axenic dietary restriction or ADR), which has been shown to induce a DR-like phenotype with worms displaying reduced fecundity and body volume, slowed development and prolonged lifespan.

For a long time, the anti-aging effect of dietary restriction was ascribed to a reduction in metabolic rate and consequent decrease in ROS production. However, it was established that no reduction but even an increase in metabolic rate occurred and this effect was shown to be indispensable for the longevity effect of DR. Conversely, micro-array analysis of differentially expressed genes in axenically cultured *C. elegans* compared to fully fed (FF) controls suggested a downregulation of many metabolic genes. We attempted to resolve these conflicting data by assessing transcript abundance of genes involved in energy metabolism using quantitative RT-PCR and we found no evidence for a decrease in the transcriptional output of genes involved in aerobic metabolism. Our data suggest that ADR increases flux through replenishing pathways including glyceroneogenesis and possibly gluconeogenesis. Flux through glycolysis and the tricarboxylic acid cycle either displays no changes or is upregulated. These data confirm the earlier findings of our group that ADR does not decrease metabolic rate.

Considering the important role that mitochondria play in energy metabolism and the link between DR and metabolism, we tried to elucidate the effect of ADR on mitochondrial function. We showed that ADR increases mitochondrial efficiency: mitochondria are better coupled and leakiness is lower in young adult worms cultured in axenic medium. Furthermore, we found a dramatic decrease in energetic efficiency with age in FF controls, which was attenuated by culture in axenic medium. ATP production capacity of isolated mitochondria was equally high for both feeding conditions, suggesting that ADR worms are tightly coupled to produce sufficient amounts of ATP from fewer nutrients. We found a trend towards an increase in supercomplex abundance, which could explain the increased bioenergetic efficiency.

Next, we determined membrane potential and found that there is a trend towards a lower membrane potential in mitochondria form axenically cultured worms. A lowered membrane potential has been linked to reduced ROS production. Hence, we assessed mitochondrial ROS production capacity but could not confirm that ADR would establish its effects by reducing ROS production. Using an *in vivo* biosensor, no differences in hydrogen peroxide levels between young adult ADR and FF worms were found. Together, these data question the importance of early ROS-induced damage for lifespan.

Until this date, the underlying molecular mechanism of ADR-mediated longevity is unclear. In an attempt to identify more genes involved in this mechanism, we focused at the usual suspects for DR-mediated longevity. So far, except for *cbp-1* and *cup-4*, not any of the genes had an extensive impact on ADR-mediated lifespan and probably many of them are part of the downstream processes regulating the ADR-mediated effect. This suggests that the pathways DR invokes depend on specific nutritional cues and that several pathways act together as a DR network.

In conclusion, ADR induces a subtle metabolic restructuring together with a higher bioenergetic efficiency. The role of ROS in ADR-mediated longevity remains a question as are the main molecular effectors of the lifespan extension caused by culture in axenic medium. Whether these metabolic changes are required for the ADR-mediated effect and whether they are the result of signaling induced by CBP-1 are targets for further research.

Samenvatting

Veroudering is een complex fenomeen dat aan de basis ligt van de geleidelijke structurele en functionele achteruitgang met de tijd. Deze achteruitgang start op de reproductieve leeftijd en leidt onvermijdelijk tot de dood. Omwille van de aanzienlijke sociale en economische impact die menselijke veroudering heeft, werd er reeds veel moeite gedaan om het mechanisme dat aan het verouderingsproces ten grondslag ligt op te helderen.

Een van de belangrijkste theorieën die voorgesteld werd, is de Vrije Radicaal Theorie. Deze theorie suggereert dat uitgebreide schade aan cellulaire componenten door reactieve zuurstof deeltjes ('reactive oxygen species' of ROS) de basis vormt voor veroudering. Als voornaamste plaats waar ROS-productie plaats vindt, worden de mitochondriën een cruciale rol in dit proces toegekend.

Het nematode modelsysteem *Caenorhabditis elegans* is een uitstekend instrument voor verouderingsonderzoek. Zijn korte generatietijd en levensduur laten toe om snel en herhaaldelijk te screenen naar mutaties en condities die een effect hebben op de levensduur. Verder is het genoom van *C. elegans* volledig gesequeneerd en goed geannoteerd en zijn er verschillende langlevende mutanten beschikbaar.

Diëtaire restrictie (DR), of het verminderen van nutriënten zonder ondervoeding te veroorzaken, is de enige interventie in het milieu van een organisme die de levensduur consistent verlengt in een grote waaier aan organismen, van gisten, wormen en vliegen tot knaagdieren. Omdat DR het verouderingsproces vertraagt vormt het een onderwerp waar uitvoerig onderzoek naar gevoerd wordt. In *C. elegans* zijn er verschillende methoden om DR toe te passen. Eén methode bestaat erin om de wormen op te kweken in de afwezigheid van bacteriën, in axenisch medium, ook wel axenische diëtaire restrictie (ADR) genoemd. Er werd aangetoond dat ADR een DR-achtig fenotype induceert met wormen die een afname in vruchtbaarheid en lichaamsvolume, een vertraagde ontwikkeling en verlengde levensduur vertonen.

Lange tijd werd het anti-verouderingseffect van DR toegeschreven aan een afname van de metabole snelheid en vervolgens ook een verminderde ROS-productie. Er werd echter vastgesteld dat er geen afname, maar in sommige gevallen zelfs een toename, van metabole snelheid plaats vindt. Deze toename blijkt noodzakelijk voor het levensduurverlengend effect van DR. Daarentegen suggereert micro-arrayanalyse van gentranscripten dat heel wat metabole genen neergereguleerd zijn onder ADR versus ad libitum-condities. In een poging om deze tegengestelde resultaten op te helderen, hebben we de transcriptabundantie van genen die

betrokken zijn bij het energiemetabolisme bepaald aan de hand van kwantitatieve RT-PCR. We vonden geen bewijzen voor een afname in de transcriptionele abundantie van genen betrokken bij het aeroob metabolisme. Onze data suggereren een verhoogde flux doorheen aanvullende pathways, zoals de glyceroneogenese en mogelijks ook de gluconeogenese. Flux doorheen de glycolyse en de citroenzuurcyclus vertoont ofwel geen veranderingen of is opgereguleerd. Deze data bevestigen eerdere bevindingen dat ADR de metabole snelheid niet doet afnemen.

Rekening houdend met de belangrijke rol die mitochondriën spelen in het energiemetabolisme en de link tussen DR en metabolisme, probeerden we om het effect van ADR op de mitochondriale functionaliteit op te helderen. We toonden aan dat ADR leidt tot een toename in mitochondriale efficiëntie: mitochondriën zijn beter gekoppeld en vertonen minder 'lek' in jong-adulte wormen die gekweekt werden in axenisch medium. Verder vonden we ook een duidelijke afname in de bioenergetische efficiëntie met de leeftijd in ad libitum gevoede wormen welke sterk afgezwakt werd door cultuur in axenisch medium. De ATP-productiecapaciteit van de mitochondriën was even hoog voor beide voedingscondities en dit suggereert dat ADR-wormen goed gekoppeld zijn om ze in staat te stellen even veel ATP te produceren uitgaand van minder nutriënten. We vonden een trend die wijst op een toename van supercomplexabundantie die mogelijks de toegenomen bioenergetische efficiëntie zou kunnen verklaren.

Daarnaast hebben we ook de membraanpotentiaal bepaald. We vonden een trend tot verlaging van de membraanpotentiaal in mitochondriën van axenisch gekweekte wormen. Een verlaagde membraanpotentiaal wordt in verband gebracht met een verminderde ROS productie. Daarom werd ook de mitochondriale ROS-productiecapaciteit gemeten maar we konden niet bevestigen dat de effecten van ADR het gevolg zouden zijn van verlaagde ROS-productie. Gebruik makend van een *in vivo* biosensor konden we ook geen verschillen vinden in de waterstofperoxidegehaltes van jonge ADR en ad libitum-gevoede wormen. Samen stellen deze data het belang van vroege ROS-productie voor de levensduur in vraag.

Tot op heden is het onderliggende moleculair mechanisme van ADR-gemedieerde levensduurverlenging onbekend. In een poging om meer genen te identificeren die bij dit mechanisme betrokken zijn, richtten we ons op de gebruikelijke kandidaten verantwoordelijk voor DR-geïnduceerde levensduurverlenging. Behalve *cbp-1* en *cup-4*, had geen enkel van de genen een gelijkaardige impact op de verlengde levensduur. Waarschijnlijk maken deze genen allemaal deel uit van de downstream processen die het ADR effect reguleren. Dit suggereert dat de pathways die DR induceert afhangen van specifieke nutritionele signalen en dat verschillende van deze pathways samenwerken als een DR netwerk.

Concluderend kunnen we zeggen dat ADR een subtiele metabole herstructurering induceert, gecombineerd met een hogere bioenergetische efficiëntie. De rol van ROS voor ADR-gemedieerde levensduurverlenging blijft onbekend, net als de moleculaire spelers die de levensduur door axenische cultuur bepalen. Of deze metabole veranderingen noodzakelijk zijn voor het ADR-gemedieerde effect en of ze het resultaat zijn van signalisatie via CBP-1 vormen interessante vraagstukken voor toekomstig onderzoek.

PART I

INTRODUCTION

Chapter 1. General introduction and outline of the thesis

1.1. Definition and theories of aging

1.1.1. What is aging?

Aging is a complex and fascinating phenomenon. For ages, we have been trying to unravel its mechanism, since sooner or later, we all experience its effects. To be able to study aging, first we have to define what it is exactly. Arking (1998) defined aging as the series of cumulative, progressive, intrinsic and deleterious functional and structural changes that usually begin to manifest themselves at reproductive maturity and eventually culminate in death.

In an attempt to explain aging, many theories were developed. These theories can be divided in evolutionary theories, trying to explain why we age, and mechanistic theories of aging, attempting to answer the question how we age.

1.1.2. Evolutionary theories of aging

One of the first theories attempting to explain aging, posits that aging benefits the species by preventing overcrowding and so ensuring enough resources for the next generation (Weismann et al., 1889). Such a theory implies that there are processes, actively leading to aging and regulated by a genetic program, which is very unlikely. First, for most natural populations, extrinsic mortality (due to predation, starvation, disease, etc.) is the main reason for decrease of the population, rather than death because of 'old age', indicating there is no requirement for aging to remove worn-out individuals. Secondly, the idea is circular and supposes that old individuals are worn out and less reproductive, while its purpose is to explain why such a deterioration occurs (Kirkwood, 2005).

If aging is not genetically programmed, then how has it evolved? In general, evolutionary theories start from the shared idea that aging occurs because the force of natural selection declines with age. A mutation that increases mortality or decreases fertility early in life will be selected more strongly against than a mutation exerting a similar effect later in life. The Mutation Accumulation Theory proposed that mutations with deleterious effects later in life will not be selected against and can increase to substantial frequencies over many generations and contribute to aging (Medawar, 1952). In addition, pleiotropic genes with beneficial effects early in life but detrimental effects at older age may exist, since natural selection will act more strongly on the beneficial effect early in life. This Antagonistic Pleiotropy theory was postulated by Williams (1957).

From a more physiological point of view, the Disposable Soma Theory is based on the premises that somatic maintenance and repair are metabolically costly and metabolic resources devoted to reproduction are not available for maintenance and repair. Each organism should optimally allocate its energy between somatic maintenance and reproduction. As natural selection will prefer the investment in reproduction, somatic damage will progressively accumulate and eventually result in aging (Kirkwood, 1977).

1.1.3. Mechanistic theories of aging

While evolutionary theories try to explain why aging occurs, mechanistic theories focus on molecular mechanisms that cause age-related cellular senescence. Many mechanistic theories have been proposed, focusing on various forms of damage accumulating and causing aging. The Free Radical Theory of Aging (FRTA) is the most popular one and has received most attention.

1.1.3.1. Free Radical Theory of Aging

In the early 20th century, the most dominant theory of aging stated that metabolic rate (oxygen consumption) is inversely correlated to lifespan. This theory was termed the Rate-of-Living theory (Live fast, die young) and assumes that all organisms have the same amount of energy at their disposal during their lifetime (Pearl, 1928). Denham Harman provided a physiological mechanism explaining it by postulating the Free Radical Theory of Aging in 1956 (Harman, 1956). This theory is based on the fact that oxygen has toxic properties, since it is a free radical that upon single electron additions generates reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and the hydroxyl radical. The main cause of aging is here depicted as the accumulation of extensive damage to proteins, lipids and DNA caused by these ROS which are considered normal by-products of oxidative metabolism. The discovery of superoxide dismutase (SOD), an enzyme detoxifying the ROS superoxide (McCord and Fridovich, 1969), and the detection of the ROS hydrogen peroxide (H_2O_2) (Chance et al., 1979) further strengthened the theory. In a refinement of the theory, the mitochondria were highlighted as the main ROS source and assigned a crucial role in the aging process (Harman, 1972). Based on the fact that there are ROS that are not free radicals and that oxidative damage is caused by an imbalance of ROS production and detoxification and the the cell's abilities to repair oxidative damage to DNA and proteins , the theory is now often referred to as the oxidative stress/damage theory of aging (Sohal and Weindruch, 1996). This imbalance leads to a progressive accumulation of oxidative damage with age, ultimately leading to senescence. Although the FRTA provides a possible mechanism to explain it, the Rate-of-Living theory was rejected when the metabolic rate of longlived animals was found not to be decreased. In *C. elegans*, it was shown that a mutation in a mitochondrial protein or dietary restriction, conditions that both extend lifespan, do not reduce or even increase metabolic rate, indicating that the Rate-of-Living theory can not explain why aging

occurs (Braeckman et al., 2002a; Houthoofd et al., 2002c; Speakman, 2005). The past decades, immense effort was dedicated to verify the FRTA, but until now experimental studies either do not support or remain inconclusive on whether oxidative damage really is the main cause of the aging process (Gems and Doonan, 2009; Lapointe and Hekimi, 2010). Nevertheless, some gerontologists still adhere to the theory and refine it (Barja, 2013). Furthermore, it is suggested that oxidative stress is important for many pathologies, including age-associated diseases, but are not the main cause of aging .

An important weakness of these studies, so far, is the lack of direct *in vivo* measurements of the unstable superoxide or hydrogen peroxide due to the lack of reliable methods to do so. Recently, these limitations were overcome by the development of genetically encoded ROS sensors based on the green fluorescent protein GFP (Meyer and Dick, 2010), such as HyPer and Orp1-roGFP2. Genetically encoded sensors have advantages compared with chemical fluorescent dyes. They can be targeted to specific tissues, cells or subcellular locations. Furthermore, these probes are selective, sensitive, instantaneously, reversible, *in vivo* and non-invasive. Therefore, they provide a promising tool that undoubtedly will help future research to explore the involvement of ROS in the aging process (Back et al., 2012a; Back et al., 2012b). However, genetically encoded probes have some limitations that have to be considered as well. Some probes, such as HyPer, are pH sensitive, indicating that subtle pH differences may bias the results. Furthermore, they have anti-oxidant properties and are overexpressed. It is not known yet how this may affect the organism. (Lukyanov and Belousov, 2013).

1.1.3.1.1. ROS production in the mitochondria

ROS production sites

Multiple mitochondrial enzymes, not part of the respiratory chain, have been shown to produce ROS at significant rates under experimental conditions using isolated enzymes or mitochondria. However, their contribution to ROS generation under physiological conditions is unknown (Andreyev et al., 2005). The major site of ROS production lies with the mitochondrial respiratory chain, containing several redox centers capable of one-electron reduction of oxygen thereby forming superoxide (Fig. 1). The electron transport chain complexes will be discussed in more detail in 1.2.2.1. The majority of the electrons delivered at the electron transport chain (ETC) are used by Complex IV in the reduction of oxygen (O_2) to water (H_2O). However, some electrons may escape at earlier stages and it was established that 0.1-0.3% of all O_2 consumed is converted to superoxide (St-Pierre et al., 2002). The two capital sites of ROS production have been identified as Complex I, producing matrix-sided ROS, and Complex III, releasing ROS to the matrix side as well as to the intermembrane space (Li et al., 2013). It was suggested that

Complex I has two ROS producing sites. During reverse electron transport (RET) from succinate to NAD+, superoxide is produced at high rates and this ROS production is markedly diminished by rotenone (Complex IQ site inhibitor) addition. It appears that this superoxide comes from the ubiquinone binding site at Complex I, site IQ (Lambert and Brand, 2004a). Of note, the concentrations of succinate necessary to support RET are much higher than normal physiological concentrations. During forward electron transport from NAD-linked substrates, ROS production is fairly low. However, most mitochondria produce high superoxide after inhibition with rotenone or antimycin (a Complex III inhibitor). In isolated mitochondria, this production is dominated by fully reduced flavin (site IF) (Lambert and Brand, 2004a; Liu et al., 2002). Recently, the two-site model of Complex I superoxide production was supported by the findings of Treberg et al. (2011), showing a superoxide producer during RET that responds to the Q pool redox state and is not in equilibrium with the NAD reduction state. For Complex III, the main reductant has been pinpointed to the unstable ubisemiquinone molecule and high rates of ROS production only emerge in the presence of antimycin (Brand, 2010; Echtay, 2007).

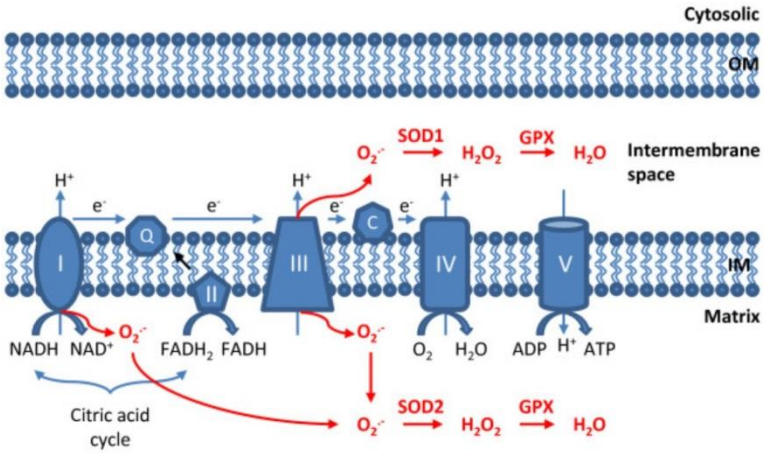

Figure 1: Schematic overview of the electron transport chain with its major ROS production sites and ROS detoxification enzymes (Li et al., 2013). I-II-III-IV represent the mitochondrial electron transport complexes , V = ATP-synthase, Q = ubiquinone, c = cytochrome c, SOD = Superoxide dismutase, GPX = Glutathion peroxidase.

Compared to maximum ROS production capacity at Complexes I and III, rates at Complex II are negligible (Chen et al., 2003; Quinlan et al., 2011; St-Pierre et al., 2002), but mutations in the enzyme can cause high ROS production rates and pathology. Recently, Quinlan et al. (2012) showed that Complex II (the flavin moiety of Complex II (site II_F) and not its ubiquinone binding site(site II_Q)) is capable of producing considerable amounts of ROS in both forward and reverse reactions under certain circumstances, when ubiquinone reoxidation through Complex I and III is prevented and succinate concentration is low. The authors even suggest a relevant role for CII in *in vivo* ROS production, when multiple substrates feed electrons but energy demand is low. The relative contribution of each site to total ROS production is unknown. Most assays to define

site specific ROS production measure maximal capacity and ROS production in the absence of inhibitors is not known. An important factor that has been shown to influence ROS production is the mitochondrial membrane potential. The high ROS production by Complex I during RET is particularly sensitive to membrane potential (Miwa and Brand, 2003) and even more sensitive to the pH gradient (Andreyev et al., 2005; Lambert and Brand, 2004b). Furthermore, metabolic state as well has an influence on ROS production rate. A detailed explanation of the metabolic states can be found in section 1.2.4.3. Under resting state 4 conditions, in the absence of ADP and low oxygen consumption, membrane potential is high and so is ROS production. Shifting this metabolic state from state 4 to state 3, in which oxygen consumption is accelerated, ATP synthesis is maximized and membrane potential is lowered, causing ROS production to decrease (Adam-Vizi and Chinopoulos, 2006).

Antioxidant defense enzymes

Actual ROS levels are determined by ROS generation as well as ROS detoxification by antioxidant defense enzymes. The primary ROS, superoxide, undergoes highly efficient dismutation to hydrogen peroxide by superoxide dismutase (SOD). Several SOD isoforms exist and they are classified according to which metal cofactor they contain. The most common SODs contain copper and zinc (CuZnSOD) and are located in the cytosol while some are found in the mitochondrial intermembrane space as well as in the extracellular space. Manganese SOD (MnSOD) is primarily located to the mitochondria of eukaryotes.

Hydrogen peroxide can be further detoxified to water by two classes of enzymes: catalases and peroxidases. Catalase directly decomposes two H_2O_2 molecules to $2H_2O$ and O_2, while peroxidase uses H_2O_2 for the oxidation of another substrate. Catalase activity is largely located to peroxisomes. In the mitochondria, H_2O_2 detoxification mainly happens by glutathione peroxidase. This enzyme couples the reduction of H_2O_2 to the oxidation of reduced glutathione. The conversion of oxidized glutathione back to its reduced form is catalyzed by glutathione reductase and uses NADPH as a substrate. Another important group of peroxidases are peroxiredoxins. Redox reactions catalyzed by these enzymes are dependent on cysteine at the active sites (Halliwell and Gutteridge, 2007).

Oxidative damage and repair

The balance between ROS production and detoxification is imperfect and results in the presence of oxidative damage to biomolecules (DNA, proteins and lipids). Oxidative DNA damage is mainly induced by the highly reactive hydroxyl radical attacking bases and (deoxy)ribose sugars, generating products such as 8-hydroxyguanine (Halliwell and Gutteridge, 2007). This damage, such as mutation and deletion, results in gene expression errors, replication errors, genomic

instability and carcinogenesis (Cooke et al., 2003). DNA errors can be repaired by two types of excision repair, nucleotide excision repair (NER) or base excision repair (BER), removing damaged nucleotides or bases respectively, and repairing the DNA (de Laat et al., 1999). In proteins, H_2O_2 can oxidize SH-groups, thereby inactivating enzymes and this kind of damage is reversible by glutaredoxin or thioredoxin activity (Halliwell and Gutteridge, 2007). The hydroxyl radical and peroxynitrite can lead to the irreversible carbonylation of aminoacids, leading to impaired function of receptors, antibodies, signal transduction, transport proteins and enzymes. These damaged proteins need to be degraded in lysosomes (autophagy) or by the proteasome, a cytosolic and nuclear system selectively degrading unwanted and oxidatively damaged proteins, marked by ubiquitin, into peptides (Friguet, 2006). In the mitochondria, the Lon protease is responsible for breakdown of damaged mitochondrial proteins, such as aconitase (Bota and Davies, 2002). Lipid peroxidation of polyunsaturated fatty acids (PUFAs) upon hydroxyl radical attacks is the main form of oxidative damage to lipids. This is promoted by the double bonds in the PUFAs. This results in a decrease of membrane fluidity, increase in leakiness and damage of membrane proteins. Lipid hydroperoxides can be reduced by phospholipid hydroperoxide glutathione peroxide (GPX) or by phospholipase, after which the released hydroperoxides can be reduced by GPX or peroxiredoxin (Halliwell and Gutteridge, 2007)

1.1.3.2. Mitochondrial theory of aging

This theory, proposed by Miquel et al. (1980), is generally considered as a refinement of the FRTA and states that aging is the result of damage caused by ROS to the mitochondrial genome in post-mitotic cells. It was the result of the discovery of mitochondrial DNA (mtDNA) and the realization that the proteins it encodes are critical subunits of the oxidative phosphorylation machinery. mtDNA is located in close proximity to the respiratory chain, lacks histones and introns and has lower rates of DNA repair, which makes it more vulnerable to oxidative damage than nuclear DNA (Lim et al., 2005; Richter et al., 1988). mtDNA mutations are proposed to accumulate with age leading to an increasing amount of dysfunctional mitochondrial encoded respiratory complex subunits with consequently increasing ROS generation and thus establishing a vicious cycle (Lenaz et al., 2002). Over the past years, correlative data supporting the theory emerged, including studies showing increased oxidative damage to mtDNA, reduced mitochondrial activity and increased ROS production with age (Figueiredo et al., 2008; Van Remmen and Richardson, 2001). Arguments against this theory were provided by the creation of a mutator mouse with increased mtDNA mutagenesis that displayed a progeroid-like phenotype without elevated ROS production (Kujoth et al., 2005; Trifunovic et al., 2004).

1.2. Energy metabolism and mitochondria

Mitochondria contain their own circular mtDNA, varying in length between organisms (16.5 kb in mammals vs. 13.7 kb in *C. elegans*) encoding 13 proteins all part of the oxidative phosphorylation (OxPhos) machinery. In the absence of mtDNA expression, OxPhos collapses (Larsson et al., 1998). Mitochondria are involved in many essential cellular processes, including energy production, redox control, calcium homeostasis and certain metabolic and biosynthetic pathways. In addition, they are the cells' main source of ROS (as described in 1.1.3.1.1) and play a pivotal role in cell death mechanisms. They do not exist as single organelles, but rather they fuse and divide into pieces by the process called fission (Bereiter-Hahn, 2013).

1.2.1. Energy metabolism (Voet et al., 2006)

Metabolism can be defined as the overall process occurring in living organisms that allows them to acquire and use the energy they need to perform their various functions. Traditionally, it can be divided into catabolism, the degradation of complex biomolecules, and anabolism, the biosynthesis of biomolecules from simpler components. Intermediary metabolism includes all reactions concerned with the storage and generation of metabolic energy required for the biosynthesis of low-molecular weight compounds and energy storage compounds. The part that consists of pathways involved in storage and generation of metabolic energy is called energy metabolism (Braeckman et al., 2009; Mathews and Van Holde, 1996).

In Fig. 2, an overview of the major pathways in intermediary metabolism is provided. A central pathway in intermediary metabolism is glycolysis. This pathway is located in the cytosol, where it converts sugars (mainly glucose as a result of the breakdown of polysaccharides) into pyruvate. In this process, small amounts of ATP are generated and the electron carrier NAD^+ (nicotinamide adenine dinucleotide) is reduced to NADH. In anaerobic conditions, pyruvate must be converted to a reduced end product to reoxidize NADH. This process is called fermentation and can be achieved by homolactic fermentation with lactate as an end product or by alcoholic fermentation with ethanol and CO_2 as the end products. Under aerobic conditions, pyruvate is shuttled into the mitochondria where it is decarboxylated and converted into acetyl-Coenzyme A (Acetyl-CoA) which in turn is oxidized in the tricarboxylic acid cycle (TCA). The TCA is the central pathway for recovering energy from several metabolic fuels, including carbohydrates, fatty acids and amino acids. It is a series of eight enzymatic reactions that conserves energy in the electron carriers NADH and $FADH_2$ (flavin adenine dinucleotide) and yields a high-energy compound in the form of ATP or GTP (adenosine and guanosine triphosphate). NADH and $FADH_2$ donate their electrons to the electron transport chain (ETC) where they pass through a series of redox centers until they finally reduce oxygen. This electron transfer causes translocation of mitochondrial matrix protons to the intermembrane space

thereby establishing a proton gradient that forms the driving force for ATP synthesis (Braeckman et al., 2009; Voet et al., 2006).

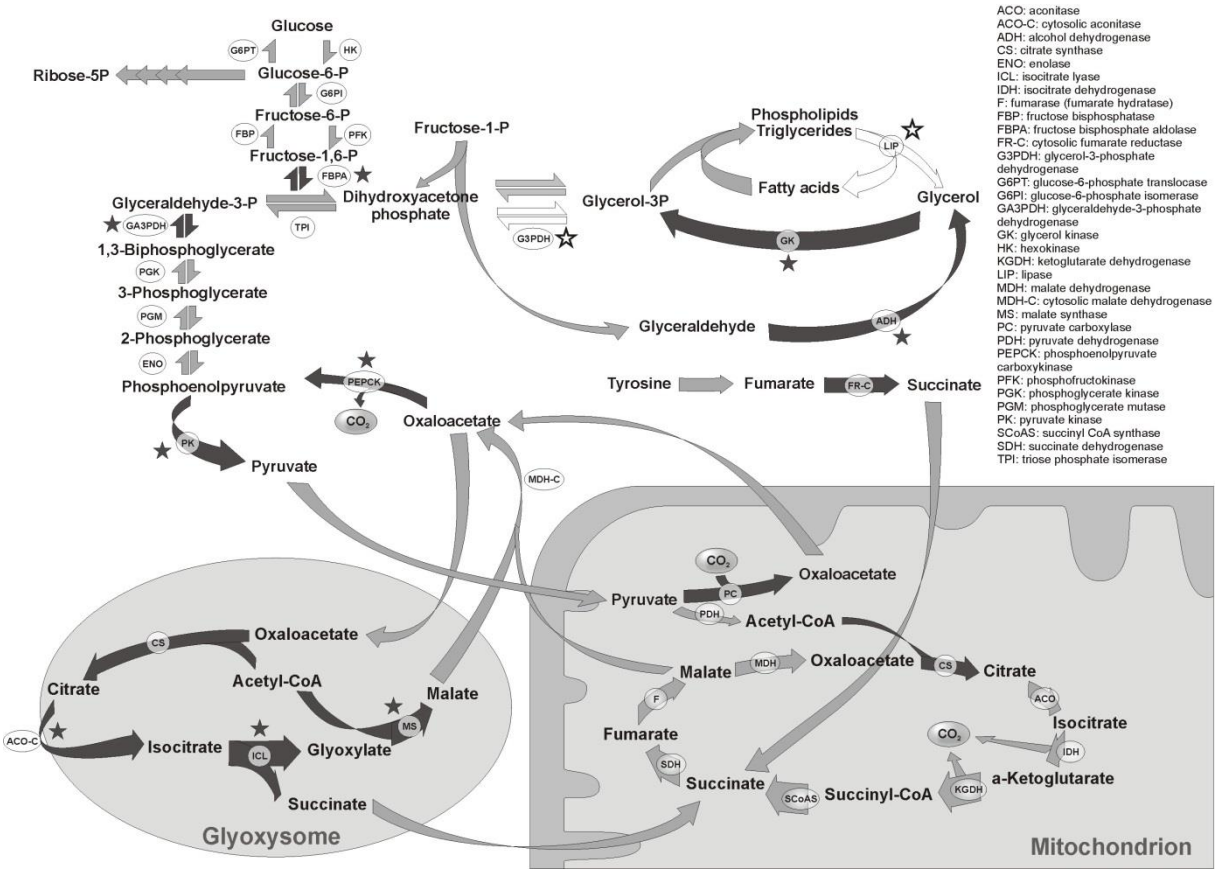

Figure 2: Schematic overview of some of the major pathways of intermediary metabolism, including glycolysis, TCA, glyoxylate cycle, pentose phosphate pathway, gluconeogenesis and lipid hydrolysis (Castelein et al., 2008).

An alternative pathway to glycolysis, the pentose phosphate pathway, oxidizes glucose-6-phosphate to generate NADPH (nicotinamide adenine dinucleotide phosphate), CO_2 and pentoses. NADPH uses the free energy of metabolite oxidation for reductive biosynthesis of fatty acids and cholesterol. Pentoses are used in nucleotide biosynthesis.

The glyoxylate cycle forms an alternative to the TCA and is found in plants, bacteria, fungi and nematodes. In plants, the glyoxylate cycle operates in a specialized peroxisome, called glyoxysome. This pathway mediates the conversion of acetyl-CoA to succinate, which can either be transported to the mitochondria for reentry in the TCA thereby making the glyoxylate cycle an anaplerotic process or it can be transported to the cytosol, converted to oxaloacetate and used for gluconeogenesis. The latter option enables conversion of fatty acids to carbohydrates.

As described above, acetyl-CoA that enters the TCA is not only the product of carbohydrate catabolism. Lipid metabolism as well, converges on the TCA. Triacylglycerols (fat or triglycerides) are the major form of metabolic energy storage in humans. They consist of

glycerol triesters of fatty acids. Before fatty acids can be catabolized for the generation of energy, they have to be released from the triacylglycerol structure and this happens by the enzymatic action of lipases. Fatty acids are activated for oxidation in the cytosol by the formation of fatty acyl-CoA. However, fatty acid oxidation occurs in the mitochondria by a progressive degradation in two-carbon units and this process involves the oxidation of the carbon atom β to the carboxyl group, hence the term β-oxidation. Each round of β-oxidation results in one NADH, one $FADH_2$ and one acetyl-CoA of which the oxidation in the TCA renders an additional $FADH_2$ and 3 NADH. Complete oxidation of fatty acids is a highly exergonic process that yields plenty of ATP molecules.

Under fasting conditions, in most organisms, glucose needs are met by gluconeogenesis from non-carbohydrate precursors such as amino acids, lactate, pyruvate and TCA intermediates. Most of the reactions of gluconeogenesis are glycolytic reactions that are carried out in the reverse direction. However, glycolysis contains three rate-determining steps that are metabolically irreversible and these reactions need to be circumvented during gluconeogenesis. The activity of pyruvate kinase is replaced by a combination of pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity. The activity of phosphofructokinase is replaced by fructose bis-phosphatase activity and hexokinase activity is replaced by glucose-6-phosphatase activity. Gluconeogenesis occurs in the cytosol, while oxaloacetate formation from pyruvate only occurs in the mitochondria and so, gluconeogenesis requires metabolite transport between mitochondria and cytosol using the malate-aspartate shuttle.

1.2.2. Oxidative phosphorylation

Mitochondria are composed of two compartments, the intermembrane space and the matrix, bound by the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The OMM is easily permeable to small metabolites, whereas the permeability of the IMM is tightly controlled to support the high electrochemical gradient, built up by the respiratory chain and necessary for ATP production. Among the many described roles for the mitochondria, the most significant one is oxidative phosphorylation (OxPhos). This involves coupling of electron transfer through the mitochondrial electron transport chain (ETC) to the pumping of protons to obtain an electrochemical gradient that can be harvested by ATP synthase for the production of energy in the form of ATP.

1.2.2.1. Electron transport chain Complexes

The ETC contains four complexes contributing to energy production: Complex I (NADH:ubiquinone oxidoreductase), Complex II (succinate:ubiquinone oxidoreductase), Complex III (ubiquinone:cytochrome c oxidoreductase), Complex IV (cytochrome c oxidase)

supplemented with ubiquinone and cytochrome c as electron carriers. Complex V (F_1F_0-ATP synthase) is also a part of the oxidative phosphorylation machinery but is not a part of the ETC.

Electrons from NADH at Complex I, or from FADH2 at Complex II, flow through the ETC and finally reduce oxygen to water at Complex IV. Meanwhile protons are translocated from the mitochondrial matrix to the intermembrane space by active pumping at Complexes I, III and IV, establishing a proton motive force that is harvested by Complex V for the production of ATP. The proton motive force consists of an electrical component, the membrane potential, and a chemical component, the pH gradient across the IMM, but is mainly dominated by the membrane potential (Adam-Vizi and Chinopoulos, 2006) (Fig. 3).

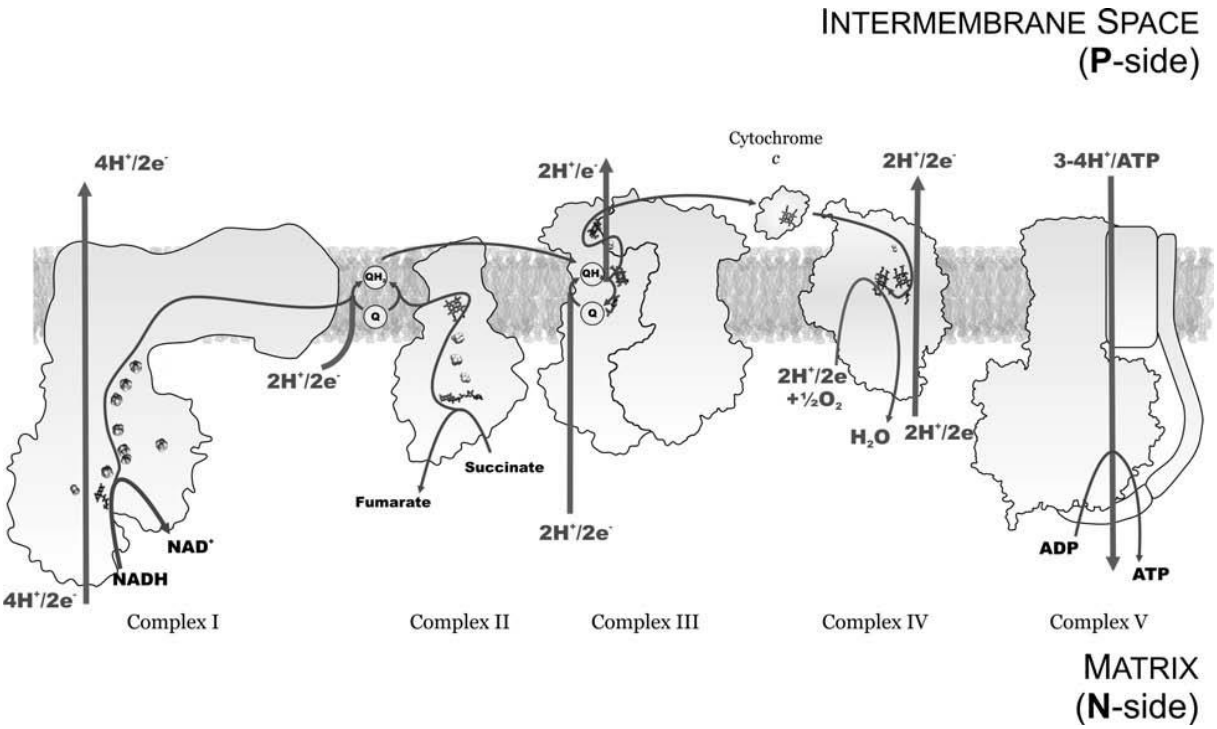

Figure 3: overview of oxidative phosphorylation. Electrons are delivered to CI and II by NADH and FADH2 respectively and passed to CIV, while establishing a proton gradient that is harvested by ATP synthase for the production of ATP (Belevich and Verkhovsky, 2008).

1.2.2.1.1. Complex I (NADH:ubiquinone oxidoreductase)

Electrons enter the ETC at Complex I. This enzyme couples electron transfer from NADH to ubiquinone to the translocation of four protons across the IMM and is considered the most complicated enzyme in the IMM. In mammals, it consists of no less than 46 subunits. Electron microscopy revealed an L-shaped architecture with two arms of equal length, and exposed both to the matrix side as to the intermembrane space (Fig. 4).

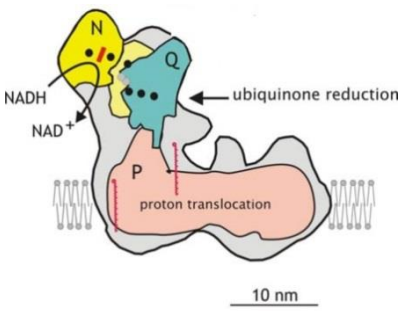

Figure 4: Schematic overview of the structure of Complex I, showing the NADH oxidation, ubiquinone reduction and proton translocation sites. Adapted from Zickermann et al. (2009)

All redox-active groups, a flavin mononucleotide (FMN) and eight iron-sulfur clusters, reside in the peripheral arm and this is also the location where binding and oxidation of NADH and reduction of ubiquinone take place (Zickermann et al., 2009). How electron transfer is coupled to proton pumping is an unresolved question (Brand, 2010). It is suggested that the proton translocating site is situated in the membrane arm, since its hydrophobic subunits are homologous to Na^+/H^+ antiporters. But exactly which subunits form the proton pumping unit still remains unknown (Zickermann et al., 2009). Complex I can be inhibited at the Q_0 site using rotenone.

1.2.2.1.2. Complex II (succinate:ubiquinone oxidoreductase)

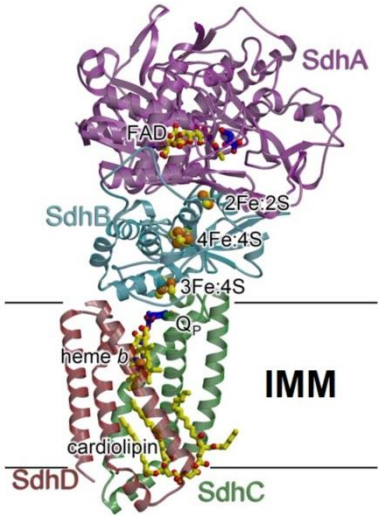

Figure 5: : Overview of the structure of Complex II, showing the four subunits and sites of electron translocation. Adapted from Iverson (2013).

Complex II forms the second entrance point for electrons in the ETC and passes electrons from succinate to ubiquinone. It contains the tricarboxylic acid cycle (TCA) enzyme succinate dehydrogenase, thus coupling the TCA directly to the ETC. Its redox groups include three Fe-S clusters and one cytochrome b_{560}. The complex consists of four subunits and has a large soluble domain and a smaller membrane-integrated domain (Fig. 5). It is at the soluble domain that the interconversion of succinate to fumarate proceeds, while the interconversion of quinone and quinol proceeds at membrane spanning regions. Complex II does not shuttle protons from the matrix to the intermembrane space and consequently does not attribute to the build-up of the membrane potential. This enzyme can also operate in the opposite direction as a fumarate reductase. Important to note is that, although their names suggest otherwise, Complex I and II do not operate in series (Iverson, 2013; Voet et al., 2006). Inhibition of Complex II can be achieved using malonate.

1.2.2.1.3. Complex III (Ubiquinone:cytochrome c oxidoreductase)

Electrons from Complex I and Complex II are passed to Complex III, also known as cytochrome bc_1, by the fat-soluble ubiquinone, and from there they are passed on to cytochrome *c*. Complex III has a dimeric structure, incorporating 4 metal centers in each monomer. The cytochrome *b* subunit contains 2 *b* hemes (b_H for high potential and b_L for low potential) and another heme, heme c_1, is located in the cytochrome c_1 subunit. The third subunit contains a Fe-S cluster known as the Rieske center or iron sulphur protein (ISP). These subunits are anchored in the IMM and extend in the intermembrane space as well as in the matrix (Crofts et al., 2008; Voet et al., 2006). Despite the complexity of this Complex, the mechanism of electron transport is better understood than for the other complexes. Complex III mediates the reduction of two molecules of cytochrome *c*, a one-electron carrier by one molecule of QH_2, a two-electron carrier. This happens in the so-called Q cycle (Fig. 6) and permits Complex III to pump protons from the matrix to the intermembrane space. The oxidation of ubiquinol (QH_2) occurs at the Q_P site (located between the ISP and heme b_L) where two electrons are diverted to two separate chains. One electron goes through the way of ISP, heme c_1 and cytochrome *c*, while the other electron passes by the hemes b_L and b_H to the Q_N site where it either reduces ubiquinone (Q) to semiquinone (SQ) or SQ to ubiquinol (QH_2). Reactions at the Q_P site need to take place twice in order to fully reduce ubiquinone at the Q_N site, meaning that oxidation of 2 molecules of QH_2 results in one molecule of fully reduced ubiquinone (Q) (Crofts et al., 2008; Voet et al., 2006). The localization of the two Q-sites at opposite sides of the membrane, gives rise to the proton translocating mechanism that is supported by a redox center itself (QH) being the proton carrier. The net reaction of the Q-cycle indicates that oxidation of QH_2 results in the appearance of two reduced cytochrome c molecules and four protons at the outer side of the IMM. Complex III can be inhibited by antimycin A, myxothiazol and stigmatellin.

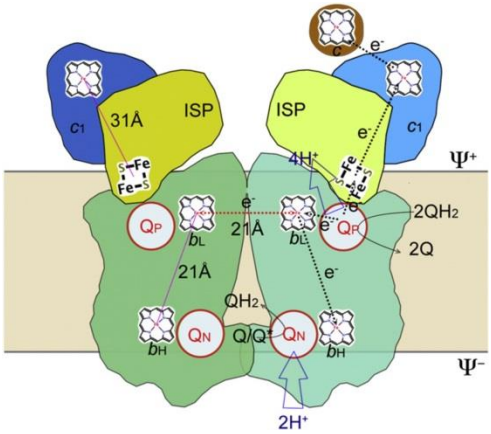

Figure 6: Schematic overview of the structure of Complex III, with a representation of the Q cycle (Xia et al., 2012). ISP = iron-sulphur protein. Green = cyt *b* subunit, yellow = ISP, Blue = Cyt *c1* subunit.

1.2.2.1.4. Complex IV (cytochrome c oxidase)

Complex IV is the terminal complex of the ETC and catalyzes the one-electron oxidation of four consecutive cytochrome *c* molecules thereby reducing one O_2 molecule to water and couples this to the translocation of four protons across the IMM. Complex IV is a homodimer and each monomer contains 13 subunits, embedded in the IMM. Its largest subunits I, II and III, forming its core, are encoded by the mitochondrial genome. Complex IV contains four redox centers:

subunit I houses cytochrome *a*, cytochrome a_3 and a copper atom Cu_B while subunit II contains a pair of copper atoms known as the Cu_A center as the redoxactive center. Electrons flow from cytochrome *c* to the Cu_A center, then to heme *a* and finally to heme a_3 and Cu_B. It is at the cytochrome a_3-CU_B binuclear complex that the reduction of oxygen to water proceeds. Complex IV has two proton translocating channels. The K-channel takes up protons at the matrix side and does not appear to be connected to the intermembrane space and is therefore thought to provide protons for O_2 reduction. The D-channel extends to the vicinity of the a_3-CU_B center where it connects to the exit channel, communicating to the intermembrane space and thus responsible for proton pumping from the matrix to the intermembrane space (Belevich and Verkhovsky, 2008; Voet et al., 2006) (Fig. 7).

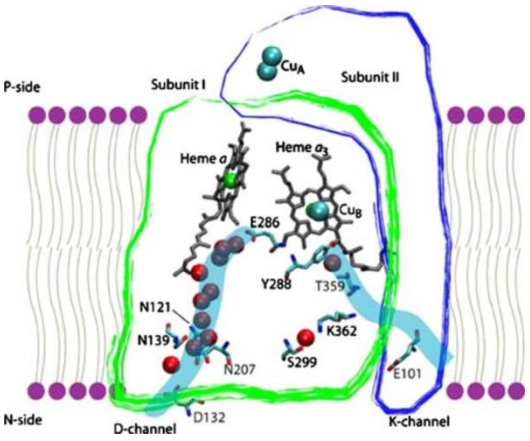

Figure 7: Schematic representation of the CIV I and II subunits, showing the metal centers and two proton input channels (Brzezinski and Gennis, 2008).

1.2.2.1.5. Complex V (F_1F_0-ATP synthase)

The electron transport through Complexes I-IV releases free energy that is conserved in the form of an electrochemical gradient of protons across the IMM and that is coupled to ATP synthesis by Complex V. The use of the energy provided by the proton gradient for ATP synthesis is termed oxidative phosphorylation. ATP synthase catalyzes ATP synthesis from adenosine diphosphate (ADP) and inorganic phosphate (P_i) and consists of 2 rotary motors: F_1, the water-soluble part of the enzyme, and F_0, embedded in the IMM. The enzyme contains eight different subunits found in the composition $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10\text{-}15}$. $\alpha_3\beta_3$ form the F_1 sphere by alternating arrangement of α and β subunits and are connected to the γ subunit as the rotor shaft. The γ subunit binds to the ε subunit that provides the connection between the rotor parts of F_1 and F_0. δ and b_2 form the peripheral stalk. A ring of 10-15 c subunits is located in the mitochondrial membrane and is connected to the a subunit (Okuno et al., 2011) (Fig. 8). ATP is synthesized in the F_1 moiety of Complex V. To this end, energy is derived from the protons being translocated back from the intermembrane space to the mitochondrial matrix by the F_0 motor. The release of energy is coupled to a rotation of the ring of c subunits in the F_0 motor and the γ, δ and ε subunits of the F_1

motor. It is the rotation of the γ subunit within the $\alpha_3\beta_3$ hexamer that provides energy for ATP synthesis (Jonckheere et al., 2012). This can be explained by the 'binding-change' mechanism first proposed by Boyer (1975). The catalytic sites for ATP synthesis are located at the three αβ subunits, each in a different conformational state: one that binds substrates and products loosely (L), one that binds them tightly (T) and one that does not bind them at all (open state or O). While the γ subunit rotates each of the sites undergoes conformational changes: an O state changes to a L state, binding ADP and P_i, a L state changes to a T state, binding the substrates more tightly and converting them to ATP and a T state transfers to an O state, releasing the synthesized ATP (Voet et al., 2006).

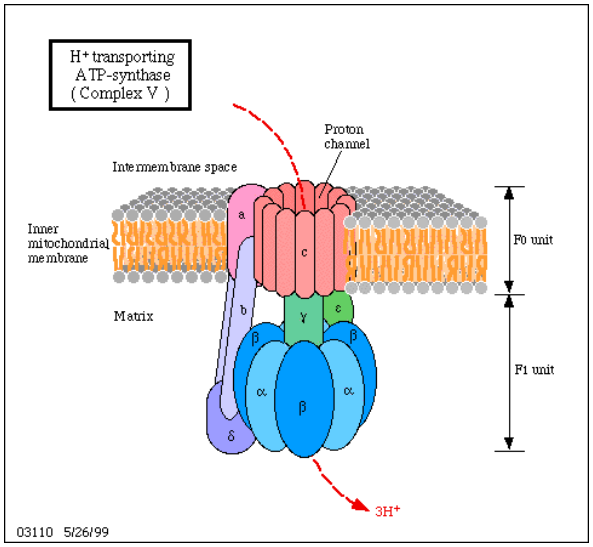

Figure 8: Schematic overview of the structure of Complex V (KEGG Regulatory pathways, http://www.biologie.uni-hamburg.de).

1.2.2.2. Supercomplex organization of ETC Complexes

The view on bioenergetic processes became more precise by the discovery of the higher order organization of the individual ETC Complexes into supercomplexes. In contrast to the random collision model, where all ETC complexes were considered as independent entities (Hackenbrock et al., 1986), evidence was found that Complexes I, III and IV are mutually associated. Chance and Williams (1955a) already established the view of the ETC as a solid state assembly of flavins and cytochromes in a protein matrix (Lenaz and Genova, 2010). A breakthrough in this research area happened with the application of the blue-native polyacrylamide gelelectrophoretic (BN-PAGE) technique to yeast and mammalian digitonin-solubilized mitochondria. Originally, these supercomplexes were termed respirasomes (Schagger and Pfeiffer, 2000) and it has been suggested that they may even be connected forming respiratory strings (Wittig et al., 2006) (Fig. 9). Studies with bovine mitochondria showed supercomplexes containing complexes I, III and IV, with almost all Complex I observed in supercomplexes rather than in its individual form. In yeast, Complex IV exists only in a monomeric state while in bovine mitochondria it can be isolated in dimeric form (Schagger and

Pfeiffer, 2000). The most common supercomplexes documented are the complexes I:III_n, I:III_n:IV_n and III_n:IV_n and they can contain up to 4 Complex IV monomers (Dudkina et al., 2011; Schagger and Pfeiffer, 2001). Most of Complex II was found in a free, non-associated form and Complex V dimer was found to co-migrate with other supercomplexes but not as a part of them (Vartak et al., 2013).

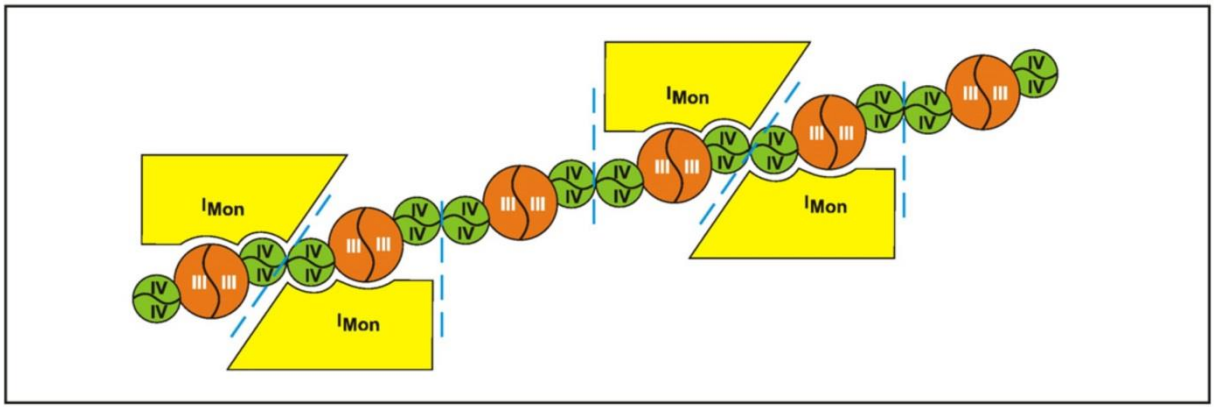

Figure 9: Respiratory string model for mammalian mitochondria, showing the organization of ETC Complexes into supercomplexes and their interactions to form larger supramolecular structures. I_{Mon}: Complex I monomer (Wittig and Schagger, 2009).

Today the evidence of supercomplex organization of the respiratory chain is convincing and the concept seems evolutionary conserved for several organisms and tissues such as rat brain (Frenzel et al., 2010; Reifschneider et al., 2006), mice (Acin-Perez et al., 2008), bovine heart (Reifschneider et al., 2006; Schagger, 2002), fish (Schäfer et al., 2007), the fungal model *Podospora anserina* (Groebe et al., 2007; Maas et al., 2009), *Drosophila* (Le Pecheur et al., 2009) and *Caenorhabditis elegans* (Suthammarak et al., 2010; Suthammarak et al., 2009). Proposed functions for these supercomplexes are substrate channeling, the direct transfer of an intermediate between the active sites of 2 enzymes catalyzing consecutive reactions, and stabilization of Complex I (Lenaz and Genova, 2012). Indirect evidence suggests that substrate channeling facilitates electron flow and thus helps keeping the redox components in the oxidized state with as net result decreased ROS production (Seelert et al., 2009). In *C. elegans*, it was shown that mutations in certain subunits of Complex IV reduce Complex I enzymatic activity and Complex III mutations inhibit Complex I by several mechanism involving supercomplex stability (Suthammarak et al., 2010; Suthammarak et al., 2009). Factors that influence supercomplex association are the lipid content and composition of the IMM. Cardiolipin, a phospholipid, is a major constituent of the IMM and has been shown to be indispensable for the activity of cytochrome c, Complex I , Complex III and Complex IV (Fry and Green, 1981) as well as for the stability of respiratory supercomplexes (Paradies et al., 2010; Schagger, 2002; Zhang et al., 2002). Not only the ETC Complexes are organized in higher order structure. Complex V as

well is often isolated in dimeric and oligomeric forms. These structures play a role in the formation of the cristae, the fold in the IMM. Multiple dimers, arranged as dimer ribbons at the apex of the cristae, induce a strong curvature. Complex V multimers may favour substrate channeling as well and allow efficient cooperation of the ETC complexes (Davies et al., 2012; Wittig and Schagger, 2009).

1.2.2.3. Regulation of oxidative phosphorylation

As the need for ATP is not constant, e.g. in resting versus active conditions, a strict coordination of the pathways that produce ATP is necessary. The reactions of the ETC from NADH to cytochrome *c* operate near equilibrium and are mainly controlled by the ATP concentration. Cytochrome *c* oxidation is an irreversible reaction and is regulated by the concentration of reduced cytochrome *c*, which depends on the [NADH]/[NAD+] and [ATP]/[ADP][P_i] ratio. Cytochrome *c* can be allosterically inhibited by ATP at high ATP/ADP ratios (Kadenbach, 2003). The [ATP]/[ADP][P_i] ratio on its turn is dependent on transport proteins: voltage dependent anion channel (VDAC) in the OMM and adenine nucleotide translocase (ANT) and a P_i/H^+ symporter in the IMM. In particular the ATP/ADP translocator and the Pi transporter play a role in the regulation of OxPhos. Furthermore, the protein IF_1 is a specific inhibitor of ATP synthase that is activated at a pH below 6.5. Also, glycolysis and the TCA are coordinately coordinated according to the need for OxPhos (Voet et al., 2006).

1.2.3. Mitochondria, aging and dietary restriction

Since the formulation of the FRTA, the role of mitochondria in the aging process has been the topic of intense interest. In most organisms, changes in structure and function of mitochondria during aging have been observed. Whether these changes are a consequence or a cause of aging remains difficult to answer, since most of the evidence is correlative (Bereiter-Hahn, 2013).

1.2.3.1. Mitochondria and aging

Several mitochondrial changes occur during the aging process. A decrease in mitochondrial number is observed, associated with an enlargement of the remaining mitochondria, more irregular structure and loss of cristae structure (Miquel et al., 1980). Protein levels and mtDNA copy number have been shown to be reduced in mammalian cells (Stocco et al., 1977). In other organisms, such as *C. elegans*, *D. melanogaster*, and mice, decreased protein levels were found as well (Li et al., 2009; Sohal et al., 2008; Yang et al., 2008). mtDNA levels were found unchanged with age in *C. elegans* (Brys et al., 2010). Recent work shows that mitochondria are dynamic organelles and rather than existing as individual structures, they form complex tubular networks through fusion and fission. Abnormalities in mitochondrial dynamics play an important role in the pathophysiology of neurodegenerative diseases (Chen and Chan, 2009)

and it is thought that dysfunction in this process plays a role in cellular senescence (Lee et al., 2007). Furthermore, somatic mtDNA mutations were found to accumulate with age, including large-scale deletions, point mutations and tandem duplications (Lee and Wei, 2012). The mtDNA mutator mice, deficient in mtDNA proofreading, display a progeroid phenotype and provide evidence for mtDNA mutations being the driving force in aging (Kujoth et al., 2005; Trifunovic et al., 2004). The mtDNA deleter mice accumulate large-scale mtDNA deletions and have progressive respiratory chain dysfunction. However, they do not display the progeroid phenotype and have a normal lifespan, suggesting that mtDNA deletion accumulation and respiratory chain dysfunction are insufficient to cause aging (Tyynismaa et al., 2005).

Bioenergetic studies on isolated mitochondria of humans and animals suggest that respiratory function declines with age (Boveris and Navarro, 2008; Fannin et al., 1999; Hempenstall et al., 2012; Kim et al., 1988; Lanza et al., 2012; Navarro and Boveris, 2008; O'Toole et al., 2010; Paradies and Ruggiero, 1990; Ventura et al., 2002). Some of these studies show that ADP/O and RCR (defined in 1.2.4.4) remain unaffected during aging (O'Toole et al., 2010; Paradies and Ruggiero, 1990), while others report that RCR declines with age (Kim et al., 1988; Navarro and Boveris, 2008). In *C. elegans*, Brys et al. (2010) showed that state 3 respiration (defined in 1.2.4.3) does not decrease with age, contradictory to the other model organisms described above, and that LEAK respiration (defined in 1.2.4.3.2) increased and RCR decreased with age. In other model organisms, Complex I and IV activity was shown to decrease with age (Lenaz et al., 1997; Petrosillo et al., 2013; Ren et al., 2010) and ROS production rates from mitochondria were found to be increased with age as well in the brain, heart and kidney of mice (Sohal et al., 1994). Although it is clear that aging affects mitochondrial functionality, there is no consensus on what the general impact of aging is. Another interesting link between respiratory function and aging was established with the discovery of *C. elegans* mutants defective in essential ETC components displaying lifespan extension (Dillin et al., 2002; Feng et al., 2001; Lee et al., 2003b). Similarly, downregulation of ETC component subunits in mice, flies and nematodes was found to extend lifespan (Lapointe and Hekimi, 2008; Rea et al., 2007; Zordan et al., 2006).

1.2.3.2. The effect of dietary restriction on mitochondrial function.

Dietary restriction (DR) has been shown to extend lifespan in a wide variety of species, ranging from yeast to mammals (Masoro, 2005). How DR exerts its effect on lifespan remains currently unresolved. Initially, it was thought that DR increases lifespan by reducing metabolic rate. However, today it is commonly accepted that the metabolic rate of DR rodents is not decreased (Masoro et al., 1982) and these findings were confirmed in *C. elegans* and *D. melanogaster* as well (Bishop and Guarente, 2007; Houthoofd et al., 2002b; Houthoofd et al., 2002c; Hulbert et al., 2004; Schulz et al., 2007). Using isolated mitochondria, it was shown that DR prevents the age-

induced decline in mitochondrial respiratory capacity (Bevilacqua et al., 2005; Hagopian et al., 2005; Hempenstall et al., 2012; Lanza et al., 2012; Lopez-Lluch et al., 2006; Sohal et al., 1994) and that ROS production rates are decreased by DR, as are the steady-state levels of oxidative stress (Gredilla et al., 2001; Hagopian et al., 2005; Lopez-Torres et al., 2002; Sohal et al., 1994). On the other hand, some studies show that DR leads to increased ROS production and suggest that this leads to a mitohormetic response by upregulating ROS defense mechanism which ultimately extends lifespan (Schulz et al., 2007; Sharma et al., 2011). These discrepancies can be explained by the fact that different modelorganisms were used during these studies. The Uncoupling-to-Survive theory postulates that mild uncoupling protects against aging and oxidative damage by reducing the mitochondrial membrane potential, consequently limiting excessive ROS production and extending lifespan (Brand, 2000). It was suggested that such a mechanism could be at the molecular basis of the DR-induced reduction of mitochondrial ROS generation (Ash and Merry, 2011). However, there is controversy on how DR changes mitochondrial function in rodents: some studies pointed out that DR increases mitochondrial proton leak (Lambert and Merry, 2004), while others found that DR generally decreases mitochondrial respiration but maintains equally high ATP synthesis (Lopez-Lluch et al., 2006) and still others report that DR decreases LEAK respiration (Bevilacqua et al., 2005; Lal et al., 2001; Sohal et al., 1994). In addition, DR has been shown to induce mitochondrial biogenesis in rodents and humans and it was suggested that this effect may contribute to the maintenance of high respiration, while decreasing membrane potential and ROS production (Civitarese et al., 2007; Lopez-Lluch et al., 2006; Nisoli et al., 2005). These results are contested by studies reporting no such induction (Hempenstall et al., 2012; Lanza et al., 2012). Possibly, the use of different tissues and application of slightly differing DR protocols may provide an explanation for the opposing results.

1.2.4. High Resolution Respirometry (HRR)

The metabolism of animals can be estimated using various techniques, including assessment of heat dissipation, ATP levels, and respirometry. During the process of oxidative phosphorylation, mitochondria consume oxygen presenting the opportunity to use this as a parameter for their functionality. One approach is to use a polarographic, Clark-type oxygen sensor in a closed-chamber system which can be applied for intact living animals as well as for isolated mitochondria. The advantage of working with isolated mitochondria is that potentially confounding factors of the cellular environment can be excluded. The principle involves the determination of changes in the oxygen concentration, a decline over time as a result of respiratory processes. Plotting the oxygen concentration against elapsed time gives an estimate of the rate of oxygen consumption and the standard for this approach was set 50 years ago by

Chance and Williams (1955b). In ideal circumstances, the oxygen decline is strictly due to the sample's oxygen consumption. However, the results may be troubled by oxygen backdiffusion in the medium and correction is needed. As small changes in cellular respiration and respiratory control ratio's (explained in 1.2.4.4.) may point to significant mitochondrial defects, high resolution and accuracy are required to face this challenge and conventional approaches were replaced by the new concept of high resolution respirometry with the Oroboros Oxygraph(O2k) (Gnaiger, 2001; Hutter et al., 2006). This technique was developed especially for measurement of small amounts of cells, tissues or isolated mitochondria.

1.2.4.1. O2k design

The O2k design is optimized to obtain high performance. Appropriate chamber materials that are chemically inert, such as glass for the chambers and PEEK (polyetheretherketone) stirrers instead of teflon, were selected to secure minimal background diffusion. Furthermore, chamber volume is optimized to a larger volume, leading to extended experimental time and decreased side effects. Electronics attribute to the improved performance as well. Simultaneous online recordings of the oxygen concentration and its time derivative, oxygen flux, allow for immediate evaluation of small changes in this flux (Fig. 10). Oxygen consumption by the polarographic oxygen sensor (POS) and backdiffusion at low oxygen pressure contribute to background effects. By determining this background over the experimental oxygen concentration range (background calibration), corrections can be applied, contributing to the accuracy of the measurements (Gnaiger, 2001; Gnaiger, 2011; Hutter et al., 2006).

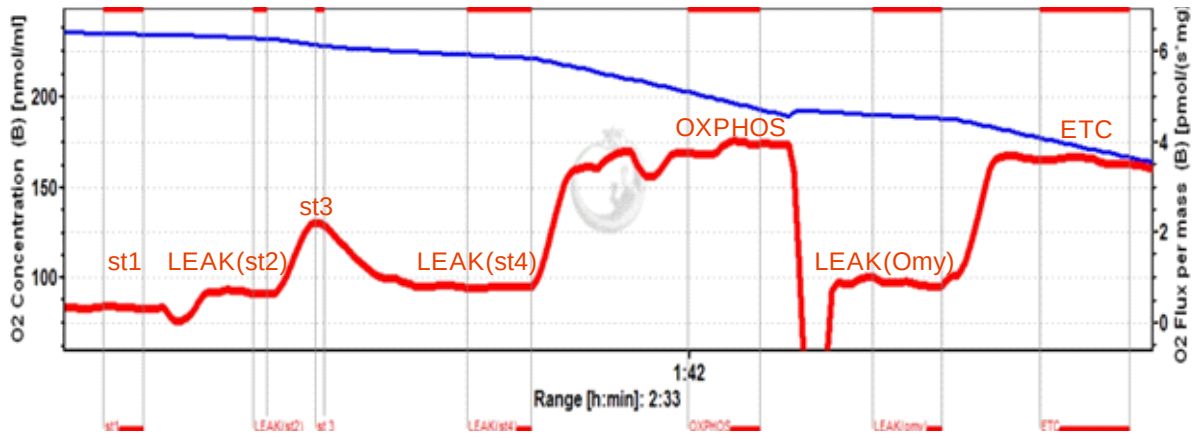

Figure 10: Typical trail obtained after an experiment using isolated mitochondria. The left Y-axis and blue trail represent changes in oxygen concentration, while the right Y-axis and red trail represent changes in oxygen flux and thus respiration rate. St1 =state1, St3=state 3, ETC = electron transport chain capacity. A more detailed definition of the respiratory states can be found in 1.2.4.3.

1.2.4.2. O2k-Multisensor setup

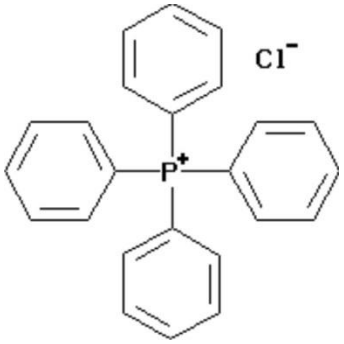

Figure 11: Structure of tetraphenylphosphonium-chloride.

The O2k can be extended with extra sensors such as a fluorescence module, enabling e.g. H_2O_2 measurements using Amplex Red, ion-selective electrodes (ISE) for measurement of e.g. membrane potential or pH and amperometric sensors, e.g. for the detection of nitric oxide. The ISE-setup enables measuring membrane potential by using a tetraphenylphosphonium (TPP^+)-sensitive electrode and consists of separate measuring and reference electrodes that are inserted in the chambers through the stopper and are connected to the potentiometric channels of the system, allowing simultaneous measurement of oxygen and TPP^+-concentration. TPP^+ is a cation that accumulates in the mitochondrial matrix as a function of membrane potential (Fig. 11). Before measurements using TPP^+Cl^- can be started, the inhibitory effect of this chemical on the experimental system and the optimum concentration have to be determined. Furthermore, chemicals may have a substantial effect on the potentiometric signal increasing the importance of performing a chemical background experiment in the absence of biological sample and correcting for these effects. TPP^+ electrode membranes are very sensitive to the adherence of lipophilic inhibitors. Therefore, in our experimental setup, we decided to avoid transfer of inhibitory effects between experiments by using simplified respiratory protocols in the absence of these inhibitors.

1.2.4.3. Respiratory states (Gnaiger, 2012)

For the respirometric analysis of oxidative phosphorylation, a good definition of respirometric states and control ratios is a prerequisite. Respiratory states have been defined by Chance and Williams (1955c) and the classical titration protocol starts with the addition of isolated mitochondria to air-saturated respiration medium, called state 1. Addition of ADP exhausts endogenous substrates and induces state 2 respiration. State 3 is established by the addition of substrates prompting high respiration and ADP depletion by phosphorylation to ATP. Thereafter, there is a transition to a ADP-depleted state 4, where respiration drops. Traditionally, the state 3-state 4 transition is repeated several times until oxygen is depleted. An alternative protocol, often used in several laboratories, defines state 2 and state 3 slightly different than the description above: state 2 is a state after the addition of substrates, followed by state 3 induction by the addition of ADP. The use of HRR prompted an extended terminology. The next section will give a detailed description of the terminology used for the analysis of mitochondrial function in this thesis.

1.2.4.3.1. Electron gating

Electrons can enter the ETC either at Complex I, Complex II or at other sites which we will not discuss here. The choice of the appropriate substrates gives the possibility to distinguish between Complex I or Complex II fueled mitochondrial function. Using NADH-linked substrates such as pyruvate and malate, in particular Complex I function will be assessed. Using succinate/rotenone (rotenone is a Complex I inhibitor), electrons are entered at Complex II, allowing exclusive monitoring of changes affecting Complex II function. It is interesting that convergent electron flow through Complexes I and II results in an additive effect on respiratory flux.

1.2.4.3.2. LEAK respiration

The aim of this state is to assess respiration that is compensating for LEAK (proton leak and electron slip), in non-phosphorylating respiration of the mitochondria. Such a state occurs in the presence of substrate and absence of ADP or under conditions of Complex V inhibition. This means that the classical state 2 and state 4 can be considered LEAK states and they will be termed LEAK(st2) and LEAK (st4) respectively. An extra LEAK state can be induced by the inhibition of ATP synthase using the inhibitor oligomycin and is termed LEAK(omy) (Fig. 12).

Under LEAK conditions, due to the absence of ADP, respiration is usually low leading to high membrane potential. Because of electron stalling, ROS production is expected to be highest in these conditions.

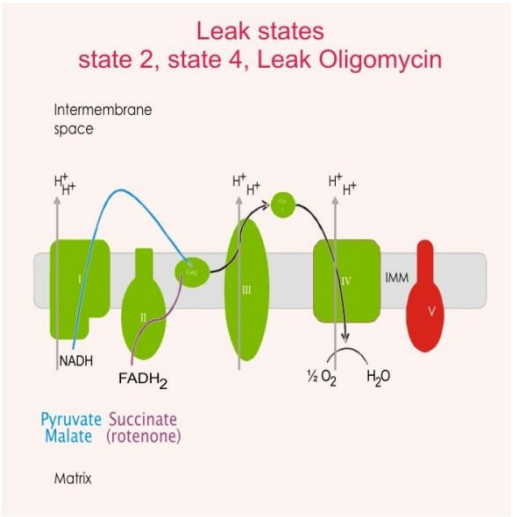

Figure 12: Schematic overview of LEAK state respiration. Electrons are delivered to Complexes I and II and pass through the ETC to finally reduce oxygen at Complex IV. Without ADP to start OxPhos, electron transfer and oxygen consumption remain low and membrane potential is high.

1.2.4.3.3. OXPHOS capacity

Stimulation of the mitochondria by the addition of saturating amounts of ADP in the presence of substrates yields a state that can be used to assess oxidative phosphorylation (OXPHOS) capacity. It is different from the classical state 3 because of the saturating ADP concentration,

allowing stimulation of maximal OxPhos and is characterized by high respiratory rates. Mitochondria respiring under OXPHOS capacity generate a proton gradient by proton pumping that is used by ATP synthase for the synthesis of ATP. In this thesis this state is induced after the state 3-state 4 transition (Fig. 13). In OXPHOS conditions, respiration is maximized and membrane potential is lowered because protons are used by CV. Electrons can flow rapidly through the ETC and ROS production is expected to be very low.

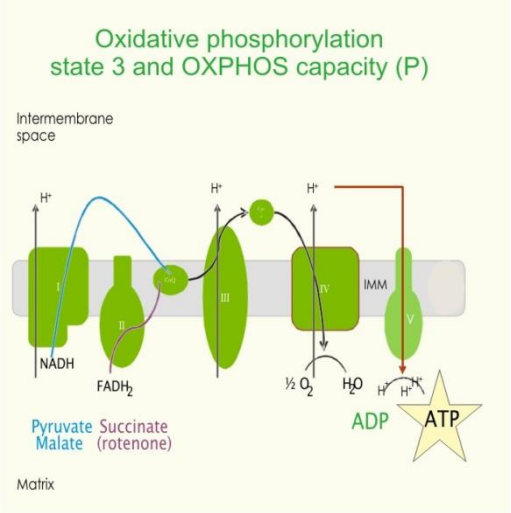

Figure 13: Schematic overview of oxidative phosphorylation. Providing ADP for ATP synthesis prompts high electron flow rates and consequently high respiratory rates. Membrane potential is lowered, since protons are used for ATP synthesis.

1.2.4.3.4. Electron transport chain capacity (ETC capacity)

The capacity of the electron transport chain can be assessed by establishing a non-coupled state using protonophores such as the chemical uncoupler FCCP at optimum concentration for stimulation of maximum flux. This state is different from OXPHOS capacity because it does not depend on the rate of ATP synthesis to reach its maximum capacity and thus ETC capacity potentially exceeds OXPHOS capacity (Fig. 14). In non-coupled conditions, electron transfer and thus respiration is maximized while membrane potential is decreased leading to very few opportunities for premature electron escape and formation of ROS.

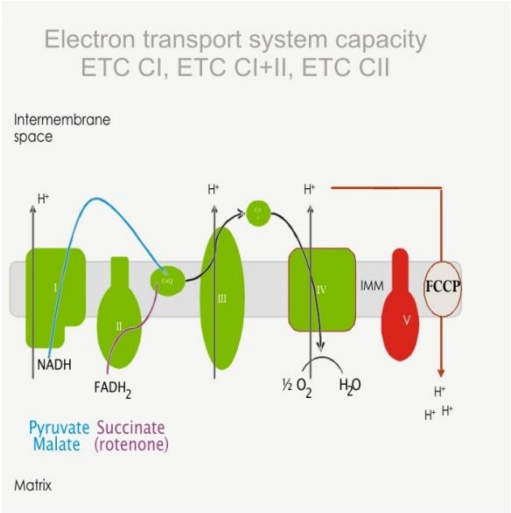

Figure 14: Schematic overview of ETC capacity, which can be estimated by using chemical uncouplers (e.g. FCCP). These protonophores shuttle protons back to the mitochondrial matrix in the absence of ATP synthesis, maximizing electron transfer and respiration.

1.2.4.4. Control ratios

1.2.4.4.1. Respiratory Control Ratio (RCR)

The RCR reflects the control of oxygen consumption by phosphorylation (Lesnefsky and Hoppel, 2006) and illustrates the coupling between respiration and ATP synthesis. This ratio is calculated by dividing classical state 3 respiration by state 4 respiration. The RCR starts at minimum 1 and is unlimited.

1.2.4.4.2. LEAK control ratio (LEAK/ETC)

Unrelated to uncoupling, OXPHOS capacity and hence RCR are lowered in the case of a low capacity of the phosphorylation system (adenine nucleotide transferase (ANT), phosphate carrier, ATP synthase). This is the case when ETC capacity exceeds OXPHOS capacity. In such a case, Gnaiger (2012) suggests that it may be better to use ETC capacity as the reference state for defining an index of uncoupling. The LEAK control ratio expresses the 'leakiness' of the mitochondria (Gnaiger, 2012).

1.2.4.4.3. Phosphorylation system control ratio (OXPHOS/ETC)

This ratio expresses how close partially coupled oxidative phosphorylation approaches the capacity of non-coupled electron transfer. If this ratio is smaller than one, this means that the phosphorylation system exerts control over OXPHOS capacity (Gnaiger, 2012).

1.2.4.4.4. ADP/O ratio

The ADP/O ratio measures the oxygen consumed to phosphorylate a determined amount of exogenous ADP and is an index of the efficiency of oxidative phosphorylation (Lesnefsky and Hoppel, 2006).

1.3. *C. elegans* as a model for aging research

Caenorhabditis elegans (Rhabditida) is a free-living soil nematode, feeding predominantly on bacteria and was chosen as a model organism for genetic studies by Sydney Brenner based on its short life cycle and generation time, transparency, high fecundity and simple cellular assembly (Brenner, 2003). These features allow for rapid, easy and inexpensive storage and culturing. Moreover, *C. elegans* has a fully sequenced genome and genetic alterations are easily introduced using various techniques, generating thousands of transgenic and mutant strains that are publicly available. The popularity of this small roundworm for research led to a vast amount of data that is made freely available on the internet (www.wormbase.com; www.wormsatlas.org; www.wormbook.org).

1.3.1. Life cycle and development

A population of *C. elegans* consists predominantly of hermaphrodites and only 0.2% are males. Males arise by the rare event of meiotic non-disjunction, leading to one X chromosome in diploid somatic cells while hermaphrodites have two. The mature hermaphrodite produces eggs for about four days and after this fertile period it lives for an additional 10-15 days. Self-fertilization leads to a progeny of approximately 300 nematodes and in the case male mating occurs, this number can increase to 1200-1400. After hatching, the L1 larva will arrest its development if no food is available. In the presence of food, the larva develops rapidly through four larval stages (L1 to L4) separated by molts. The rate of development depends on the environmental temperature. Under adverse conditions, such as limited food supply, overcrowding or higher temperatures, an alternative pathway in the lifecycle is chosen. Rather than molting to L3, the L2 larva will develop into a dauer stage, a hypometabolic non-eating larva with high fat content, able of enduring adverse conditions for a prolonged period of time. When conditions improve the dauer larva will exit the dauer stage and molt to an L4 larva and continue its normal life cycle and development (Braeckman et al., 2004) (Fig. 15).

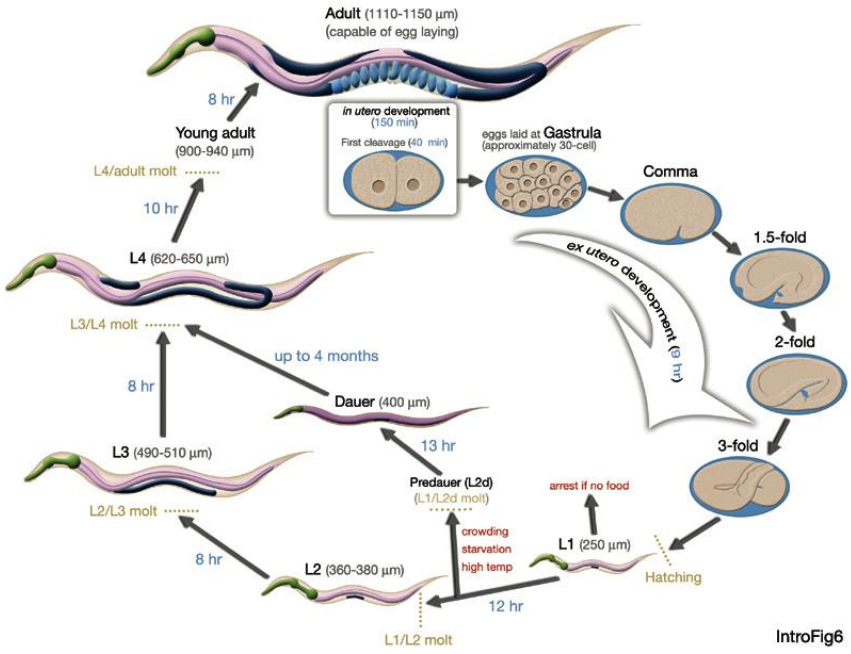

Figure 15: *C. elegans life cycle* (Wormatlas)

1.3.2. Mechanisms of lifespan extension in C. elegans

1.3.2.1. Dietary Restriction

1.3.2.1.1. Definition

Since its first description in the 1930's (McCay et al., 1935), dietary restriction or the reduction of nutrients without malnutrition is the only environmental intervention shown to extend lifespan in a wide variety of species ranging from yeast, worms and flies to rodents (McDonald and Ramsey, 2010). Mammals that are subjected to dietary restriction display delayed onset and reduced severity of age-associated diseases such as cancer, neurodegenerative and autoimmune diseases and diabetes. So, DR does not only prolong life of aged individuals, but maintains animals in relatively healthy and youthful state (Mair and Dillin, 2008). Two studies in non-human primates are ongoing and although lifespan data are still years away, preliminary results show that parameters as glucose intolerance and body composition indicate a similar delay in aging as found in rodents (McDonald and Ramsey, 2010).

For a long time, it was common belief that DR extends lifespan by reducing metabolic rate and consequently ROS production and damage as well (Lakowski and Hekimi, 1998). However, measurement of oxygen consumption and heat production rates indicated no changes or even an increase of metabolism occurred (Houthoofd et al., 2002b; Houthoofd et al., 2002c) and it was shown that the induced lifespan extension by some DR methods requires increased respiration (Bishop and Guarente, 2007; Schulz et al., 2007). Other mechanisms that were suggested to underlie the DR-mediated lifespan extension include a mitohormetic response and the Uncoupling-to-Survive Theory. Schulz et al. (2007) showed that glucose-restricted *C. elegans* displays increased ROS production, leading to the upregulation of oxidative stress defense mechanisms and extended lifespan and termed this mitohormesis. The Uncoupling-to-Survive theory postulates that lowering membrane potential limits ROS production and was suggested as a mechanism underlying the DR effect. Lemire et al. (2009) showed that membrane potential is indeed lowered in long-lived *C. elegans* mutants, including the *eat-2* mutant and concluded that these results support the Uncoupling-to-Survive theory. However, it is unclear whether the lowered membrane potential in these mutants leads to decreased ROS production. Using *in vivo* biosensors for H_2O_2, Back et al. (2012b) found a gradual age-dependent increase in H_2O_2 levels, which is significantly postponed by DR, but H_2O_2 levels in young worms were equal to fully-fed controls.

1.3.2.1.2. Chemosensation and dietary restriction in *C. elegans*

The chemosensory system of *C. elegans* is highly developed and enables it to detect volatile (olfactory) and water-soluble (gustatory) cues associated with food, danger or other animals. This is regulated primarily by the amphid chemosensory organ in the head region, containing eleven pairs of chemosensory neurons that penetrate the cuticle to expose their sensory cilia to the environment. Upon stimulation, the chemosensory neurons express secreted peptides of the TGF-β, the insulin and neuropeptide family that can act at a distance to affect both neuronal and non-neuronal cells. *C. elegans* chemotaxes to bacteria, its natural food source, by following both water-soluble and volatile cues. Most of the attractive compounds known are natural products of bacterial metabolism (Bargmann, 2006).

Chemosensory neurons have been shown to be involved in the DR response. Loss of SKN-1 in the ASI neurons impaired the response to dietary restriction (Bishop and Guarente, 2007). Furthermore, a neuropeptide-like protein expressed in anterior neurons, NLP-7, has been shown to be indispensable for DR-mediated longevity, as was the expression of TRX-1 in the ASJ neurons. The role of these genes is discussed in more detail in 1.3.2.1.5. The activation of these genes in the neurons results in signaling to peripheral tissues, such as the intestine, ultimately resulting in lifespan extension. Furthermore, it was shown by Smith et al. (2008) that dietary deprivation (DD)can extend lifespan when initiated at an age when animals no longer consume bacteria and it was suggested its mechanism relies on calorie restriction as well as reduced food sensing. Which neurons, signaling pathways and hormone signals mediate this effect is currently unknown. Possibly, the expression of nuclear hormone receptors (NHR) in the intestine may play a significant role in transducing the signal from the neurons to the intestine. NHR-49 has been shown to be involved in the starvation response (Van Gilst et al., 2005b), while NHR-62 has recently been identified as a mediator of the DR longevity response (Heestand et al., 2013).

1.3.2.1.3. The *C. elegans* intestine

The intestine represents a good candidate for mediating DR effects in *C. elegans* but the biology of digestion represents somewhat of a blind spot in an otherwhise well characterized organism (Walker et al., 2005). It is one of the major organs in *C. elegans* and is responsible for digestion and food uptake as well as synthesis and storage of macromolecules. *C. elegans* is a filterfeeder, taking up fluids containing suspended bacteria and spitting out the fluid while retaining the bacteria. The food particles are transported from the mouth to the intestine through the pharynx, which is responsible for the uptake, concentration and first digestion of the food particles by grinding the bacteria (Avery and Thomas, 1997). The intestine is a tubular structure and the intestinal cells have microvilli at their luminal side while the basolateral membrane is in contact with the pseudocoelomic cavity allowing transport of nutrients to tissues (Avery and

Thomas, 1997). In the intestine, a small number of lysozymes are expressed, probably secreted and responsible for the degradation of the bacterial cell wall, the next step in the digestion process. Furthermore, proteases and peptidases are highly expressed and the luminal pH is optimal for their activity (pH 5) (McGhee, 2007). Nutrients are taken up by the intestinal cells either by endocytosis or by dedicated transporters. Early studies found that the addition of precipitated material in semi-defined media improved nematode growth suggesting a role for endocytosis in nutrient uptake (Vanfleteren, 1974). These findings were visually confirmed when it was found that fluorescently labeled dextran or protein accumulates in the gut granules, suggesting fluid phase pinocytosis (Walker et al., 2005). Intestinal transporter proteins contribute to nutrient uptake as well. Sodium-coupled transporters are responsible for the uptake of di-/tricarboxylates such as succinate and citrate. PEP-2 mediates the H^+-dependent uptake of di-and tripeptides, leading to an acidification of the cellular cytoplasm. This acidification is counteracted by the activity of the NHX-2, effecting the electroneutral exchange of intracellular H^+ with extracellular Na^+. Deletion of these intestinal transporters has been shown to lead to longevity and it was suggested they induce a DR-like state (McGhee, 2007). Some genes important for pharynx development and function, such as *pha-4* and *eat-2*, or expressed in the intestine, such as *trx*-1, have been shown to play a role in DR-mediated longevity. Their role in DR is discussed in more detail in 1.3.2.1.5.

1.3.2.1.4. DR methods

In *C. elegans*, there are several ways to impose DR and they all extend lifespan to a different degree (reviewed in Greer and Brunet (2011)). The standard food source for *C. elegans* in laboratory cultures is the bacterium *Escherichia coli*, of which the OP50 strain is most often used (Brenner, 1974). The most straightforward DR method is reduction of the amount of bacteria, termed bacterial dilution, which can be conducted either in liquid (BDR) (Klass, 1977) or on solid agar culture (sDR) (Greer et al., 2007). Other plate methods are peptone dilution (PD) which leads to reduction of bacterial growth (Hosono et al., 1989), dietary deprivation (DD) or the total absence of bacteria (Kaeberlein et al., 2006) and intermittent fasting (IF), in which worms are fed only every other day (Honjoh et al., 2009). Mutation in the gene *eat-2* decreases food intake by reducing pharyngeal pumping rate and is often used as well (Avery, 1993; Lakowski and Hekimi, 1998). DR-like phenotypes are also induced when using chemically defined or undefined liquid media in the absence of bacteria, called axenic dietary restriction (ADR) (Vanfleteren, 1976). In addition, a number of chemical compounds, such as resveratrol, metformin and rapamycin, have been described to act as 'DR mimetics'. These compounds are of particular interest because they could help to overcome the difficulties of implementing dietary

restriction, resulting in the beneficial effects of DR without the need for diet limitation (Lee and Min, 2013).

1.3.2.1.5. Signaling pathways mediating the response to DR

As described above, there are several methods to dietary restrict *C. elegans* but the notion whether all of these methods converge on a common molecular pathway to extend lifespan or whether independent pathways are stimulated depending on the DR method used is only beginning to emerge. Next to description of the observed DR regulating genes, Fig. 16 attempts to provide an overview of the DR regulatory network.

Energy sensors: DAF-2, AAK-2, SIR2.1, TOR

Insulin levels are regulated by nutrients, which raises the possibility that insulin signaling may play a role in mediating the DR effect. *daf-2* is the *C. elegans* insulin/IGF receptor ortholog and a mutant of this receptor still displays lifespan extension in response to BDR, ADR (Houthoofd et al., 2003), sDR (Greer et al., 2007), *eat-2* (Lakowski and Hekimi, 1998) and DD (Kaeberlein et al., 2006) suggesting that insulin/IGF signaling is dispensable for this effect. The lifespan of one allele of *daf-2*, *daf-2(e1368)*, was not extended by an alternate *eat-2* mutation (Iser and Wolkow, 2007) and IF only mildly increases *daf-2* mutant lifespan (Honjoh et al., 2009). These results suggest that lifespan extension due to an *eat-2* mutation and IF may involve insulin signaling, but this pathway does not seem to be the major effector of DR-induced longevity.

The energy-sensing AMP-activated protein kinase (AMPK) is activated by low energy levels and other stimuli increasing the AMP:ATP ratio. DR has been shown to increase this ratio (Greer et al., 2007) and *aak-2*, one of the catalytic subunits of AMPK, was found to be necessary for the lifespan extending effect of sDR (Greer and Brunet, 2009; Greer et al., 2007), but not for the longevity induced by *eat-2* (Greer and Brunet, 2009) or IF (Honjoh et al., 2009). Lifespan extension via bacterial dilution shows a partial dependence on AAK-2 (Greer and Brunet, 2009; Mair et al., 2009).

SIR-2.1 is a NAD-dependent protein deacetylase of the sirtuin family and has been proposed to sense the cell's metabolic state via changes in NAD:NADH ratio (Guarente, 2005). The *sir-2.1* gene was found to be necessary for lifespan extension induced by the *eat-2* mutation in one study (Wang and Tissenbaum, 2006), while another study showed that this gene was not important for *eat-2* induced longevity (Hansen et al., 2007). SIR-2.1 is not necessary for lifespan extension induced by sDR (Greer et al., 2007), DD (Kaeberlein et al., 2006), IF (Honjoh et al., 2009) and BDR (Mair et al., 2009). Taken together, it cannot be excluded that SIR-2.1 is a possible node in the DR network, but its effect on DR-induced longevity is limited.

The target of rapamycin, TOR, is a nutrient-sensing molecule and a central regulator in cell growth and cell division (Stanfel et al., 2009). Reduction of the *C. elegans* TOR ortholog *let-363* by RNAi increases lifespan, but this increase is not further enhanced by the *eat-2* mutation, suggesting TOR involvement for the *eat-2* mediated effect (Hansen et al., 2007). However, this is controversial since Henderson et al. (2006) did not find this TOR involvement. TOR is also partially required for IF and sDR induced lifespan extension and knockdown of RHEB-1, a regulator of TOR is necessary as well for the effect of these DR methods (Honjoh et al., 2009). Recently, DAF-16 and SKN-1 were identified as downstream effectors of TOR signaling (Robida-Stubbs et al., 2012), emphasizing the importance of TOR as a central regulator of DR.

Transcriptional regulators: CBP-1, SKN-1, HIF-1, HSF-1, DAF-16 and PHA-4

A large number of transcriptional regulators have been shown to play a role in lifespan extension. The histone acetyltransferase CREB-binding protein, or CBP-1, was the first gene product shown to be completely indispensable for the ADR-mediated lifespan effect in *C. elegans*. Furthermore, this protein was found to be of importance in other DR regimens, such as *eat-2* and BDR, as well. Longevity of the insulin/IGF mutant *daf-2* was also shown to be dependent on CBP-1, suggesting that the importance of CBP-1 is not strictly specific to DR (Zhang et al., 2009).

The forkhead transcription factor FoxA/PHA-4 is important for pharynx development in worms (Mango et al., 1994) and was shown to be indispensable for lifespan extension induced by *eat-2* mutation or BDR (Panowski et al., 2007) but is not necessary at all for the effects of sDR and IF (Greer and Brunet, 2009; Honjoh et al., 2009). Upstream regulators of *pha-4* are found to be the TOR pathway (Sheaffer et al., 2008) and the E3 and E2 ubiquitin ligases *wwp-1* and *ubc-18*. *wwp-1* was found to be necessary for the longevity effects of sDR, BDR and *eat-2* (Carrano et al., 2009)

SKN-1 is an important transcription factor involved in the control of the oxidative stress response and because oxidative stress is considered a key cause of aging, it was proposed that SKN-1 may be a determining factor for aging (An and Blackwell, 2003). Indeed, loss of SKN-1 in the ASI neurons impaired the response to dietary restriction by BDR and *eat-2* mutation (Bishop and Guarente, 2007). However, SKN1 is not necessary for sDR- or IF-induced longevity (Greer and Brunet, 2009; Honjoh et al., 2009). Park et al. (2009) identified two downstream targets of SKN-1, NLP-7 and CUP-4, that are required for normal lifespan and appear to be partially necessary for sDR- and *eat-2*-induced lifespan extension. Interestingly, *cup-4* is necessary for upregulation of *pha-4* mRNA, suggesting that this gene is upstream of *pha-4* (Park et al., 2010).

The forkhead transcription factor FoxO/DAF-16 lies downstream of the Insulin/IGF pathway and is dispensable for longevity triggered by ADR (Houthoofd et al., 2003), *eat-2* (Lakowski and Hekimi, 1998), DD (Kaeberlein et al., 2006) and a sDR variant (Bishop and Guarente, 2007;

Panowski et al., 2007). The lifespan extension induced by IF and BDR were found to be partially dependent on DAF-16 (Greer and Brunet, 2009; Honjoh et al., 2009) and the effect of sDR was shown to be completely depending on DAF-16, which was found to act downstream of AAK-2 in this context (Greer et al., 2007).

The hypoxia inducible transcription factor, HIF-1, is critical for the response to low oxygen concentrations and is a downstream target of the TOR pathway. A loss-of-function mutation, *hif-1*, normally extends lifespan. Chen et al. (2009) showed that under sDR this lifespan extension is no longer present and that the upstream negative regulator of *hif-1*, *egl-9*, is partially necessary for longevity by sDR.

The heat shock transcription factor HSF-1 regulates the expression of many heat-inducible target genes, such as small heat shock proteins, and is involved in DR-induced lifespan extension by DD. It was suggested that it concerts its action by improving protein homeostasis (Steinkraus et al., 2008). Furthermore, it was found that *hsf-1* shortens lifespan in FF conditions.

Other genes: mitochondrial, oxidative stress, autophagy and other genes

CLK-1 is a mitochondrial protein involved in ubiquinone synthesis and the *clk-1* gene was found necessary for the longevity induced by the *eat-2* mutation and sDR (Greer and Brunet, 2009; Lakowski and Hekimi, 1998). The role of other mitochondrial proteins in DR-induced longevity is unclear.

trx-1 encodes a thioredoxin expressed in the ASJ neurons. Thioredoxins act as anti-oxidants by reducing disulfide bonds in targeted proteins. Furthermore, they were shown to be involved in the aging process, since their overexpression extends lifespan in *C. elegans* and *trx-1* deletion was proven to shorten adult lifespan combined with an increased sensitivity to oxidative stress (Jee et al., 2005; Miranda-Vizuete et al., 2006). Fierro-Gonzalez et al. (2011) showed that TRX-1 is indispensable for lifespan extension induced by the *eat-2* mutation, as well as by DD.

Autophagy induces the degradation of cellular components through the lysosomal machinery and can provide nutrients in times of scarcity. Downregulation of three autophagy genes, *bec-1*, *atg-7* and *vps-34*, using RNAi prevents lifespan extension induced by the *eat-2* mutant (Hansen et al., 2008; Jia and Levine, 2007). Since autophagy is regulated by TOR, it is well possible that a part of the TOR effect on DR-induced longevity may be effected by autophagy.

Four more dietary restriction response (*drr*) genes, *drr-1*, *drr-2*, *sams-1* and *rab-10* were identified from a genome-wide RNAi screen (Hansen et al., 2005). *sams-1* encodes S-adenosyl-methionine synthetase, an important enzyme in methionine metabolism. This gene is involved in *eat-2* and sDR induced lifespan extension (Ching et al., 2010; Hansen et al., 2005) and is

important for the lifespan extending effect of the DR mimetic metformin as well (Cabreiro et al., 2013). *rab-10* encodes a Rab-like GTPase and was also shown to be necessary for lifespan extension by the *eat-2* mutant, as are *drr-1* and *drr-2* (Hansen et al., 2005). Ching et al. (2010) identified *drr-2* as an ortholog of human eukaryotic translation initiation factor 4H (eIF4H) and confirmed the role of *drr-2* in DR mediated longevity using sDR. Furthermore, they established that DRR-2 acts downstream of TOR, SAMS-1 and RAB-10 and in parallel to PHA-4.

Conclusion

In conclusion, these studies suggest that different DR regimens induce mostly separate pathways. However, the genetic pathways are capable of extensive cross-talk and this suggest that rather than forming completely independent pathways, they may form a DR network (Greer and Brunet, 2011). As to why the various DR methods evoke different genetic pathways, it was suggested that some nutrients may be more limiting than others depending on the DR method. The timing of DR-initiation may play a pivotal role in the decision which pathways are invoked by a particular DR method, as well as tissue specificity: different methods may be sensed by different tissues (e.g. neurons or intestine) and thus require specific genes (Greer and Brunet, 2011).

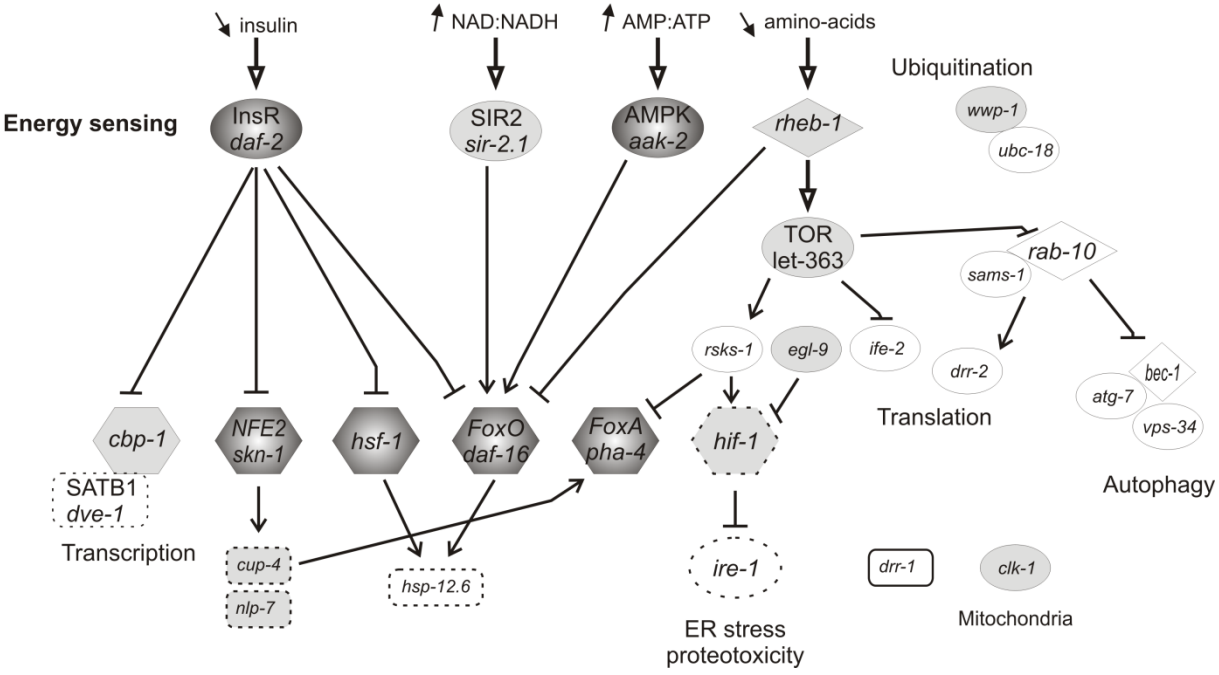

Figure 16: *C. elegans* DR network. Circles = enzymes; hexagons = transcriptional regulators; diamonds = small G proteins; rectangles = other proteins. Dark grey: genes tested in five to nine DR methods; light grey: genes tested in two to four DR methods; white genes: tested in one DR method. Dotted shapes represent genes partially necessary in all methods tested, or that have a similar effect on lifespan under DR and ad libitum conditions. Redrafted and adapted from Greer and Brunet (2011).

1.3.2.1.6. Axenic culture

Culture

One method to dietary restrict *C. elegans* is by culture in axenic medium, i.e. in the absence of *E. coli* bacteria. The basal medium consists of 3% soy peptone and 3% yeast extract. Since *C. elegans* is not capable of haem synthesis, supplementation with 0.05% haemoglobin is required (Vanfleteren, 1974; Vanfleteren, 1976) and to allow normal development rates in axenic medium, 20% skimmed milk is added (Houthoofd et al., 2002b). Lenaerts et al. (2008) established that, for normal lifespan, *C. elegans* has a nutritional requirement for metabolically active microbes or possibly an unidentified, heat-labile, non-soluble component preserved in live microbes. It was shown that the addition of growth arrested *E. coli* to an axenic culture rescued its effects and this was not the case when heat-killed *E. coli* was added. Since axenic medium is rich in peptides and amino acids, carbohydrates, vitamins and minerals, it may seem counterintuitive that culturing worms in this medium leads to such an extensive lifespan extension. However, *C. elegans* is a filter-feeder and worms take in fluids containing suspended particles and spit out the fluid while retaining the particles (Avery, 1993). The absence of suspended particles of bacterial size probably presents the worms with difficulties taking up nutrients from this medium, leading to DR. Although unconfirmed, it has been suggested that axenic culture induces a failure of endocytotic uptake of nutrients in the intestinal lumen (Vanfleteren, 1974; Vanfleteren, 1980). Possibly ADR-cultured worms experience cues of a rich nutritious environment but are unable of efficiently taking up these nutrients. Previously, it was shown that ADR-induced longevity is independent of DAF-16, DAF-2 and EAT-2 (Houthoofd et al., 2003) and that culturing *eat-2* mutants in axenic medium extends lifespan even further, suggesting that both DR methods act through separate molecular mechanisms. Recently, Zhang et al. (2009) established that ADR-mediated lifespan extension as well as the extension induced by other DR methods is completely abolished in the absence of CBP-1. This was the first report on a gene indispensable for ADR-mediated lifespan extension.

In table 1, the most studied DR methods and ADR are compared with a focus on the genetic aspect as well as biochemical data. This illustrates the need for further charachterization of the genetic pathways and downstream biochemical mechanisms underlying (A)DR mediated longevity.

Table 1: Comparing several DR methods: lifespan extension, genetics and biochemistry

DR methods							
	ADR	BDR	sDR	*eat-2*	IF	DD	GR
Lifespan % increase	50-150 %	32-101 %	18-35 %	0-57 %	30-57 %	42-50 %	
daf-16	I	I/PD	D	I	PD	I	
daf-2	I	I	I	D/I	PD	I	
eat-2	I		I			D	
aak-2		I/PD	D	I	I		D
age-1		I					
cbp-1	D	PD		D			
wwp-1		PD		D			
pha-4		D	I	D/I	I		
hsf-1		D		I	PD	D	
hif-1				I		I	
clk-1			D	D	Pd		
skn-1			I	D	I		
cup-4			PD				
nlp-7			PD				
sir-2.1		I	I	D/I	I	I	I
rheb-1					D		
Respiration	up	Up/no change		up			up
Heat dissipation	up			up			
ROS							Up (whole worms)
Mitochondrial bioenergetics							
References	[1-3]	[1, 3-8]	[7,9, 10]	[2,3,5, 7,8,10 -18]	[19]	[16, 18, 20]	[21]

Table 1: summarizing and comparing the most studied DR methods with focus on genes who's importance for longevity induced by specific DR regimens has been tested and that are of interest in this thesis as well. The effect on lifespan and on biochemical changes induced by DR are included as well. D, Dependent; PD, Partially dependent; I, Independent; The DR methods are ADR, axenic dietary restriction;

BDR, bacterial dietary restriction (liquid); sDR, solid dietary restriction; *eat-2*; IF, intermittent fasting; DD, dietary deprivation; GR, glucose restriction. [1] Houthoofd et al., 2003 [2] Houthoofd et al., 2002 [3] Zhang et al., 2009 [4] Klass, 1977 [5] Panowski et al., 2007 [6] Mair et al., 2009 [7] Greer et al., 2009 [8] Carrano et al., 2009 [9] Greer et al., 2007 [10] Park et al., 2010 [11] Lakowski et al., 1998 [12] Wang et al., 2006 [13] Hansen et al., 2005 [14] Iser et al., 2007 [15] Hsu et al., 2003 [16] Kaeberlein et al., 2006 [17]Chen et al., 2009 [18] Lee et al., 2006 [19] Honjoh et al., 2009 [20] Steinkraus et al., 2008 [21] Schulz et al., 2007. Redrafted after Greer and Brunet (2011);

Phenotype of axenically cultured C. elegans

ADR worms share the typical characteristic traits of worms subjected to other DR methods: slowed development, reduced body size and fecundity and prolonged lifespan (Houthoofd et al., 2002b; Vanfleteren, 1976) (Fig .17).

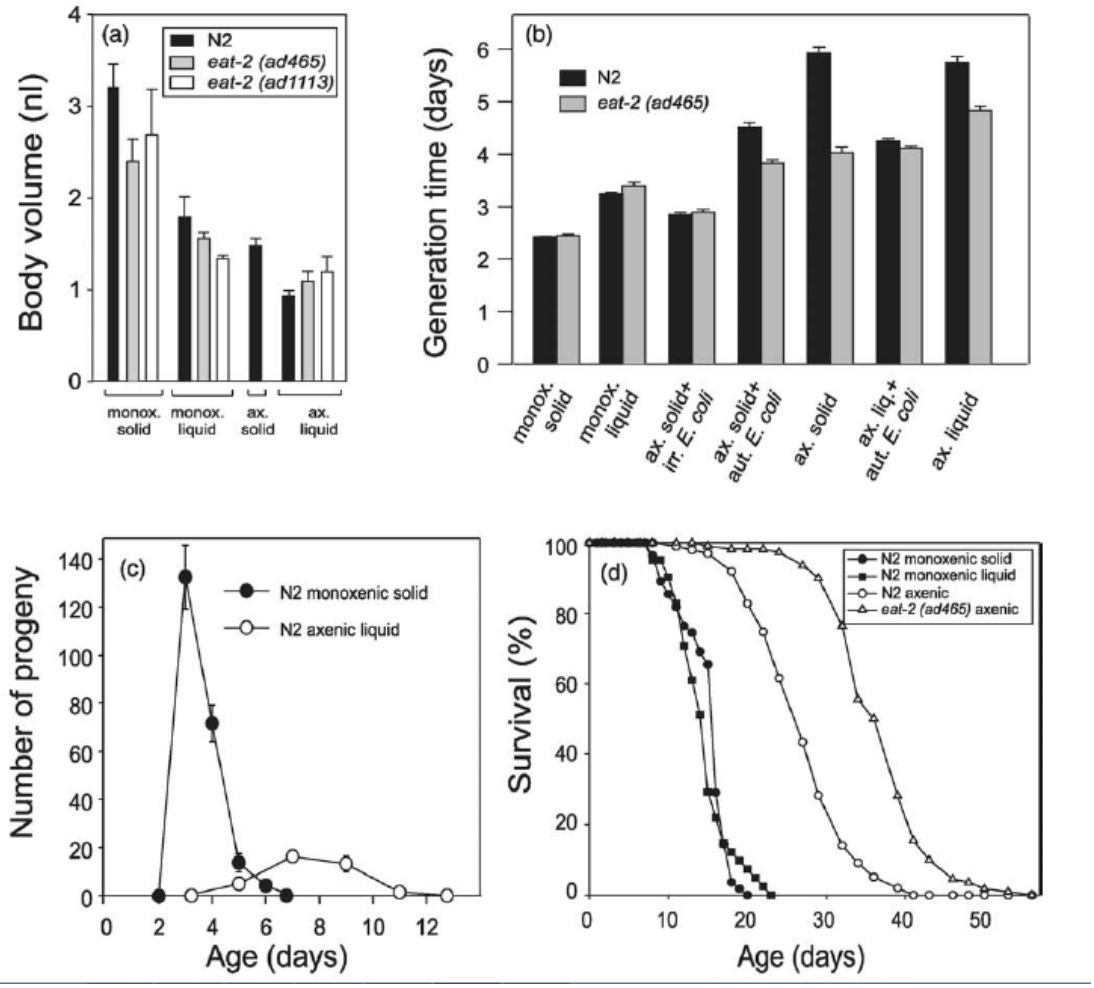

Figure 17: Effects of different nutritional regimes on size and life history characteristics at 24 8C. (a) Body volumes of adults, 2 days after the L4 to adult molt of wild type (N2) and two strains carrying mutant alleles in eat-2. Monoxenic solid indicates standard plate culture. Monoxenic liquid cultures contained $3x10^9$ E. coli cells/ml. (b) Generation times of N2 and eat-2 (ad465) raised under various culture conditions, measured as the time needed from hatching to the appearance of the first hatchlings of the next generation. Bacterial densities in liquid medium were: monoxenic 3 x 10^9 cells/ml, irradiated (irr) E. coli cells, 3 x 10^9/ml; autoclaved (aut) E. coli cells:3 x10^9/ml; monoxenic solid refers to standard plate culture; axenic solid þ irr/aut. E. coli: a concentrated slurry containing 3 x 10^9 treated cells for every ml of medium was distributed over the agar surface. (c) Fecundity of N2 grown on a lawn of bacteria and in axenic culture. Progeny were counted daily (bacterial culture) or every 2 days (axenic culture). (d) Life span of C. elegans on monoxenic plate culture, in monoxenic liquid culture and in axenic culture, at 24 °C.

Mean life span (days) ± SE (population size): N2 (monoxenic, solid): 14.4 ±0.1 (55), N2 (monoxenic, liquid): 14.0± 0.5 (41), N2 (axenic): 25.9 ± 0.6 (99), eat-2 (ad465): 35.7 ± 0.6 (110). (Houthoofd et al., 2002b)

However, when performing metabolic assays using isolated mitochondria (as in Chapter 3), large cultures are needed and worms are cultured in 250 mL fernbach flasks with aereation baffles and rotary shaking. It was noticed that the average adult lifespan of wild-type worms in such conditions can range from 5-12 days (Fig. 18) (Back et al., 2012b; Braeckman et al., 1999; Braeckman et al., 2002b; Depuydt et al., 2013; Houthoofd et al., 2002a; Houthoofd et al., 2002c).

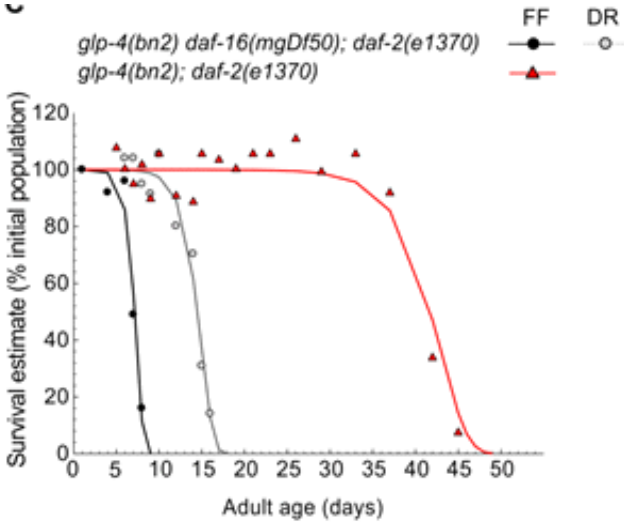

Figure 18: Representative lifespan curve of *glp-4 daf-16;daf-2* and *glp-4;daf-2* cultured in liquid fernbach cultures (Depuydt et al., 2013). *glp-4 daf-16;daf-2* has similar lifespan as WT worms and displays a mean lifespan in FF conditions of 6 days.

1.3.2.2. Other mechanisms of lifespan extension

1.3.2.2.1. The Mit phenotype

In *C. elegans*, several mutants with a defect in the ETC resulting in lifespan alteration were identified. Some mutations, such as *mev-1*, encoding the cytochrome *b* subunit of Complex II and *gas-1*, encoding a subunit of Complex I, reduce lifespan and are both hypersensitive to oxidative stress (Ishii et al., 1998; Kayser et al., 2001). Other mutations, such as *clk-1*, encoding a mitochondrial hydroxylase involved in ubiquinone synthesis (Lakowski and Hekimi, 1996), *isp-1*, encoding a Rieske iron sulfur protein (Feng et al., 2001), *gro1*, encoding a tRNA transferase (Lemieux et al., 2001) and *nuo-6*, encoding a subunit of Complex I (Yang and Hekimi, 2010a) cause lifespan extension. Furthermore, knock down of genes encoding subunits of all ETC complexes, except Complex II, using RNAi leads to lifespan extension as well (Dillin et al., 2002; Lee et al., 2003b; Rea et al., 2007). Mitochondrial (Mit) mutation and Mit RNAi leads to the same phenotype: the animals all display slowed development, decreased pharyngeal pumping, reduced defecation rates and small body size (Rea et al., 2007). The timing of administration of

Mit RNAi in order to have a lifespan extending effect was pinpointed to the developmental stages (Dillin et al., 2002). Mitochondrial respiration of some of the Mit mutants was found to be reduced (Dillin et al., 2002; Feng et al., 2001; Lee et al., 2003b; Yang and Hekimi, 2010b) while *clk-1*metabolic rate was not decreased (Braeckman et al., 2002a). It was suggested that decreased respiratory rate might lead to decreased ROS levels as the mechanism behind the lifespan extension of *isp-1* mutants (Feng et al., 2001). However, some long-lived Mit mutants (*isp-1*, *nuo6* and *clk-1*) actually display increased ROS levels (Lee et al., 2010b; Yang and Hekimi, 2010a) and it was shown that HIF-1 is necessary for this effect (Lee et al., 2010b). The mitochondrial unfolded protein response (UPR^{mt}) is a stress response initiated by the detection of unfolded or misfolded proteins, leading to the transcription of nuclear encoded genes such as chaperones that are targeted to the mitochondria to resore protein homeostasis. The UPR^{mt} increases *hsp-6* expression, was shown to be induced by mitochondrial respiratory chain defects and necessary for their lifespan extending effect, acting through unidentified signaling molecules (maybe ROS) and in different tissues (Durieux et al., 2011; Yang and Hekimi, 2010b) (Fig. 19).

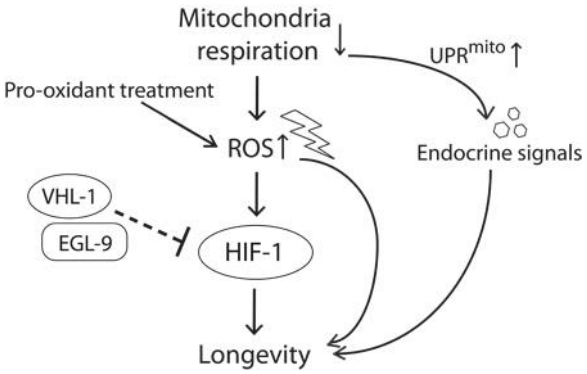

Figure 19: Model for lifespan extension by mild inhibition of mitochondrial respiration (Hwang and Lee, 2011)

1.3.2.2.2. The Insulin/IGF pathway as modulator of lifespan

age-1 and *daf*-2 were the first gerontogenes that were discovered and mutations in these genes were found to cause a twofold lifespan extension in *C. elegans* (Friedman and Johnson, 1988; Kenyon et al., 1993). Both gene products are part of the insulin/IGF pathway (IIS) (Fig. 20) which determines dauer formation, stress resistance and longevity and is evolutionary conserved (Kenyon et al., 1993; Partridge and Gems, 2002). The pathway is a phosphorylation cascade and its principal effect is to act on the downstream effector, the transcription factor FoxO/DAF-16. When the pathway is activated by the binding of insulin-like ligands to the receptor (DAF-2), DAF-16 is phosphorylated and inactivated leading to its relocation to the cytoplasm and preventing it from exerting its function. Reduced signaling through the pathway, either by environmental factors (e.g. nutrient availability) or mutations of genes involved in the pathway (such as the *daf-2* or *age-1* mutation), allows more unphosphorylated DAF-16 to reside

in the nucleus and initiate transcription of its target genes. These genes include stress response genes, antimicrobial and metabolic genes and a group of genes with unknown function (Lee et al., 2003a; McElwee et al., 2003; Murphy et al., 2003) and they all need DAF-16 for their transcription but can be regulated independently in response to environmental stressors (Hsu et al., 2003; Wolff et al., 2006). Together with the observation that nuclear localization of DAF-16 alone is insufficient for lifespan increase, this suggest that alternative pathways act in parallel to the ISS pathway (Daitoku and Fukamizu, 2007) (Fig. 20).

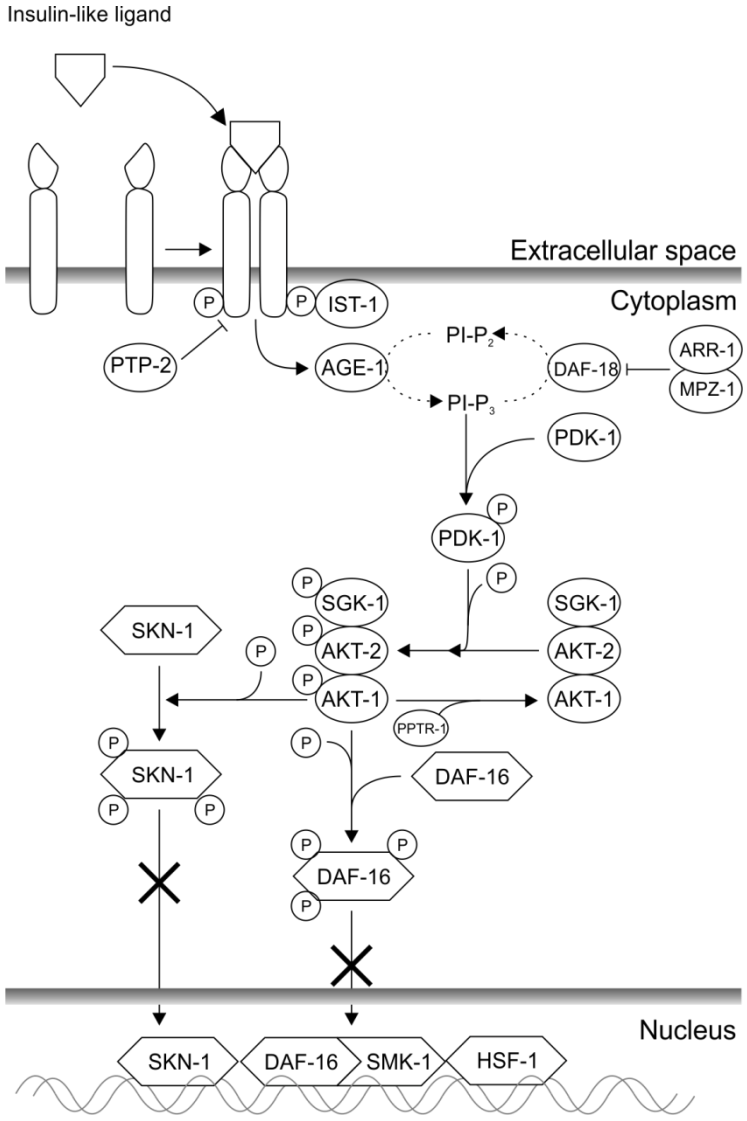

Figure 20: Overview of the Ins/IGF pathway in *C. elegans* (Depuydt, 2012).

1.3.3. Lifespan analysis

Traditionally, aging populations are evaluated by generating lifespan curves from which mean and maximum lifespan can be deduced. In these studies death is taken as a measure for aging.

Lifespan extending conditions, such as mutation in the Ins/IGF pathway or dietary restriction induce an increase in mean lifespan. Although mean and maximum lifespan are valuable parameters to compare different populations, they do not allow to distinguish whether longevity is caused by slowing down the rate of aging or by simply postponing the aging process. Therefore, age-specific mortality rate, containing information about age-specific changes, is a relevant measure of aging.

The exponential increase in morality rate with age is a typical characteristic of aging and can be expressed using the mathematical Gompertz model.

$$m(t) = A_0 e^{Gt}$$

In this formula m(t) is mortality rate, A_0 represents the age-unrelated death rate or initial mortality rate constant (IMC) and G represents the Gomperts mortality rate constant (GMC), expressing the age-specific increase in mortality. This formula can also be expressed as

$$\ln[m(t)] = \ln(A_0) + Gt$$

and yields a linear curve (Fig. 21).

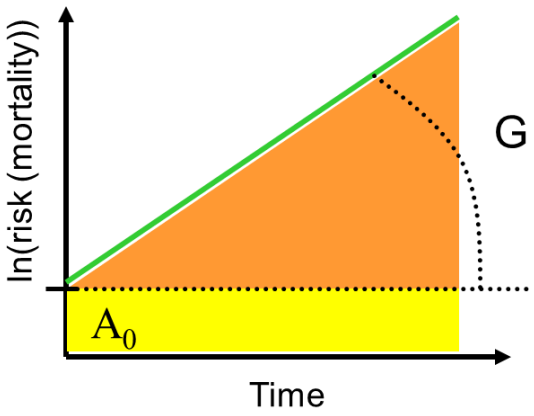

Figure 21: Representation of the linear curve generated by the Gompertz model. A_0 represents the age-unrelated death rate or initial mortality rate constant (IMC) and G represents the Gomperts mortality rate constant (GMC).

An increase in lifespan may result from either a delay in the onset of aging (delay in the increase of mortality with age) (Fig. 22 shift from the blue to the red curve) or from slowing the rate of physiological decline after its onset (a slowing of the rate of increase in mortality rate) (Fig. 22: difference in slope between the red and green curve) (Gems et al., 2002).

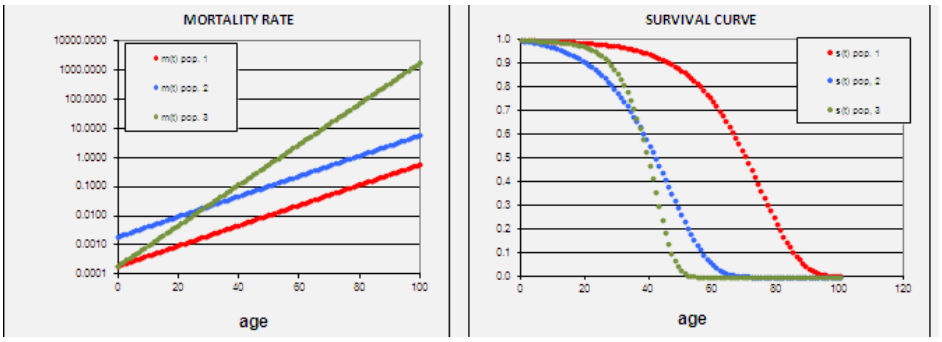

Figure 22: Representative curves presenting changes in mortality rate and the subsequent changes in survival curve (Braeckman, 2013).

However, the relationship between the slope of the survival curve and the slope of the mortality rate is not easily grasped by visual inspection (Masoro, 2006).

When assessing lifespan, little attention is being paid to whether or not lifespan extending conditions extend health span as well. Health-span studies in *C. elegans* have focused on age-associated decline characteristics and the reasoning is that if any of the age-related changes can be related to the causes of aging, then the change will occur either ealier (for short-lived strains) or later than in wild-type (WT) worms (for long-lived mutants). A number of neuromuscular changes have been examined in WT as well as long-lived and short-lived strains. The rate of body movements can be tracked on solid or in liquid media, correlates with deterioration of the body muscle wall and displays an age-related decline. Pharyngeal pumping rate as well has been studied as a phenotype that can be easily measured and shows age-related changes. However, signaling genes that affect healthy aging (assessed by prolonged swimming ability) do not necessarily show increased mean and maximum lifespan or reduced mortality rates. Only few studies have been done on older long-lived animals to examine if their extended lifespan results in extend health as well. Therefore, the connection between lifespan and healthspan needs to be further explored (Tissenbaum, 2012).

1.4. Aims and outline of the thesis

Dietary restriction by culture in axenic medium (ADR) induces a twofold lifespan extension. Despite the fact that its beneficial effects are well known, the underlying molecular mechanism of the ADR-mediated effects remains unclear. ADR and other dietary restriction worms display increased metabolic rates, suggesting a link between dietary restriction and metabolism. The Free Radical Theory of Aging depicts an essential role for mitochondria in the aging process. Since mitochondria harbor important energy providing pathways, we hypothesize that their function may be affected by ADR to induce lifespan extension. In this study, we aimed to 1) examine the putative role of energy metabolism in ADR-mediated longevity and 2) identify the molecular mechanism underlying the ADR-mediated effect.

In **Chapter 2**, the objective was to determine how energy metabolism is affected by culture in axenic medium. Axenic dietary restriction has been shown to induce energy expenditure but analysis of differentially expressed genes using microarray pointed towards downregulation of many metabolic genes in axenic medium (Szewczyk et al., 2006). Attempting to resolve these conflicting data, we describe the observed changes in transcript abundance patterns of 49 genes playing a role in energy metabolism, using a qRT-PCR approach.

Chapter 3 aims to further elucidate the effect of ADR on mitochondrial aerobic energy production. We hypothesized that the reduced nutrient uptake may be compensated by more efficient mitochondria. We use high resolution respirometry, supplemented with established metabolic assays and BN-PAGE electrophoresis, to generate a detailed impression of the effects of ADR on the respiratory system of young worms and try to explain why the observed changes occur. In addition, changes in bioenergetic competence and supercomplex organization with age are evaluated.

In **Chapter 4,** the purpose is to resolve the question which signaling pathways are involved in ADR mediated longevity. The molecular mechanism underlying DR-mediated lifespan extension was unknown for a long time but recently, successful efforts were made to elucidate these pathways. However, for ADR-mediated lifespan extension, the role of only few genes was established. We try to identify more genes underlying the ADR-mediated effect. This was done by a large screen for genes that affect lifespan under ADR conditions.

Chapter 5 provides a general discussion of the results with the emphasis on the role of metabolic changes and ROS in dietary restricted *C. elegans*, supplemented with some perspectives for future research.

PART II

Results

Chapter 2. Dietary restriction by growth in axenic medium induces discrete changes in the transcriptional output of genes involved in energy metabolism in Caenorhabditis elegans.

Personal contribution: Experimental work

Data analysis

Published as :

Dietary restriction by growth in axenic medium induces discrete changes in the transcriptional output of genes involved in energy metabolism in *Caenorhabditis elegans*

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Abstract

Dietary restriction increases life span in a wide range of species, including the nematode worm *Caenorhabditis elegans*. The mechanism by which it does so remains largely unknown, although it is commonly thought that a reduction of ROS plays a pivotal role. More specifically for *C. elegans*, it has been proposed that food restriction reduces energy expenditure, possibly in conjunction with an anaerobic shift in energy production, with consequent reduction in the formation of ROS. We have measured differential transcript abundance of 49 genes known to play roles in energy metabolism in axenic culture medium, which causes a nutritional deficit and leads to a substantial increase of life span. We found no evidence for a reduction in metabolic rate or a shift to anaerobic metabolism in axenic culture. Major changes induced by growth in axenic medium include downregulation of lipid degradation and upregulation of glyoxylate cycle activity glyceroneogenesis and, possibly, gluconeogenesis. The activities determined in worm extracts for pyruvate kinase, phosphoenolpyruvate carboxykinase and isocitrate lyase followed a similar trend. We conclude that growth in axenic culture is marked by a general upregulation of replenishing pathways.

Keywords: *Caenorhabditis elegans* · Dietary restriction · Energy metabolism · Transcription profile

2.1. Introduction

The nematode *C. elegans* is widely used as a model organism for studying aging. Numerous genes were identified that, when mutated or silenced by RNAi treatment, cause increased longevity (Age phenotype). Genes of the nematode insulin/IGF-1 signaling pathway and genes involved in mitochondrial bioenergetics constitute two major classes of life span controlling genes giving rise to the Ins/IGF-1 and Mit classes of longevity mutants. Rea and Johnson (2003) proposed a hypothesis that unifies the effects of these two classes of genes on longevity. They suggested that most, if not all, life span extending mutations in *C. elegans* induce anaerobic shifts in energy metabolism with consequent reduction in the formation of toxic reactive oxygen species (ROS), in agreement with the free radical theory of aging (Harman, 1956).

C. elegans is usually grown on agar plates seeded with a lawn of *E. coli* cells as a food source, but the animals can also be propagated in axenic (no other species present) liquid medium consisting of a complex mixture of chemically defined constituents (CbMM (Buecher et al., 1966) or CeMM (Lu and Goetsch, 1993; Szewczyck et al., 2003)) or undefined medium composed of 3% soy peptone, 3% yeast extract and 0.05% hemoglobin (Vanfleteren, 1999). Culture of *C. elegans* in axenic medium also generally results in a 50-80% increase in life span relative to monoxenic culture conditions (Vanfleteren, 1999; Walker et al., 2005). It is generally accepted that this effect is due to food restriction since axenic culture also causes delayed development, greatly reduced fertility, reduced body size and lean appearance although it has not been demonstrated that axenic medium causes these effects solely by imposing dietary restriction (Houthoofd and Vanfleteren, 2006; Lenaerts et al., 2008; Walker et al., 2005). Although a genetic pathway mediating the effects of food restriction was recently discovered (Bishop and Guarente, 2007; Panowski et al., 2007), virtually nothing is known about the downstream biochemical processes that extend survival.

The antiaging effects of dietary restriction were commonly ascribed to reduced energy expenditure with a consequent reduction in ROS formation (Beckman and Ames, 1998; Roth et al., 2004; Sohal and Weindruch, 1996). Heilbronn et al. (2006) showed that calorie restriction induced a decrease of energy production in humans although there was no unequivocal effect on markers of oxidative stress. Increased energy expenditure is not necessarily linked to increased ROS production, however. Although ROS production and the inner mitochondrial membrane potential are positively correlated, both are inversely related with respiratory activity: high in resting mitochondria and low in actively respiring mitochondria (Brand, 2000; Nicholls, 2004). In fact, the effects of dietary restriction on metabolic rate, oxidative stress and life span may be more complex and greatly species-specific. Rea and Johnson (2003) and Rea (2005) suggested that a shift to anaerobic metabolism underlies the increased longevity seen in long-lived mutants

of *C. elegans* with reduced insulin/IGF-1 signaling or mitochondrial function by virtue of decreased ROS production. Clearly, food restriction mediated life span could also be explained by this hypothesis.

We have previously shown that axenic culture increased energy expenditure of *C. elegans* as measured by oxygen consumption and heat production (Houthoofd et al., 2002b). Similar findings were reported for animals subjected to dietary restriction by bacterial food dilution (Bishop and Guarente, 2007) and glucose restriction by exposure to 2-deoxy-D-glucose (Schulz et al., 2007). However microarray analysis of genes differentially expressed between axenically and monoxenically grown worms seemed to indicate that many metabolic genes are downregulated in axenic culture (Szewczyk et al., 2006).

In an attempt to resolve these conflicting data we measured the transcript abundance patterns for 49 genes that play roles in energy metabolism using a quantitative RT-PCR (qRT-PCR) approach. We found no evidence for a decrease in the transcriptional output of genes involved in aerobic respiration or a shift to anaerobic metabolism caused by growth in axenic culture, but our findings suggest that this nutritional regime increases the flux through replenishing pathways including glyceroneogenesis and, possibly, gluconeogenesis.

2.2. Materials and methods

2.2.1. Strains and culture methods

The nematode strain *glp-4(bn2ts) daf-16(mgDf50)* was used in this study. Gravid wild-type (N2) animals contain many more embryonating eggs in monoxenic than axenic culture. *glp-4*(bn2ts) animals are germline defective and long-lived when raised from eggs at the restrictive temperature (24 °C). This effect is suppressed by mutation in the gene *daf-16*, but the longevity effect of dietary restriction is still retained in the double mutant. The *E. coli* strain K12 was used as a food source as it is more suitable for growing bulk cultures of *C. elegans* than the uracil auxotroph OP50 which is the standard food source in *C. elegans* research. Nematode populations were scaled up by growth on nutrient agar (Oxoid, Basingstoke, England) plates and treated with hypochlorite and NaOH to isolate eggs (Sulston and Hodgkin, 1988). The eggs were washed with S buffer (0.1 M NaCl, 0.05 M potassium phosphate, pH 6.0) and treated once more with the alkaline hypochlorite mixture (and more extensively washed to remove all traces of bleach) to guarantee perfect sterile conditions. The eggs were allowed to hatch overnight in S buffer at 24 °C. The resulting synchronized L1 larvae were concentrated by centrifugation and added to Fernbach flasks containing approx. 250 ml axenic or monoxenic culture medium at 1000 worms/ml.

Axenic basal medium (200ml) contained 3% soy-peptone (Sigma-Aldrich, St Louis, MO, USA) and 3% yeast extract (Becton-Dickinson, Franklin Lane, NJ, USA). Axenic basal medium (200 ml) was autoclaved under standard conditions (15 psi, 15 min) and, after cooling, supplemented with 2.5 ml hemoglobin (bovine, Serva, Heidelberg, Germany) solution, 0.125 ml cholesterol solution (1 g/100 ml ethanol) and 50 ml skimmed milk (UHT sterilized). The hemoglobin supplement was added from a 100x stock in 0.1 N KOH that was autoclaved for 10 min. For monoxenic culturing, the L1 larvae were added to 250 ml S buffer containing 5 µg/ml cholesterol and 10^{10} E. coli cells/ml. This concentration was maintained constant by monitoring the absorbance at 550 nm and adding fresh E. coli cells as needed.

The cultures were grown at 24 °C with rotary shaking (120 rpm). This temperature drastically repressed germ-line proliferation (*glp-4* phenotype) thereby abolishing progeny production. The animals were harvested when they reached day 2 of adulthood. They were cleaned for RNA extraction by flotation on dense sucrose followed by several washes in S buffer (Braeckman et al., 1999). Both axenic and monoxenic cultures were grown in parallel four times generating 4 independent biological replicates to account for biological variation.

2.2.2. Quantitative PCR

RNA was extracted using the RNeasy Midi (Qiagen, Venlo, The Netherlands) kit according to the manufacturer's instructions. All samples were treated with DNase (Zymo Research, Orange, CA, USA). First strand cDNA was synthesized using an oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Fermentas, Vilnius, Lithuania) at 42 °C for 1 h.

Full-length gene sequences were extracted from WormBase [http://www.wormbase.org] and primers were designed by the Primer3 software [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi] and tested for specificity using NCBI BLAST. The targets amplified by the primer pairs were evaluated with MFOLD software [http://www.bioinfo.rpi.edu/applications/mfold/] in order to check for the formation of secondary structures at the site of primer binding. Primers were purchased from Invitrogen. Primer and amplicon information is listed in Tables 1 and 2.

Quantitative RT-PCR was carried out using a Rotor-Gene 2000 centrifugal real-time cycler (Corbett Research, Mortlake, Australia) using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA) as described in detail in Hoogewijs et al. (2008). A single melt peak for each reaction confirmed the identity of each PCR product. Each assay included a no-template control for every primer pair.

2.2.3. Data analysis

The threshold cycle (Ct) values of the Rotor-Gene software version 6.0 (Corbett Research) were exported to qBase version 1.3.5, a free program for the management and automated analysis of qPCR data (Hellemans et al., 2007) for further analysis. All measurements were produced in duplicate or triplicate, and for each primer set, reaction efficiency estimates were derived from standard curves that were generated using serial dilutions of a cDNA pool of all nematode samples. These were then used by qBase to transform the Ct values to relative quantities, which were normalized using the geometric mean of three reference genes identified by the geNorm 3.4 software (Vandesompele et al., 2002). The geNorm VBA applet for Microsoft Excel determines the most stable reference genes from a set of genes (Table 2) in a given panel of cDNA samples. Next, the normalized expression levels were converted into logarithmic values to calculate the mean expression per nematode sample and its 95% confidence interval. Finally, all values were linearized again using a power function, and plotted in a graph. Differential gene expression was considered significant when the 95% confidence interval of the mean expression levels did not overlap (equivalent to $P < 0.05$).

2.2.4. Enzyme assays

Two ml microcentrifuge tubes containing approx. equal amounts of frozen animals, glass beads (0.248-0.318 µm) and 50 mM Na/K-phosphate buffer (pH 7.0) were shaken at 5000 strokes/min in a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA) for 1 min. Chaps was added at 1% final concentration to the resulting homogenate and the mixture was kept on ice for 15 min and centrifuged at 14,000 rpm for 10 min. The supernatant was next cleared by a second centrifugation step.

The enzymes were assayed against the appropriate blanks at 25 °C in microtiter plate wells using the Spectramax 190 plate reader (Molecular Devices, Wokingham, Berkshire, UK). Isocitrate lyase was assayed using the method of Dixon and Kornberg (1959). This assay measures the increase in absorbance at 324 nm by glyoxylic acid hydrazone ($\varepsilon 324 = 1.7x104$) formed by isocitrate and phenylhydrazine in the presence of isocitrate lyase. Pyruvate kinase activity was assayed according to the method of Bergmeyer et al. (1974), following the Sigma protocol. This assay monitors the decrease at in A340nm consequent upon the formation of lactate and NAD from pyruvate and NADH (ε 340= 6,220) by lactate dehydrogenase, in conjunction with the formation of pyruvate and ATP from phosphoenolpyruvate and ADP by the action of pyruvate kinase. Aconitase was assayed in a coupled reaction with isocitrate dehydrogenase by monitoring the rate of NADPH (ε340 nm= 6.220) formation from NADP. Phosphenolpyruvate carboxykinase catalyzes the conversion of oxaloacetate to phosphoenolpyruvate formed is converted to pyruvate, and then to lactate. The consumption of

NADH and the consequent decrease in A340nm by the former reaction was monitored to assay PEPCK activity.

All enzyme activities were scaled to the protein concentration of the worm extracts, estimated by the bicinchoninic acid (Pierce, Rockford, IL, USA) method (Smith et al., 1985).

Table 2 Primer sequences for metabolic genes

Enzyme	Coding sequence	Forward primer	Reverse primer	Size (bp)
GLYCOLYSIS				
Hexokinase	Y77E11A.1	GTGCGACGAGTACTTTCTCAACTG	CTAGAGATGACGTCACACACTTCTC	230
	F14B4.2	GAGTAGAGGGTGTTGATGTTGTCAC	GGTACCTGTTCCCACAATGACT	167
	H25P06.1	GGTGTCTACAGTAATACCCGACAGA	GTTCTCGAATCTTCTCGCACAC	247
Glucose-6-phosphate isomerase	Y87G2A.8 (a+b)	CTCTGTCTCAAGACGCCACATTTG	CGAGACGCATCAGAAGTTGGAATG	206
	Y87G2A.8 (b)	GCTCGGCTACCTTTCTGACCTAATTG	CTCGAATCTAGCTGGATCCTTCTC	171
Phosphofructokinase	Y71H10A.1	GATATCTGGCTCTAGTTGCTGCTCT	CTTCTCCTTGACAGCAGTCTTCAC	221
	C50F4.2	GGCACAGAAGCTGAAAGAAGAG	GAGACTGTGCAGTCATAACAAGACC	224
Fructose-1,6-bisphosphate aldolase	F01F1.12 (a)	GTGCTCTTCAAGCTTCTGTGCTCAAG	GGAATGCTTGGCAACGAAGAGAG	175
	T05D4.1	GCACAAGATCTCATCTACGACTCC	GAGAGCGTGGTACACATATGAGAG	198
Triosephosphate isomerase	Y17G7B.7	GGAGAACATTGTGATCGCCTAC	CAGCAGTCACAGATCCTCCATA	173
	C33D9.9	CGTTCATCGCCTGCTAGAGATA	GTTCCACTAGAACTATGGGAGTTGG	161
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	T09F3.3	GGACGACATCAAGAAGGTAGTCAAGG	AGGTCGACAACACGGTTCGAGTATC	215
	K10B3.8	CTCCATCGACTACATGGTCTACTTG	AGCTGGGTCTCTTGAGTTGTAGAC	151
Enolase	T21B10.2 (a)	CAGCCTTCAACGTCATCAATGG	GTGGCATCGAGTCCATATCTCTTC	171
	T21B10.2 (a+b)	TCCATCTGGAGCTTCTACTGGAGTTC	GAAGTCGTCAATGTCCTTCTGTGC	174
Pyruvate Kinase	F25H5.3	GCTTTCTGGAGAGACTGCTAAAGG	GGCAGCGATAGCAATAGTATGAGAC	168
	ZK593.1	AGCGTGTAGTGATGTAGAGACACTG	TCAGGTCCTTTGGTGTCAAGAG	176
GLUCONEOGENESIS				
Pyruvate Carboxylase	D2023.2	TCCAACTACTCCTCTTGCTACTGAC	GTGATCATACATCCTGGTCTACTGC	167
Phosphoenolpyruvate carboxykinase	R11A5.4	GACGGAAGACTCTACGCTATCAAC	CAGAAGTACTCTCCGTTAGCAGTCT	151
Fructose-1,6-bisphosphatase	K07A3.1	GCTCTCTATGGATCTGCTACTATGG	GTGTACGAATATACTCGGCGAAACC	195
Glucose-6-phosphate translocase	F47B8.10	TACTACTGGGACTTGTCACTTGTGC	GTTCCGTAGGTAGACTTGGAGTACC	168
CITRIC ACID CYCLE				
Pyruvate Dehydrogenase E1α	T05H10.6	CCACCTATAGATACCATGGACACTC	GAGAACACCGTCTGATGTAGCA	223
Pyruvate Dehydrogenase E1β	C04C3.3	GCTCAGTACGACGGAGCTTACAAG	CTCCATTTGGTCCACGGAATACG	267
Citrate Synthase	T20G5.2	CTCGACAACTTCCCAGATAACC	GGTACAGGTTGCGATAGATGATAGC	202
Aconitase, mitochondrial	F54H12.1	GATCAACCTCGACACTCTTACTCC	GGTCTTAGCCTTGAGTCCCTTATC	219
Aconitase, cytosolic	ZK455.1	GATAGCCGTACCATTGACTACCTC	GGAGACACTTGGCACTACACTTC	176
Isocitrate Dehydrogenase (NAD)	F43G9.1	CTGCCCTCTACCCAGATATCAAGTTC	CAACAAGACCAGCGCACAAATC	146
Isocitrate Dehydrogenase (NADP)	F59B8.2	GTCTTCGACTTCAAAGGTCCAG	GCCTCATATTCTGGGTAGATCTCAG	199
Ketoglutarate Dehydrogenase	T22B11.5	GGTTTGGAGAGAACACTGAAGG	GCAGACTGTGTATTCTCCTTGTCC	248
Succinyl CoA Synthetase	C05G5.4	GTTAAGTCGCGTCTTCTCAAGC	GTAGTCTGGTGCACAGCTTCATAG	178

Succinate Dehydrogenase (iron-sulphur protein)	F42A8.3	CGGAAGTGTCATTCAGTGAGAG	GAGTTCGTCAAAGGTGGTCTTG	157
Succinate Dehydrogenase (cytochrome b)	T07C4.7	GGAACATCGATCGTCACCAAGTC	GATTCTATGGAATCCGGAGAGCATC	158
Fumarase	H14A12.2	CCAGTTATCCACGCTTTCGGAATC	CATGTTGGACTGAGTTCCTGAACC	182
Malate Dehydrogenase (organelle)	F20H11.3	CTCGTGACGATCTCTTCAACAC	GTCATAGACACCAGCCTTCTTGAG	161
Malate Dehydrogenase (cytosolic)	F46E10.10	GTGGAGGAGTCATCATTGAGAAGAG	CGTCGAAGCTAAGACCTTGAAC	227
LIPID HYDROLYSIS				
Lipase	F28H7.3	CAGCTTCTATGCTAGCCAGTTACAC	GTGTATCCAGAGCACACATCGT	187
Glycerol Kinase	R11F4.1	GCAGATGAAGCTATCTCGAGAACTGC	GTAAGCTTCCAGATGAGCCAAGTGTC	196
Glycerol-3-Phosphate Dehydrogenase	K11H3.1	GCTGCTGGATTTACTGATGGTC	CACCTTACGATTACGACCTCCATAG	189
	F47G4.3	GATCAGCCATCGCTTGTGTAGTTG	TGTGGGACGACTAGGATCAGAATG	252
FATTY ACID OXIDATION				
3-Hydroxyacyl-CoA Dehydrogenase	F54D5.7	CACGTGTCACAGAAGCTTACAGAAC	CTGATCCTCACTTCCGTAGTTGTAG	239
Enoyl-CoA Hydratase	F38H4.8	GAGCTCTATCAAGGAAACTCGGTTG	GTTTATCAGACCCGCTCTCTGTAGT	213
Acyl-CoA Synthase	C46F4.2	GGAGACTATCACTGGAGAAGCTATG	GAACTGCTTCGTCTCCAAGAGTAG	208
GLYOXYLATE CYCLE				
Isocitrate Lyase/Malate Synthase	C05E4.9	GACTACGAGGCTGGAAGAACGATTG	GTAGGCGAACATCTTGTCTGGGTAC	200
ANAEROBIC PATHWAYS				
Malic Enzyme	Y48B6A.12	GAGCAACAAGCCTACCGTATCATCAC	ACAATTGCTCGGACGTTCTCTGTC	298
Cytosolic SDH/Fumarate reductase	F48E8.3	CTATGTGGTGGGCATTCTGTACCAC	GCTTCTGGATTCTCCGATTGCTTC	136
Lactate Dehydrogenase	F13D12.2	GAGAGAAGACTGACAACGAACACTG	GAACGACTGGAAGAGAAAGGTAGAC	231
Alcohol Dehydrogenase	K12G11.3	GAAGGAGCTGGAAGTGTTGTTC	CTCCACGTATAGTGAGGTACTCCTG	202
PENTOSE PHOSPHATE SHUNT				
6-Phosphogluconolactonase	Y57G11C.3	CTCCACCATCTCGTATCACACTTAC	GAGAGTCAGAATCCGACGAATC	228
Ri(bul)ose-5-Phosphate Isomerase	B0280.3	CTGATGAAGTTGACGGACAGTTCAC	GTAGAAGTGGTTGAGCAGCAAGAG	191
Transketolase	F01G10.1	CTTCTCGGAGATGAAGTACGATGTCG	CATCGATGAAGTTCAAGCGAGGAG	202
Transaldolase	Y24D9A.8 (a+b)	GCTCTTCAACTTTGAGCAAGCAGTC	AGTAACACTGACAACTCCTGGATCG	150
	Y24D9A.8 (a)	ATCAGTCGTTGTTGCGGGATACTG	CTTGGAGCACTTCTTGATGTCCAC	181

Table 3 primer sequences for reference genes

Gene	Coding sequence	Forward primer	Reverse primer	Size (bp)
act-1	T04C12.6	GCTGGACGTGATCTTACTGATTACC	GTAGCAGAGCTTCTCCTTGATGTC	114
ama-1	F36A4.7	CCTACGATGTATCGAGGCAAA	CCTCCCTCCGGTGTAATAATG	139
mlc-3	F09F7.2	GAGTTCAAGCGTAAGGGAGAGAAG	GATCTTTCCAGTCTCCTCCTTGTC	147
csq-1	F40E10.3	AACTGAGGTTCTGACCGAGAAG	TACTGGTCAAGCTCTGAGTCGTC	111
mua-6	W10G6.3	CAAAGGAGAACACTCCAGCAGAAC	GATTGGAATGGGTGGAGGATGTAG	219
pat-10	F54C1.7	GACGGAAAGCTTCACGAAGTTC	CCTTCGTAAACTGATCCGCAAG	140
tba-1	F26E4.8	GTACACTCCACTGATCTCTGCTGACAAG	CTCTGTACAAGAGGCAAACAGCCATG	194
unc-15	F07A5.7	GAAGAAGAAGTACCAGGCTGAGATCG	CGTTGAGTATCCTCCAATGAGGCTTG	144

2.3. Results and discussion

We selected 49 genes that encode enzymes involved in various pathways of energy production including glycolysis, gluconeogenesis, the citric acid and glyoxylate cycles, the pentose phosphate pathway, oxidation of fatty acids and anaerobic pathways of energy production (Fig. 1). qRT-PCR identified several genes that showed differential transcript abundance in axenic culture medium (Fig. 2).

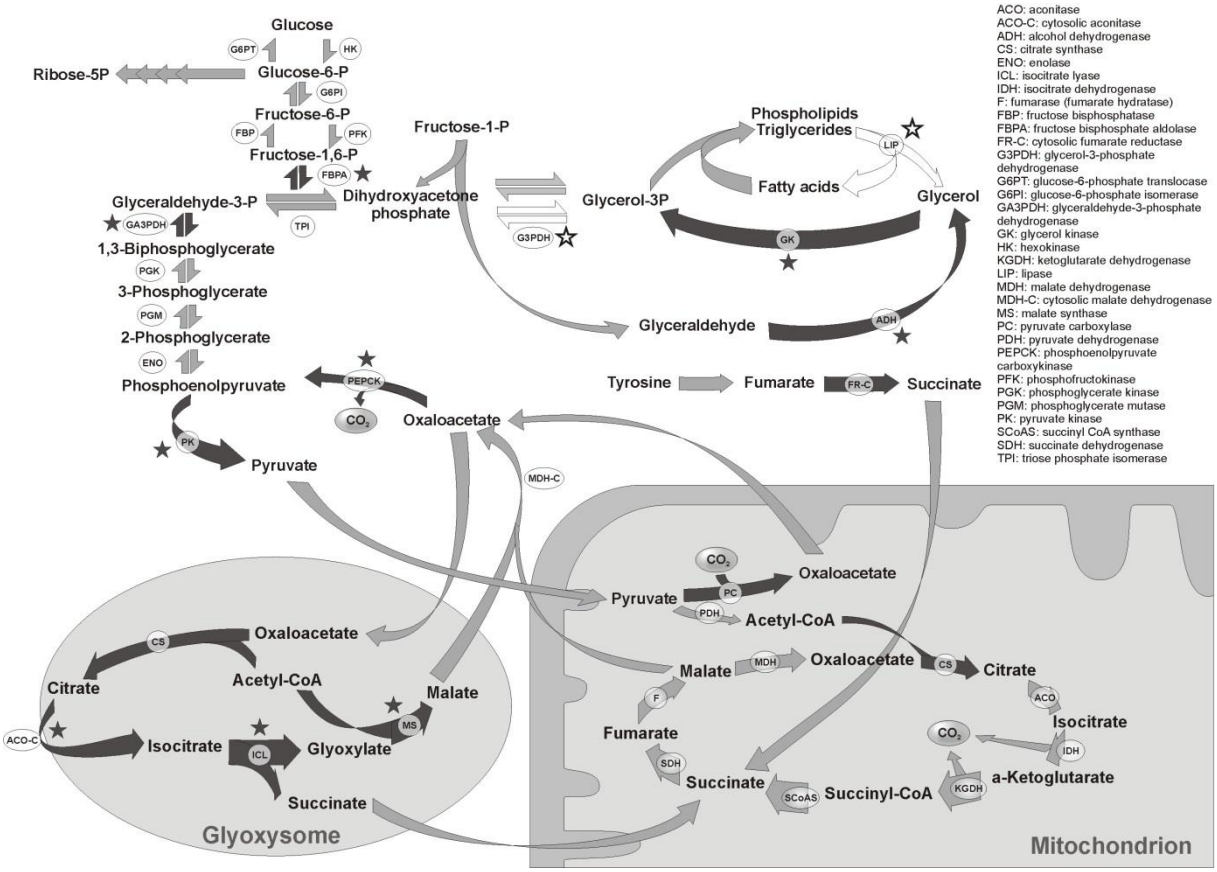

Figure 1: Diagram of metabolic pathways discussed in this study. Up-regulation in black, down-regulation in white; reactions that showed no changes or were not studied are depicted in gray tone. Stars indicate that at least one gene encoding this enzyme was significantly up- or down-regulated.

The transcripts for hexokinase, glucose-6-phosphate isomerase, phosphofructokinase, triosephosphate isomerase and enolase showed no significant alteration. ZK593.1, which encodes a pyruvate kinase isoenzyme, and T05D4.1, encoding a fructose-1,6-bisphosphate aldolase isoenzyme were upregulated in axenic medium (Fig. 2a). There are 4 genes encoding glyceraldehyde-3-phosphate dehydrogenase. gdp-1 (T09F3.3) and gdp-4 (F33H1.2) are nearly identical and encode minor GAPDH isoenzymes. Their expression (both transcripts cannot be distinguished) was reduced in axenic medium but the difference was not significant. gdp-2 (K10B3.8) and gdp-3 (K10B3.7) encode the major GAPDH isoenzymes and are also nearly identical (indistinguishable transcripts). We found that the abundance of transcripts correlated

with these genes was elevated in axenic culture (Fig. 2a). Since pyruvate kinase catalyzes the ultimate and irreversible reaction in glycolysis, upregulation of its expression would be predicted to facilitate enhanced flux through this pathway. Direct measurement of the enzyme activity in worm extract did not reveal any significant change of activity in response to the nutritional regime, however (Fig. 3). Taken together these results confirm that axenic culture does not affect, or possibly increases, the flux through glycolysis.

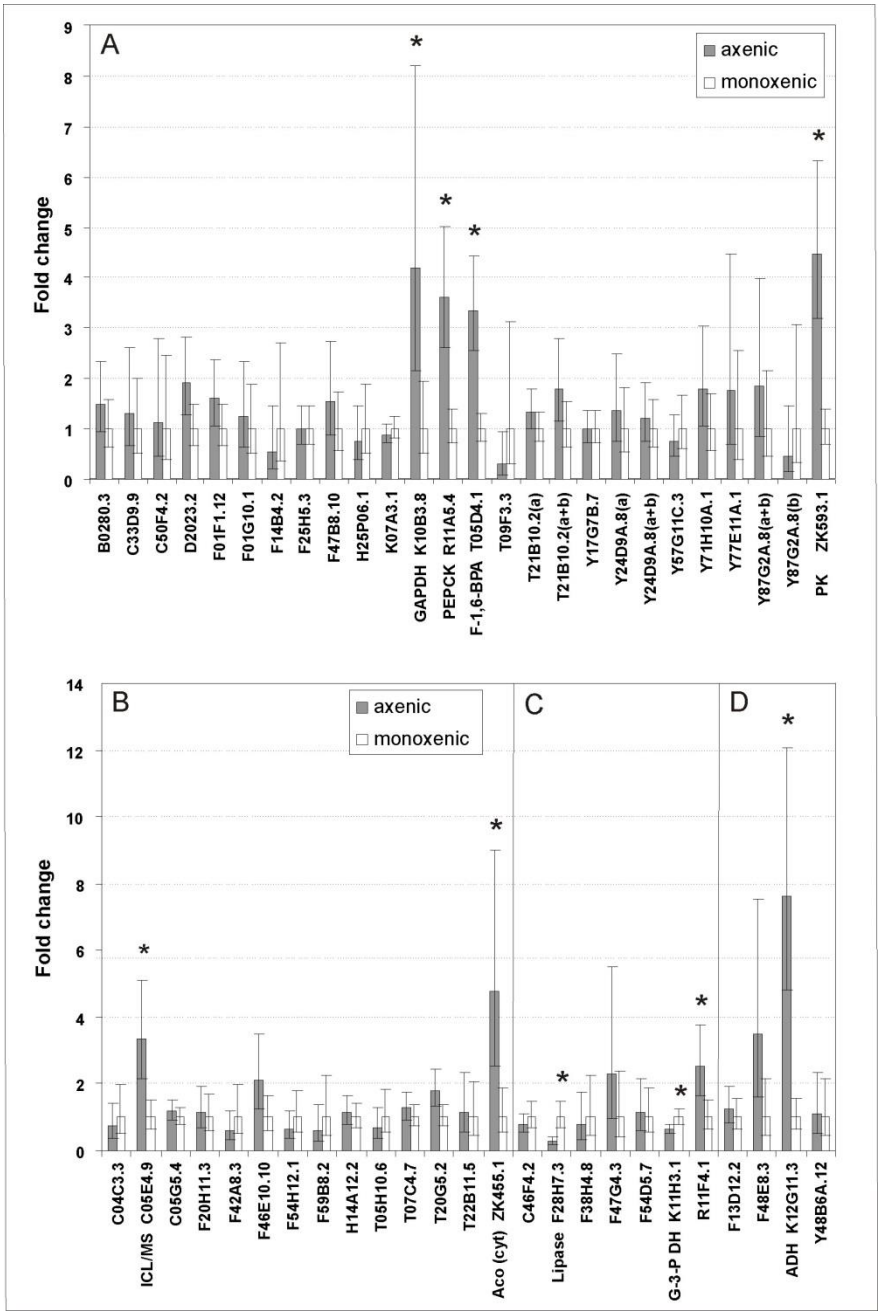

Figure 2: Differential transcription regulation of 49 metabolic genes in response to culture in axenic medium. The profiles are derived from four independent experiments where axenic and monoxenic worms were grown in parallel culture (quadruple biological replicas) and each cDNA sample was run in duplicate or triplicate (duplicate/triplicate technical replicas). The expression levels of these genes in both monoxenic and axenic medium were first normalized using the geometric mean of the three most stable

reference genes. These were: for biological replica 1: *unc-15*, *mua-6* and *tba-1*; for replica 2: *mua-6*, *unc-15* and *csq-1*; for replica 3: *pat-10*, *act-1* and *ama-1*; for replica 4: *pat-10*, *act-1* and *mua-6*. It should be noted that the differences in expression stability among these reference genes as determined by geNorm were very small. (A) Genes encoding enzymes that are involved in glycolysis, gluconeogenesis and the pentose phosphate pathway. (B) Genes of the citric acid and glyoxylate cycles. (C) Genes functioning in lipid metabolism. (D) Genes functioning in anaerobic pathways. The y-axis indicates transcript abundances in axenic relative to monoxenic culture. The bars indicate the 95% confidence interval of the mean (i.e., $P<0.05$ for non-overlapping intervals). * = $P < 0.05$.

Expression of R11A5.4, a gene encoding phosphoenolpyruvate carboxykinase was significantly upregulated in axenic culture (Fig. 2a). The activity level of this enzyme tended also to be higher in animals raised in axenic culture (Fig 3). Pyruvate carboxylase (D2023.2) was borderline upregulated and the expression of fructose-1,6-bisphosphatase (K07A3.1) and glucose-6-phosphate translocase (F47B8.10) was unchanged (Fig. 2a). Together these data suggest that animals grown in axenic medium upregulate a pathway to replenish important carbohydrate compounds for intermediary metabolism.

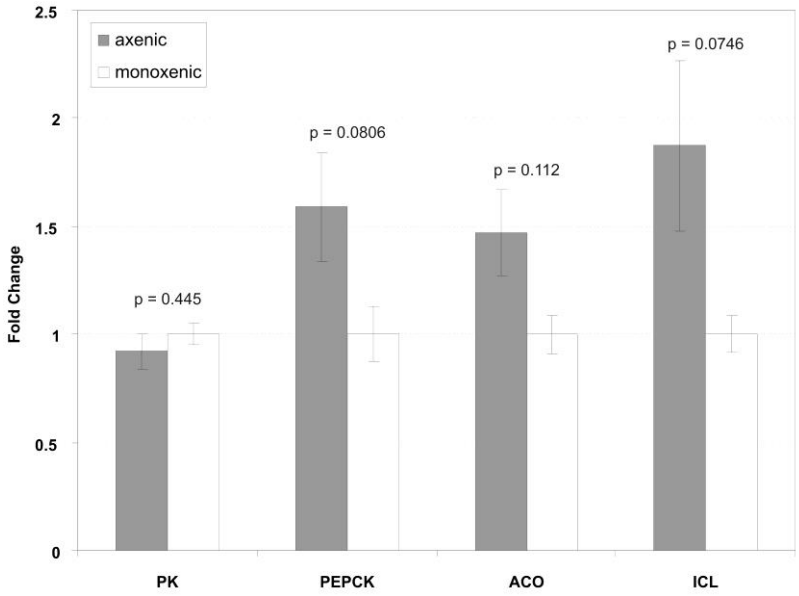

Figure 3: Activities of pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), NADPH dependent aconitase (ACO) and isocitrate lyase (ICL) in extracts from *C. elegans*. Error bars represent SEM; the p values were obtained using a two-tailed t-test.

The pentose phosphate pathway generates ribose-5-phosphate for the synthesis of nucleotides and reducing power in the form of NADPH, which is needed for numerous anabolic reactions. None of the 4 enzymes assayed (Y57G11C3.2, B0280.3, F01G10.1, Y24D9A.8) were differentially expressed (Fig. 2a).

Two genes (T05H10.6, C04C3.3) encoding subunits of pyruvate dehydrogenase, which links glycolysis to the citric acid cycle via conversion of pyruvate to acetyl-CoA showed no change in expression (Fig. 2b). Citrate synthase (T20G5.2) was slightly upregulated (Fig. 2b). Since this is a

key rate-limiting enzyme within the citric acid cycle and the expression of the other citric acid cycle genes was unchanged these data point to upregulation of citric cycle activity in axenic medium, consistent with earlier analysis of oxygen consumption by animals raised in axenic culture (Houthoofd et al., 2002b). Interestingly mRNA levels of cytosolic aconitase (ZK455.1) were also higher in axenic culture (Fig. 2b). Consistently, we measured higher NADP dependent aconitase activity extracts from axenic worms, although the increase was not significant (Fig. 3). Cytosolic aconitase catalyzes the conversion of citrate to isocitrate which can enter the glyoxysomes where it is cleaved to succinate and glyoxylate by isocitrate lyase. Malate synthase condenses glyoxylate with acetyl-CoA to form malate. Both key enzyme activities of the glyoxylate cycle are joined in a single bifunctional enzyme in *C. elegans*. The transcript of one encoding gene (C05E4.9) was also enriched by culture in axenic medium (Fig. 2b). Taken together these data strongly suggest that glyoxylate cycle activity is upregulated by culture in axenic medium. This conclusion was strengthened by assaying the enzymatic activity for isocitrate lyase, although the increase was not significant at the 95% level (Fig. 3). Malate produced by this bifunctional enzyme can be transported to the cytosol and converted to oxaloacetate for further synthesis of glycerol-3-phosphate and derived compounds, and gluconeogenesis.

Three enzymes of fatty acid oxidation (encoded by F54D5.7, F38H4.8 and C46F4.2) did not show altered mRNA levels in axenic culture. Animals raised in axenic medium expressed reduced levels of lipase mRNA (F28H7.3). This is not surprising since axenic medium contains low amounts of lipids. A need for enhanced synthesis of lipid compounds in this medium is shown by the upregulation of glycerol kinase (R11F4.1) which catalyzes the phosphorylation of glycerol to glycerol-3 phosphate. This compound is required for the synthesis of phospholipids and triacylglycerols (lipid biosynthesis). Glycerol-3 phosphate dehydrogenase catalyzes the interconversion of glycerol-3-phophate and dihydroxyacetone phosphate . One isoenzyme (K11H3.1) was downregulated, but another glycerol-3-phosphate dehydrogenase isoform (F47G4.3) was up in 3 out of 4 biological replicates (Fig. 2c). This might indicate that these isoenzymes are expressed in different compartments (tissues or cells) favoring the synthesis of glycolytic intermediates or glycerol derived compounds as needed. In conclusion these data point to a decrease of lipid degradation and recycling of glycerol for de novo biosynthesis of phospholipids and triacylglycerols.

Many parasitic nematodes have evolved anaerobic pathways in addition to oxidative phosphorylation for ATP production [30]. These pathways are also activated in the dauer stage of *C. elegans* (Burnell et al., 2005; Holt and Riddle, 2003)and their existence prompted Rea and Johnson (2003) and Rea (2005) to propose that a shift to anaerobic metabolism underlies the

increased longevity seen in many, if not all, long-lived mutants by virtue of decreased ROS production. Since axenic culture also increases life span substantially (Houthoofd et al., 2002b; Vanfleteren, 1999) we wondered if these anaerobic pathways would also be increased by growth in axenic medium. We measured a higher transcript abundance for cytosolic fumarate reductase (F48E8.3) in axenic animals, although the difference was not significant due to large variation between the biological replicas (Fig. 2d). This enzyme catalyzes the conversion of fumarate to succinate in a reaction that consumes two electrons (FADH2). In the cytosol, fumarate can be produced from tyrosine by tyrosine aminotransferase. Succinate can enter the mitochondrion where it is converted to malate via the citric acid cycle, and malate can be transported back to the cytosol for conversion to oxaloacetate and use in gluconeogenesis/glyceroneogenesis in an anabolic process that recycles the carbon atoms from tyrosine.

Alcohol dehydrogenase (K12G11.3) was strongly upregulated in axenic culture (Fig. 2d). This enzyme can reduce acetaldehyde produced by pyruvate decarboxylase to ethanol. However, the *C. elegans* genome does not seem to encode pyruvate decarboxylase (Holt and Riddle, 2003). A more likely hypothesis is that this enzyme converts glyceraldehyde to glycerol for further phosphorylation and subsequent phospholipid and triacylglycerol production. Alcohol dehydrogenase catalyzes this reaction (Voet et al., 2006) and this explanation is consistent with the upregulation of glycerol kinase (R11F4.1).

2.4. Concluding remarks

We found no evidence for a decrease of overall metabolic activity caused by growth in axenic culture. In particular, our mRNA profiles implied either no change, or up-regulation of the flux through glycolysis and the citric acid cycle mediated by increased transcript abundance of pyruvate kinase and citrate synthase. Our results also implied reduced lipid degradation, which tallies with the low levels of lipid in axenic medium, and enhanced glyoxylate cycle activity, glyceroneogenesis and, possibly, gluconeogenesis in axenic culture. We interpret these changes as adaptations to replenish important carbohydrate compounds of intermediary metabolism. Interestingly, a recent study reported that overexpression of a cytosolic form of PEPCK (PEPCK-C) in skeletal muscle retarded aging and increased metabolic rate, physical activity and life span in the mouse (Hakimi et al., 2007). At the cellular level, overexpression of PEPCK-C enhanced glyceroneogenesis, mitochondrial biogenesis and the flux of intermediates though the citric acid cycle. A comparable re-patterning of energy metabolism also occurs in insulin/IGF-1 mutants of *C. elegans* and leads to greater longevity (McElwee et al., 2006). Thus it may possible that such metabolic re-patterning is a conserved mechanism of longevity assurance but more research will have to point out whether this really is the case.

We considered whether growth in axenic culture could induce anaerobic shifts of energy production. Axenic culture appeared to induce increased abundance of cytosolic fumarate reductase and alcohol dehydrogenase mRNA was substantially elevated. At a first glimpse these changes are consistent with the hypothesis that a shift to fermentative metabolism is the predominant means of life span extension in *C. elegans* (Rea and Johnson, 2003). We do not believe that such shift underlies the extension of life span in axenic culture, however. Indeed, we found no evidence of lactate fermentation and the absence of a gene encoding pyruvate decarboxylase which is needed for the formation of acetaldehyde (a substrate for alcohol dehydrogenase) from pyruvate casts doubts about potential upregulation of ethanol fermentation caused by upregulation of alcohol dehydrogenase. On the contrary we assume that increased abundance of this enzyme functions in an anabolic pathway contributing to glyceroneogenesis.

Our mRNA profiles deviate substantially from those reported by Szewczyk et al. (2006) [supplementary material online]. The reason for this discrepancy is not clear. Poor agreement between SAGE and microarray results has been reported (Griffith et al., 2005; Ruzanov et al., 2007; van Ruissen et al., 2005). qRT-PCR is often used to validate these global transcription platforms but also reveals considerable variation between biological replicates. Improved probe design, normalization and use of sufficient biological replicates may improve agreement between the various approaches. Szewczyk et al. (2006) used a chemically defined medium (CeMM), but we have noticed very similar phenotypes of development, reproduction, size and life span using chemically defined medium and axenic medium where soy peptone and yeast extract substitute for the amino acids and vitamins/growth factors complement of chemically defined medium, respectively (unpublished information). Thus it is unlikely that this difference is relevant. Szewczyk et al. (2006) compared animals grown in liquid (CeMM) culture with standard agar plate cultures whereas we used liquid monoxenic culture as a reference. In addition, these authors used the wild-type strain N2, which carries more developing eggs on agar plates, and apparently prepared RNA from mixed stage populations where we used the synchronized germline-defective *glp-4 daf-16* adults. It is possible that these experimental differences contribute to the poor agreement between both studies.

Furthermore, it is important to realize that an upregulation in transcriptional profiles does not necessarily relate with an increased protein abundance. Proteomic versus transcriptomic analysis have been shown not to correlate entirely (Depuydt et al., 2013; Schwanhausser et al., 2011). In order to confirm the importance of the observed results, an additional proteomics study would be interesting but such studies are labour-intensive and expensive.

2.5. Acknowledgements

We thank David Gems for providing the strain *glp-4(bn2ts) daf-16(mgDf50)*. This work was supported by Ghent University (12050101), the F.W.O.-Vl (G.0025.06) and the European Community (LSHM-CT-2004-512020).

Chapter 3. Mitochondrial efficiency is increased in axenically cultured *Caenorhabditis elegans*

Personal contribution: Experimental work and design

Data analysis

Writing the manuscript

Submitted as:

Mitochondrial efficiency is increased in axenically cultured *Caenorhabditis elegans*

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Abstract

Culturing *C. elegans* in axenic medium leads to a twofold increase in lifespan and considering the similar phenotypical traits with dietary restricted animals, it is referred to as axenic dietary restriction (ADR). The free radical theory of aging has suggested a pivotal role for mitochondria in the aging process and previous findings established that culture in axenic medium increases metabolic rate. We asked whether axenic culture induces changes in mitochondrial functionality of *C. elegans*. We show that ADR induces increased electron transport chain (ETC) capacity, enhanced coupling efficiency and reduced leakiness of the mitochondria of young adult worms but not a decrease of ROS production capacity and *in vivo* H_2O_2 levels. The age-dependent increase in leak respiration and decrease in coupling efficiency is repressed under ADR conditions. Although ADR mitochondria experience a decrease in ETC capacity with age, they succeed to maintain highly efficient and well-coupled function compared to fully fed controls. This might be mediated by combination of a limited increase in supercomplex abundance and decreased individual Complex IV abundance, facilitating electron transport and ultimately leading to increased mitochondrial efficiency.

Keywords: *Caenorhabditis elegans*; bioenergetics; mitochondria; axenic; dietary restriction; ROS

3.1. Introduction

In the mitochondrial electron transport chain (ETC), complexes I-IV couple electron transfer between electron donors, such as NADH and $FADH_2$, and oxygen to the transport of protons from the mitochondrial matrix to the intermembrane space, establishing an electrochemical gradient harvested by ATP synthase. Usually, this process is well-coupled but some protons may leak across the mitochondrial membrane back to the matrix without contributing to ATP production. The function of this leak is not entirely understood, although it has been suggested to play a role in modulation of reactive oxygen species (ROS) production (Brand, 2000). The free radical theory of aging proposes that mitochondria are the main source and prime target of ROS and it is a widely held view that this might cause or at least contribute to the aging process (Harman, 1956; Harman, 1972). Recently, a growing body of evidence argues against this theory (reviewed in Back et al. (2012a)).

Dietary restriction (DR), the reduction of nutrients without malnutrition, extends lifespan in a wide variety of species (Masoro, 2005). In the nematode model *Caenorhabditis elegans*, several ways to impose dietary restriction have been described (Greer and Brunet, 2009). While the standard food source of *C. elegans* consists of bacteria, it is possible to culture these worms in sterile axenic medium as well (axenic dietary restriction; ADR) (Vanfleteren, 1976). ADR worms share the typical characteristic traits of worms subjected to other DR methods: slowed development, reduced fecundity and prolonged lifespan (Houthoofd et al., 2002b; Vanfleteren, 1999). Despite the fact that the beneficial effects of DR are well established, the underlying molecular mechanism is still unclear. Although it was first hypothesized that DR may slow down energy metabolism, it was shown that this was not the case in *C. elegans*. On the contrary, ADR worms display higher respiration rates, heat dissipation rates and stress resistance (Houthoofd et al., 2002b). Increased respiration was confirmed for other DR methods and was shown to be indispensable for the longevity phenotype (Bishop and Guarente, 2007; Houthoofd et al., 2002c; Schulz et al., 2007). Do these high metabolic rates result in increased ROS production in DR animals? Schulz et al. (2007) showed indeed that ROS production is increased under glucose restriction leading to a mitohormetic stress response which is responsible for lifespan extension. On the other hand, by using *in vivo* biosensor analyses, our group could not detect increased H_2O_2 levels in young worms raised in diluted bacterial suspension, but instead the age-dependent increase in H_2O_2 was significantly delayed under these conditions (Back et al., 2012b). Several *C. elegans* studies pointed out that DR induces changes in the expression of genes involved in energy metabolism. Although not always in agreement, these studies clearly indicate that DR and energy metabolism are closely interlinked (Castelein et al., 2008; Mouchiroud et al., 2011; Szewczyk et al., 2006; Yuan et al., 2012).

Since ADR causes a twofold lifespan extension and considering the link between DR and metabolism, we aimed to further elucidate its effect on mitochondrial function. We hypothesized that 1) mitochondria from ADR worms would be more efficient in order to compensate for the reduced nutrient uptake and 2) that they experience an attenuation of age-induced changes similar to *daf-2* mutant mitochondria (Brys et al., 2010). We used high resolution respirometry, supplemented with established metabolic assays and Blue Native Polyacrylamide gel electrophoresis (BN-PAGE), to generate a detailed impression of the effects of ADR on the respiratory system.

We found that ADR increased the capacity of the electron transport chain (ETC), enhanced coupling efficiency and reduced leakiness of the mitochondria of young adult worms. The age-dependent increase in leak respiration and decrease in coupling efficiency is repressed under ADR conditions. Although ADR mitochondria experience a decrease in ETC capacity with age, they succeed to maintain highly efficient and well-coupled function compared to fully fed controls, that might be helped by a slightly increased higher order organization of ETC units into supercomplexes.

3.2. Materials and Methods

3.2.1. Strains and culture conditions

Wild type worms contain more embryonating eggs in monoxenic than in axenic culture. Therefore we chose to work in the sterile *glp-4(bn2*ts*)* background. As germline-deficient mutants may show altered lifespan due to activation of the FOXO transcription factor DAF-16 (Hsin and Kenyon, 1999), we added the null allele *mgDf50* in our control strain. The lifespan extension from ADR is independent of DAF-16 (Houthoofd et al., 2003). Control strains similar to our *glp-4(bn2ts) daf-16(mgDf50)* have been used in previous studies and other groups (McElwee et al., 2004; TeKippe and Aballay, 2010). For determination of *in vivo* H_2O_2 levels, the strain *jrIs10[unc119(+) rps-0p::roGFP2-0rp1]*, constructed in a similar way as described in Back et al. (2012), was crossed into our control background *glp-4(bn2*ts*) daf-16(mgDf50)*. Mitochondrial density was estimated using our reporter strain *jrIs5[unc-119(+) rps-0p::mls::Dendra2]*.

Culture conditions were similar as described previously (Castelein et al., 2008). To avoid contamination, 100 µg/mL ampicillin was added to both ADR and fully fed (FF) cultures. The animals were harvested at the second day of adulthood. They were cleaned by flotation on dense sucrose followed by three washes in S-buffer.

For the aging series, young worms were sampled on the second day of adulthood, while other sampling dates were chosen at 50% survival of the FF animals. For these experiments, three independent cultures were sampled. Due to high contamination risk and required worm mass, we were able to take only one sample when ADR worms reached 50% survival.

3.2.2. Mitochondrial isolation

Mitochondria were isolated as described in Brys et al. (2010). Mitochondrial protein content was determined using the Bradford assay and mitochondria were diluted to a concentration of approximately 10 µg/µL protein unless otherwise stated. Fresh mitochondria were immediately used for respiratory measurements, ATP and ROS production assays and determination of membrane potential. The remaining mitochondria were aliquoted, frozen at -75°C and used for quantification of protein content , Western blotting and 2D BN-PAGE.

3.2.3. Oxidative phosphorylation measurements

Mitochondrial respiration was monitored polarographically using a Clark-type electrode (Oxygraph 2k, OROBOROS INSTRUMENTS, Innsbruck, Austria). An aliquot of 250 µg mitochondria was incubated in 2 mL of air-saturated MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA, pH 7.1) at 24°C.

The sequential titration protocols were as follows:

1) Respiration was prompted by the addition of 5 mM of the NADH-linked substrates pyruvate and malate (Complex I (CI) substrates) or 10 mM succinate (Complex II (CII) substrate) with 2 µg rotenone (CI inhibitor): this represents the classical state 2 (st2), also called LEAK(st2)

2) To obtain classical state 3 (st3) respiration, ADP was added (25 µM final concentration (f.c.)).

3) After consumption of all ADP and return to the classical state 4 (st4) or LEAK(st4), a saturating ADP concentration (300 µM f.c.) was added to stimulate maximal oxidative phosphorylation (OXPHOS), here designated as OXPHOS capacity.

4) To assess the intactness of the outer mitochondrial membrane cytochrome c (10 µm f.c.) was added.

5) Inhibition of ATP synthase by the addition of oligomycin (Omy; 1 µg/ml f.c.), results in respiration rates representing LEAK(omy).

6) The chemical uncoupler FCCP was titrated in 0.5 µM steps to obtain uncoupling and ETC capacity.

7) The protocol for CI fueled respiration was further extended with the addition of succinate (10 mM f.c.) and rotenone (1µg/ml f.c.) to assess non-coupled CII fueled ETC capacity.

Oxygen concentration and oxygen flux were recorded using DatLab Software (OROBOROS INSTRUMENTS, Innsbruck, Austria). All respiration data were normalized to mitochondrial protein content.

3.2.4. Determination of mitochondrial membrane potential

Simultaneously with respiration, membrane potential was determined using tetraphenylphosphonium (TPP^+)-selective electrodes, in a multisensor setup of the O2k apparatus following the protocols as described by Renner-Sattler et al. (2010). TPP^+ accumulates in the mitochondria according to the membrane potential. Before each measurement, a calibration in 5 steps, ranging from 1-2 µM TPP^+Cl^-, was performed. Since hydrophobic inhibitors were hard to remove from the electrode membranes after measurements, simplified respiration protocols were used. 500 µg of mitochondrial protein was incubated in the respiration medium followed by the sequential addition of substrates (pyruvate/malate or succinate/rotenone), ADP (25 µM and 300 µM) and cytochrome c. The influence of different chemicals on the TPP^+ signal was corrected with values obtained in a blank protocol (Renner-Sattler et al., 2010).

3.2.5. Quantification of ATP synthesis rate and H_2O_2 production rate

ATP synthesis and ROS production rates were determined as described by Brys *et al.*(2010).
ROS production was measured in OXPHOS conditions. The wells of a black microtiter plate contained 4 mM ADP, 10 Units of Cu/ZnSOD and either 5 mM pyruvate and 5 mM malate or 10 mM succinate, in the presence or absence of 2 µg rotenone and 2 µg oligomycin.

3.2.6. Determination of in vivo H_2O_2 levels

H_2O_2 levels in live worms were determined as described by Back *et al.* (2012). Fluorescence was measured for approximately 15 min, at 25°C with a Wallac Victor2 Multilabel Counter (PerkinElmer, Boston, MA, USA) with 490-nm (reduced roGFP2-Orp1) and 405-nm (oxidized roGFP2-Orp1) excitation filters and a 535-nm emission filter. Data shown are averages of four biological replicates. For each biological replicate, at least three technical replicates were averaged over the time measured.

3.2.7. Western blotting

Western blot of mitochondrial fractions was carried out as previously described by Brys et al. (2010). Primary antibodies against complex I NDUFS3 subunit (dilution 1:2000), complex V subunit beta (dilution 1:2000) and adenine nucleotide transferase (dilution 1:1000) were

purchased from Mitosciences (OR, USA). Primary antibody against voltage dependent anion channel (VDAC1/2/3) (dilution 1:1000) was purchased from Santa Cruz biotechnology, inc. (Heidelberg, Germany). LI-COR IRDye 800CW conjugated Goat anti-rabbit (dilution 1:10 000) and Goat anti-mouse antibodies and IRDye 680LT conjugated Goat anti-mouse (dilution 1:15 000) secondary antibodies were purchased from Westburg b.v. (Leusden, The Netherlands). Immunoblots were visualized using the LI-COR Odyssey infrared imaging system (Westburg b.v., Leusden, The Netherlands).

3.2.8. Mitochondrial density

Mitochondrial density of young adults was analyzed using a Nikon Eclipse T*i* confocal microscope. Worms that had reached second day of adulthood were mounted on agar pads on glass slides. For each condition, a picture of muscle cells from at least six different worms was made at 400x magnification. Mitochondrial density was quantified using an ImageJ routine described in (De Vos et al., 2010).

3.2.9. Assessment of the organization of OXPHOS complexes

Solubilization and blue-native electrophoresis (BN-PAGE) were performed as described previously (Krause and Seelert, 2008; Maas et al., 2009; Neff and Dencher, 1999). Isolated frozen mitochondria were thawed on ice and centrifuged at 20,000 g for 8 min. The pellet was suspended in solubilization buffer containing 50 mM NaCl, 50 mM imidazole/HCl (pH 7.0), 10% (v/v) glycerol and 5 mM 6-aminocaproic acid (final concentration). Mitochondria were solubilized with digitonin (Acros Organics, A0280740) using a detergent/protein ratio of 8 g/g at a final detergent concentration of 1% (w/v). The samples were incubated for 30 min at 4 °C with slight agitation, followed by centrifugation at 20,000 g for 10 min. The extracts were directly loaded onto native gels.

For BN-PAGE, linear 4–13% polyacrylamide gradient gels overlaid with a 3,5% stacking gel were run in a Hoefer SE 600 system (18.00 × 16.00 × 0.15 cm^3) with electrophoresis conditions as described previously (Krause and Seelert, 2008; Maas et al., 2009; Neff and Dencher, 1999). 200 µg mitochondrial protein (before solubilization) was always applied per lane. The apparent molecular masses of the OXPHOS complexes and their supercomplexes were calibrated according to digitonin-solubilized bovine heart mitochondria applied to the same first-dimension BN-gel. Lanes from the first-dimension BN-PAGE were then excised and used for a second-dimension 13% SDS-PAGE (Krause and Seelert, 2008; Maas et al., 2009; Reifschneider et al., 2006) with subsequent fluorescence staining by SYPRO Ruby. Quantification of protein abundances was performed using a CCD camera system (image reader LAS-3000, Fuji) in combination with the software Delta2D, version 4.3 by Decodon (Greifswald, Germany) for image analysis. The results are read-out from the gray values of the respective spots, normalized

to the total gray value of the image. The supercomplexes were assigned according to their characteristic subunit compositions revealed in 2D SDS-PAGE and apparent molecular masses. Additionally, some of the subunits of the *C. elegans* OXPHOS complexes were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described (Reifschneider et al., 2006).

3.2.10. Statistical analysis

To test whether parameters change in function of the diet in young animals, we used Student's *t*-test. To assess whether parameters changed with time and diet during aging, we used Linear Mixed Models (LMM; PROC MIXED) as described by Back et al. (2012b).

3.3. Results and discussion

3.3.1. ADR mitochondria display increased efficiency.

Mitochondria produce energy in the chemical form of ATP, given that they are provided with sufficient amounts of combustible substrate and ADP. Freshly isolated mitochondria lack both and therefore consume only small amounts of oxygen (state 1 (st1) respiration). Adding substrate only is not very helpful and thus respiration remains low (LEAK(st2) respiration). Providing the mitochondria with both substrates and ADP unlocks complex V, permitting protons to flow back into the matrix. Consequently, electron transport to complex IV and the reduction of oxygen to water is increased (st3 respiration) as is the production of ATP (oxidative phosphorylation). When ADP is depleted, respiration returns to resting LEAK(st4) conditions. Saturating amounts of ADP can stimulate respiration even further and allow estimating maximal oxidative phosphorylation (OXPHOS) capacity. Addition of the complex V (CV) inhibitor, oligomycin, slows respiration and allows estimation of LEAK(omy). Since FCCP is able to shuttle protons back to the mitochondrial matrix independently from complex V, titrations with this protonophore are very useful to assess the capacity of the electron transport chain (ETC capacity). Fig. 1 demonstrates a typical trail from a polarographic measurement and respiratory states are explained in more detail in chapter 1.2.4.3.

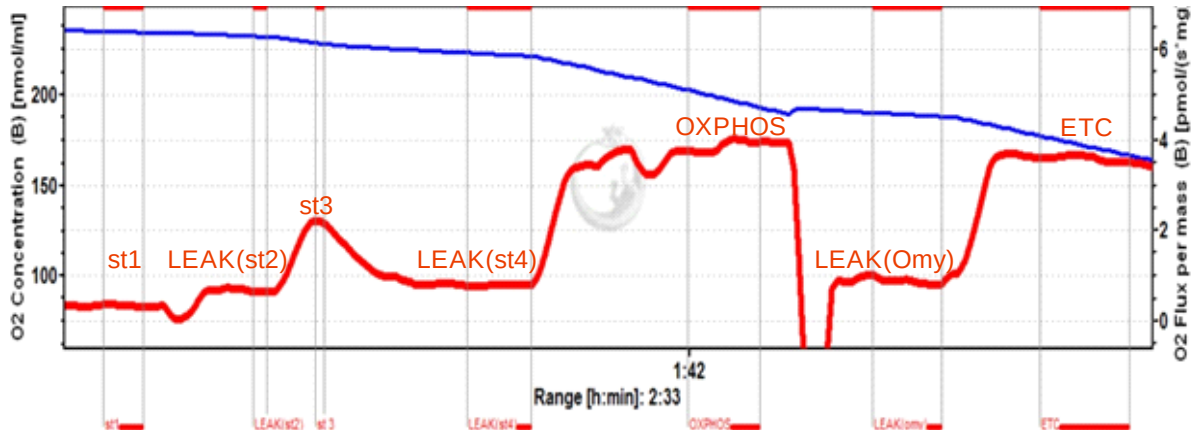

Figure 1: Typical trail obtained after an experiment using isolated mitochondria. The left Y-axis and blue trail represent changes in oxygen concentration, while the right Y-axis and red trail represent changes in oxygen flux and thus respiration rate.

We found that LEAK(st2 and st4) (P = 0.0002 and P = 0.003 respectively), but not LEAK(omy) (P = 0.16), are significantly decreased in ADR mitochondria fueled with NADH-generating substrates (Fig. 2). Except for LEAK(omy) (P = 0.002), LEAK is not significantly different when CII is fueled (LEAK(st2) P = 0.6, LEAK(st4) P = 0.37; Fig. 2).

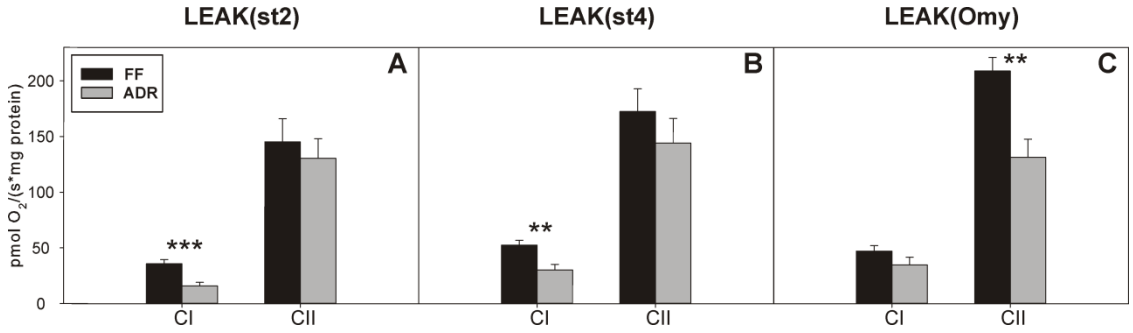

Figure 2: LEAK respiration under FF and ADR conditions. Mitochondria were fueled with pyruvate/malate to stimulate CI respiration and with succinate/rotenone to stimulate CII respiration. A) LEAK(st2): LEAK respiration in the presence of substrate only, without ADP. B) LEAK(st4): LEAK respiration after all ADP has been depleted and respiration returns to resting state 4. C) LEAK(omy): LEAK respiration after the addition of the CV inhibitor oligomycin. Values represent means of at least 5 biological replicates ± S.E.M. **P<0.01, ***P<0.001 (Student's *t*-test).

State 3 respiration is equally high under both feeding conditions, independent of the substrate used (CI: P = 0.34, CII: P = 0.35; Fig. 3A). Together, state 3 and state 4 can be used to derive the respiratory control ratio (RCR, see Chapter 1.2.2.4), illustrating the coupling between respiration and ATP synthesis (Lesnefsky and Hoppel, 2006). We found a significantly increased RCR for CI-fueled ADR mitochondria, whereas CII-fueling had no effect on RCR (P = 0.0008 and P = 0.94 resp; Fig. 4A). Furthermore, ADR mitochondria display significantly decreased OXPHOS capacity when CII is involved (CII: P = 0.028; Fig. 3B) but ETC capacity is significantly increased

(CII: P = 0.028; Fig. 3C). Although not statistically significant, a similar trend is noticeable under CI fueling conditions (P = 0.13 and P = 0.25 for OXPHOS and ETC capacity resp.; Fig. 3B and C).

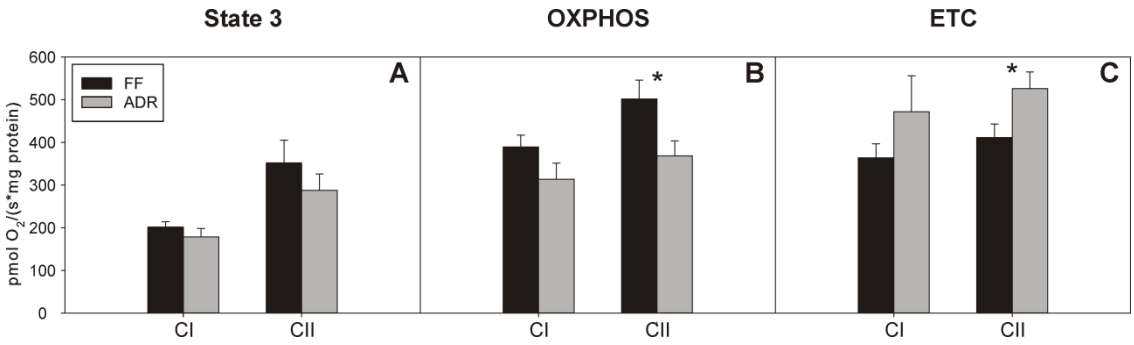

Figure 3: State 3 respiratory, OXPHOS and ETC capacity under FF and ADR conditions. Mitochondria were fueled as described in Fig.1. A) State 3 respiration in the presence of a limited amount of ADP. B) OXPHOS capacity: respiratory capacity in the presence of saturating ADP. C) ETC capacity: respiratory capacity of the ETC, uncoupled from ATP synthesis by titration of FCCP. Values represent means of at least 5 biological replicates ± S.E.M. *P<0.05 (Student's *t*-test).

Consequently, ADR mitochondria display significantly lower OXPHOS/ETC ratios (CI: P = 0.034, CII: P = 0.0068; Fig. 4B). OXPHOS/ETC (see chapter 1.2.4.4)indicates how close the capacity of coupled oxidative phosphorylation approaches the capacity of non-coupled electron transfer. If this ratio is less than one, a decreased capacity of the phosphorylation system, consisting of adenine nucleotide translocase, CV and a phosphate carrier, limits OXPHOS capacity. When this is the case and ETC capacity exceeds OXPHOS capacity, LEAK(Omy)/ETC (see chapter 1.2.4.4) gives a better estimate of the coupling and expresses the leakiness (Gnaiger, 2012), which is generally significantly decreased by ADR (CI: P = 0.0006, CII: P < 0.0001; Fig. 4C).

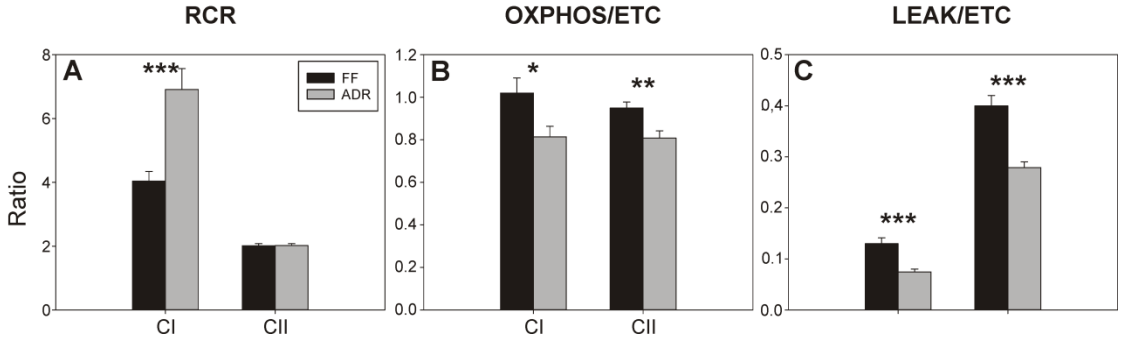

Figure 4: Control ratios under FF and ADR conditions. Mitochondria were fueled with pyruvate/malate to stimulate CI-dependent respiration and with succinate/rotenone to stimulate CII-dependent respiration. A) Respiratory control ratio (state 3/state 4) is an index for coupling. B) LEAK/ETC expresses leakiness of the mitochondria. C) OXPHOS/ETC indicates how close the capacity of coupled oxidative phosphorylation approaches the capacity of non-coupled electron transfer. Values represent means of at least 5 biological replicates ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 (Student's *t*-test).

These data point towards an increase in bioenergetic efficiency for ADR mitochondria. Using NADH-linked substrates, the increased coupling can be completely ascribed to the decrease in LEAK respiration, indicating that mitochondria from ADR worms are capable to idle at low rates

and to respond very well when CI-linked substrates are available by the efficient production of ATP. Although OXPHOS is decreased under CII fueling, ATP synthesis rate remains equally high in axenic culture (see 3.3.3), indicating higher bioenergetic efficiency as well. Independent of substrate supply, there is a general decrease of leakiness of mitochondria from ADR worms. There is controversy on how caloric restriction (CR) changes mitochondrial function in rodents: some studies pointed out that CR increases mitochondrial proton leak (Lambert and Merry, 2004), while others found that CR generally decreases mitochondrial respiration but maintains equally high ATP synthesis (Lopez-Lluch et al., 2006) and still others report that CR decreases LEAK respiration (Bevilacqua et al., 2005; Lal et al., 2001; Sohal et al., 1994). Our results are in agreement with the latter studies and the increase in mitochondrial efficiency seems to be a common outcome, making sense considering the fact that these animals are confronted with lower nutrient availability but no depression in metabolic rate.

3.3.2. ADR decreases the abundance of an outer mitochondrial membrane channel.

Because of decreased LEAK respiration and the control of the phosphorylation system over OXPHOS capacity, we were wondering whether important players in these processes such as CI, CV and adenine nucleotide translocase (ANT) possibly display differential abundance under ADR conditions. The phosphorylation system is an important determinant for OXPHOS capacity and consists of several components: CV, ANT and a phosphate carrier. A decreased abundance of these proteins may explain the decreased OXPHOS capacity but we found no changes in this direction for CV and ANT (Fig. 5A and C). Since ATP synthesis under ADR conditions is not decreased (see 3.3), the equal abundance of CV protein in control and ADR mitochondria is expected. If the abundance of the phosphorylation system is not changed, it is possible that substrate transport is regulated at the outer mitochondrial membrane (OMM). VDAC is a OMM channel involved in transporting ADP, ATP, pyruvate, malate, NADH and other metabolites (Blachly-Dyson and Forte, 2001) and we found that it is downregulated in mitochondria from ADR worms, indicating that it may represent the rate-limiting factor for oxidative phosphorylation (Fig. 5C). One possible explanation for the decreased LEAK respiration could be a decrease in CI abundance, but we could not find such a change (Fig. 5B). We noticed an extra band in the FF samples and we suspect that this may be the result of proteolytical activity (Fig. 5B). Whether this is the case could possibly be confirmed by additional western blots using protease inhibitors during the sampling process.

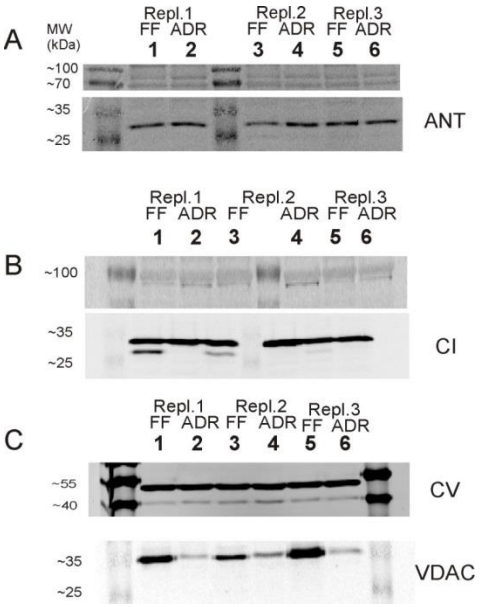

Figure 5: Western blot of ANT, CI, CV and VDAC under FF and ADR conditions. Monoclonal antibodies against A) ANT, B) VDAC and CV β subunit, and C) NDUFS3 subunit of CI, were used. MW of VDAC and CI is 30kDa, ANT is 33kDa and CV is 52kDa. Blots were performed on samples of 3 independent biological replicates and 20 µg of mitochondrial protein was loaded per sample. As loading control, aspecific staining for each gel is shown. CV and VDAC were stained on the same blot and aspecific staining bands under CV clearly demonstrate equal sample loading.

3.3.3. ADR has no effect on CV ATP synthesis.

The ADP/O ratio assesses the amount of molecular oxygen necessary to convert an exogenously added amount of ADP to ATP. This ratio may serve as an indication of the efficiency of oxidative phosphorylation (Magalhaes et al., 2005). We find that ADP/O values also point towards more tightly coupled ADR mitochondria, especially under CI-fueling (CI: $P = 0.004$, CII: $P = 0.087$; Fig. 6A). We also noticed that ADP/O values in young animals exceed the theoretically maximal attainable ratios. We reasoned that these unusual ADP/O values could be explained by additional non-OXPHOS ATP synthesis and therefore, we tested ATP synthesis rates of FF and ADR mitochondria in the presence and absence of the complex V blocker oligomycin. In the presence of non-limiting amounts of ADP (OXPHOS conditions), mitochondrial ATP synthesis rate is not significantly different for ADR mitochondria fueled with NADH-linked substrates and succinate ($P = 0.91$ and $P = 0.41$ resp.; Fig. 6B). We found that non-complex V stimulated ATP synthesis rate seems to be increased in ADR mitochondria, albeit not significantly (CI: $P = 0.08$, CII: $P = 0.09$; Fig. 6C). For all samples, rather high non-CV ATP synthesis rates were observed which probably explains the high ADP/O values obtained in this study. Adding detergent to the mitochondria did not abolish this effect, while boiling dismisses the non-complex V ATP synthesis, indicating enzyme involvement (data not shown).

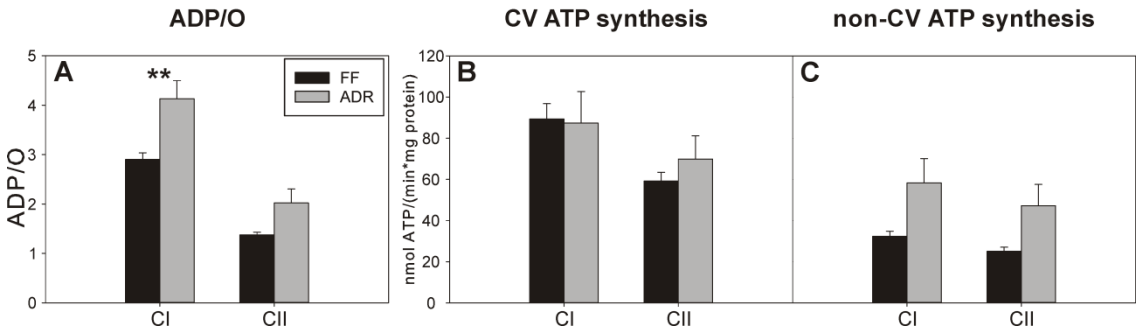

Figure 6: ADP/O and ATP synthesis rate under FF and ADR conditions. Mitochondria were fueled with pyruvate/malate to stimulate CI respiration and with succinate/rotenone to stimulate CII respiration. A) ADP/O represents the amount of molecular oxygen necessary to convert an exogenously added amount of ADP to ATP. B) CV ATP synthesis rate represents CV-specific ATP synthesis. Values are calculated by subtracting non-CV ATP synthesis rate from total ATP synthesis rate. C) non-CV ATP synthesis rates represent the residual ATP synthesis rate after inhibition of CV with oligomycin. Values represent means of at least 4 biological replicates ± S.E.M. ***P*<0.01 (Student's *t*-test).

This remarkable biochemical phenotype may be caused by upregulation of adenylate kinase or nucleoside-diphosphate kinase in ADR mitochondria thereby converting exogenously added ADP into ATP and AMP. It is very likely that this kind of ATP generation does not occur *in vivo* to this extent. ADR worms display decreased ATP standing levels (Houthoofd et al., 2002b) and our results support the conclusions that ADR worms possibly have a higher need for *de novo* synthesis of biomolecules and thus consume more ATP.

3.3.4. ADR may affect supercomplex organization.

We showed that, despite lowered VDAC abundance, ATP synthesis and state 3 oxygen consumption were not altered in ADR mitochondria. These mitochondria also showed to be well coupled which raises the question what causes this improved bioenergetic efficiency. The view on bioenergetic processes became more precise by the discovery of the higher order organization of the individual ETC complexes into supercomplexes. Chance and Williams already established this concept in 1955 (Chance and Williams, 1955a) and to this day, it is well studied (Seelert et al., 2009; Wittig et al., 2006; Wittig and Schagger, 2009) and evolutionary conserved for different organisms and tissues like rat brain (Frenzel et al., 2010; Reifschneider et al., 2006), mice (Acin-Perez et al., 2008), bovine heart (Reifschneider et al., 2006; Schagger, 2002), fish (Schäfer et al., 2007), the fungal aging model *Podospora anserina* (Groebe et al., 2007; Maas et al., 2009), *Drosophila* (Le Pécheur et al., 2009) and *Caenorhabditis elegans* (Suthammarak et al., 2010; Suthammarak et al., 2009). This higher degree organization is thought to improve the efficiency of oxidative phosphorylation since it facilitates substrate channeling and dismisses the need for diffusion of substrates between the ETC components (Lenaz and Genova, 2012). In addition, supercomplex formation enhances enzyme activity and stability of the individual complexes (Schäfer et al., 2006; Wernicke et al., 2010). More detailed information on supercomplex structure can be found in section 1.2.2.2.

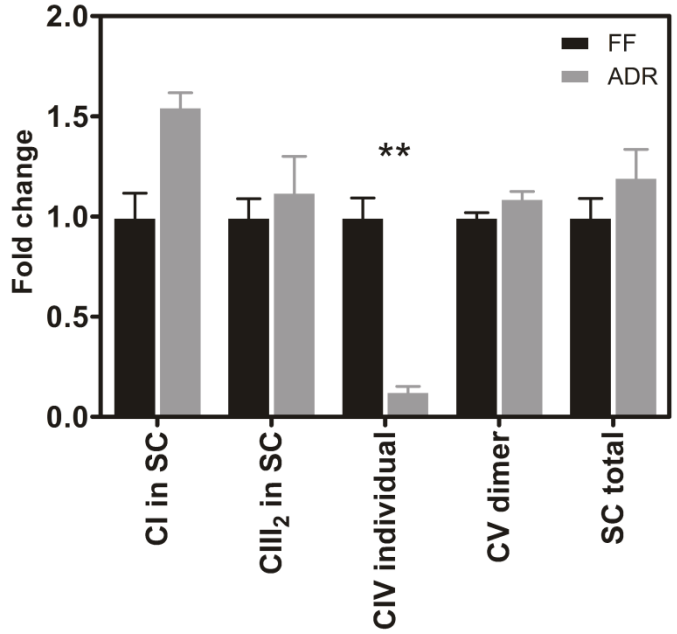

Figure 7: Supercomplex organization under FF and ADR conditions in young (day 2) adult worms relative to FF control. Abundance of CI in supercomplexes, $CIII_2$ in SC, individual CIV, CV dimer and total of supercomplex b (I-III2-IV) and c (I-III2-IV2) are represented as the mean fold change relative to FF control ± S.E.M. **P<0.01 (LMM)

Our 2D BN/SDS-PAGE analysis showed that CI can only be found as a part of the supercomplexes (Suppl. Fig. 1). The amount of CI in supercomplexes in young adult ADR-treated worms seems increased compared to age-matched fully-fed controls, although this difference borderline did not reach significance (P = 0.09). Strikingly, the same tendency was observed for $CIII_2$ in supercomplexes, for CV dimers and for the total supercomplex amount (see 1.2.2.2). This slight but consistent increase may hint at a modest supercomplex upregulation. The restriction by the technological set-up limited the number of biological replicates to three which may reduce the statistical power needed to reach significance when analyzing this dataset. In contrast to these limited changes, we observed a very pronounced decrease in the amount of individual CIV in ADR-treated worms (P = 0.002) (Fig. 7; Suppl Fig.1). Together with the slightly changing supercomplex levels, this may indicate that total CIV abundance is decreased in ADR-treated worms, similar to the repressed CIV protein expression that was found in the long-lived Ins/IGF mutant, *daf-2* (Geert Depuydt, unpublished observation). The much higher individual CIV abundance under FF conditions may provide an electron side track besides the substrate channeling way of the supercomplexes, decreasing efficiency of oxidative phosphorylation compared to ADR mitochondria that have much less individual CIV (Fig. 13). We believe that the trend towards higher level organization into supercomplexes together with the decreased individual CIV abundance under ADR conditions may contribute to the increased efficiency of their mitochondria.

3.3.5. ADR induces a modest decrease in mitochondrial membrane potential, but has no effect on ROS production capacity and in vivo H_2O_2 levels.

Since we found that ADR increases mitochondrial efficiency and may induce superior supercomplex organization, our next question is whether these observations attribute to the twofold lifespan extension under ADR. It is hypothesized and supported by experimental results that high supercomplex organization may lead to lowered ROS production (Lenaz and Genova, 2012; Schäfer et al., 2007). The Uncoupling-to-Survive theory postulates that mild uncoupling protects against aging and oxidative damage by reducing the mitochondrial membrane potential, consequently limiting excessive ROS production and extending lifespan (Brand, 2000). For various long-lived *C. elegans* strains, among which the dietary restricted *eat-2*, it was shown that membrane potential is lowered compared to the wild type control. Also, mild uncoupling, using chemical uncouplers (CCCP, FCCP), has been shown to extend lifespan (Lemire et al., 2009; Morcos et al., 2008). Seemingly in accordance with this theory, we found a trend towards lower membrane potential in ADR mitochondria but, although very close, the effect did not reach significance at the $P < 0.05$ level. This is the case for state 3 and LEAK(st4) conditions ($P = 0.07$ and P = 0.05 resp.). Together with the decreased LEAK(st4) respiration, the trend towards decreased membrane potential in LEAK(st4) conditions possibly indicates decreased proton leak across the IMM. However, in OXPHOS conditions the membrane potential is equally low in mitochondria from both feeding conditions ($P = 0.34$; Fig. 8A). The same trend was noticed under CII fueling conditions (data not shown).

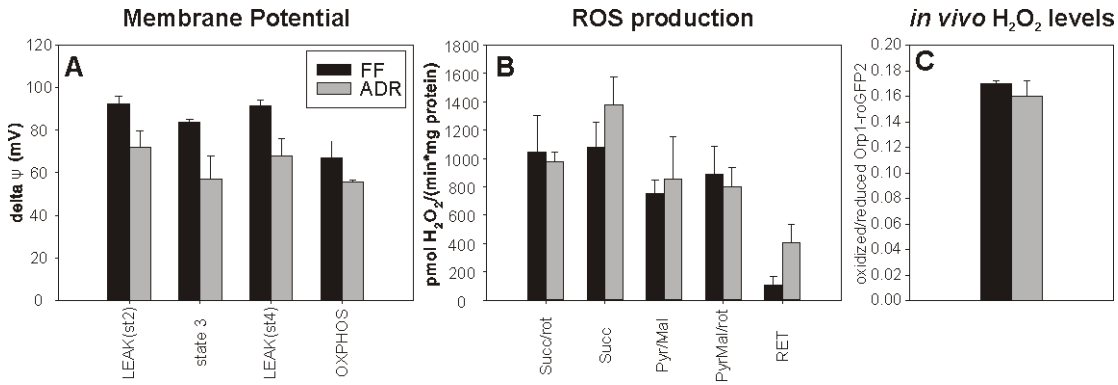

Figure 8: Membrane potential, ROS production capacity and in vivo H_2O_2 levels under FF and ADR conditions. A) Membrane potential was measured in LEAK(st2), state 3, LEAK(st4) and OXPHOS conditions using TTP+- sensitive electrodes. B) ROS production capacity was measured using an Amplex Red assay with CI- and CII-linked substrates in the presence or absence of rotenone. ROS production capacity under reverse electron transport (RET) conditions were calculated by subtracting ROS production with succ/rot from ROS production in conditions with succinate. C) in vivo H_2O_2 levels in live worms were determined using a transgenic strain expressing the H_2O_2-specific biosensor roGFP-Orp1. Values represent means of at least 3 biological replicates ± S.E.M.

In isolated mitochondria, the rate of ROS production depends on the mitochondrial membrane potential. Especially, reverse electron transport (RET) from complex II to complex I causes ROS production that is particularly sensitive to changes in the membrane potential (Miwa and Brand, 2003). Since we found a slightly decreased membrane potential in ADR mitochondria, we wanted to know whether this influences maximal ROS production capacity[1]. Combined production of superoxide and hydrogen peroxide was measured in OXPHOS conditions using an Amplex Red assay and exogenous addition of SOD. We could not detect any significant changes in ROS production capacity caused by ADR feeding. ROS production caused by RET seems higher under ADR conditions, but this is not significant (P = 0.1; Fig. 8B). Apart from *in vitro* ROS production capacity of the isolated mitochondria, we quantified *in vivo* H_2O_2 levels in intact living worms. To this purpose a transgenic strain expressing the H_2O_2-specific biosensor roGFP-Orp1 was used (Gutscher et al., 2009). Although this sensor has a lower dynamic range compared to the HyPer sensor described by our group earlier (Back et al., 2012b), it is far less sensitive to pH changes. We found that *in vivo* H_2O_2 levels are not significantly different between FF and ADR worms at young age (*P* = 0.53; Fig. 8C). Our results are in agreement with the results of Back et al. (2012b) who also showed that, at young age, there is no difference in H_2O_2 levels under DR conditions, but noted a gradual increase with age, which was attenuated by DR. Together with the decreased leakiness described in 3.1., these data are in contradiction with the Uncoupling-to-Survive theory and it is unlikely that this mechanism is at the foundation of the ADR-mediated lifespan extending effect *in C. elegans*. Also, our data are in contradiction with the findings of Schulz et al. (2007), who found that glucose restriction induces increased ROS production and states that this deregulation of ROS may induce a mitohormetic response by upregulating ROS defense mechanisms ultimately resulting in increased life expectancy.

3.3.6. ADR induces an increase in mitochondrial density

Previously, it was established that ADR increases whole-worm respiratory rate (Houthoofd et al., 2002b). However, *in vitro* mitochondrial respiratory capacity shows no such rise. One possible reason for this discrepancy is that ADR worms show a higher mitochondrial density in their tissues compared to FF worms. We quantified mitochondrial density in worms (second day of adulthood) expressing a mitochondrial fluorescent reporter and confirmed this hypothesis (*P* = 0.00026; Fig. 9). Our results are in agreement with studies in rodents and humans, where it was found that caloric restriction (CR) upregulates mitochondrial biogenesis (Civitarese et al., 2007; Lopez-Lluch et al., 2006; Nisoli et al., 2005). However, these results are contested by studies reporting no such induction (Hempenstall et al., 2012; Lanza et al., 2012). Furthermore, we have

[1] Maximum ROS production capacity refers to the capacity of the mitochondria to produce ROS in artificial, *in vitro* conditions and not to the situation in which subunits are maximally reduced.

previously shown that ADR upregulates PEPCK transcript levels and enzyme activity (Castelein et al., 2008), which, in mouse skeletal muscle, is linked to increased lifespan and mitochondrial biogenesis (Hakimi et al., 2007).

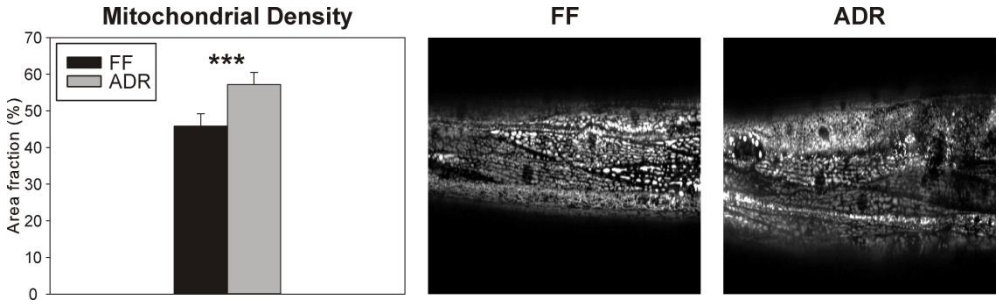

Figure 9: Mitochondrial density under FF and ADR conditions was assessed using confocal images processed with ImageJ according to De Vos et al. (2010). All pictures were taken using confocal microscopy at 400x magnification. For each condition muscle cells were selected and at least six pictures of different worms were taken. The percentage of surface occupied by the mitochondria was measured and values represent averages of mean mitochondrial areas from at least 6 individual worms ± S.E.M. ***P<0.001 (Student's t-test).

3.3.7. ADR protects mitochondria from decreasing efficiency with age.

The major change with age in mitochondria from fully fed (FF) controls is a clear increase in LEAK respiration (LEAK(st4) slope = 48.28±6.61; P = 0.0002; Fig. 10A) while state 3 respiration, OXPHOS and ETC capacity do not change (P = 0.47, P = 0.55, P = 0.99 resp.; Fig. 10B-D). This results in significantly decreased RCR (slope = -1.1±0.2; P = 0.0004; fig. 11A) and ADP/O ratios (slope = -0.74±0.19; P = 0.009; fig; 10D) and increased leakiness (slope = 0.15±0.01; P < 0.0001; Fig. 11B), indicating decreased efficiency caused by an increase in LEAK. The OXPHOS/ETC ratio does not change with age (Fig. 11C). This is in agreement with the findings published earlier by Brys et al. (2010).

Mitochondria from ADR-treated animals maintain a constant and fairly low LEAK with age (slope = -1.001±3.4; P = 0.78; Fig. 10A) and ADR attenuates the age-dependent increase in LEAK seen in FF controls (P = 0.0003). Furthermore, these mitochondria display a significant decrease in ETC capacity (slope = -38.14±10; P = 0.005; Fig. 10D). Although not significant, state 3 respiration and OXPHOS follow the same trend (state 3 slope = -9.5±6; P = 0.15; OXPHOS slope = -18.3±9.3; P = 0.06; Fig. 10B-C), resulting in a small but non-significant decrease in RCR (slope = -0.21±0.09; P = 0.054) and a subtle increase in leakiness (slope = 0.019±0.006; P = 0.01), but not to the same extent as for FF mitochondria (P = 0.003 and P < 0.0001 resp.; Fig. 11A-B). This indicates that mitochondria from ADR animals may lose some of the initial high ETC capacity with age, while remaining very efficient. This is reflected by the unchanged ADP/O ratios with age (slope = -0.15±.19; P = 0.48; Fig. 11D). These results are in contrast to wild-type and *daf*-2 animals and imply that the adaptations in mitochondrial function are specific to ADR animals,

possibly to compensate for the lower nutrient uptake. It is tempting to speculate that not changes in mitochondrial respiration per se are important for lifespan extension but that a preserved mitochondrial efficiency may play a pivotal role, at least in *C. elegans*. In mammals, it was reported that mitochondrial capacity declines with age and that CR attenuates these changes. In accordance with our data, these studies also established preserved efficiency, reflected in higher RCR and ADP/O in aging CR animals (Lanza et al., 2012).

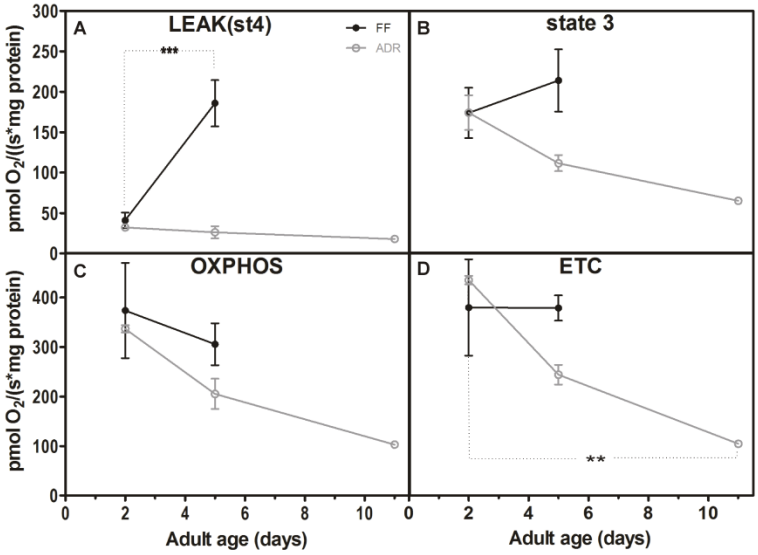

Figure 10: Age-related changes in respiratory rates in FF and ADR conditions. Mitochondria were fueled with pyruvate/malate to stimulate CI-dependent respiration. A) LEAK(st4) respiration after depletion of ADP. B) State 3 respiration, in the presence of a limited amount of ADP. C) OXPHOS capacity in the presence of saturating amounts of ADP. D) ETC capacity, after uncoupling with FCCP. Values represent mean of 3 biological replicates for samples taken at day 2 and 5 of adulthood ± S.E.M. One biological replicate was obtained for eleven-day old adults in ADR conditions. **P<0.01, ***P<0.001 (LMM).

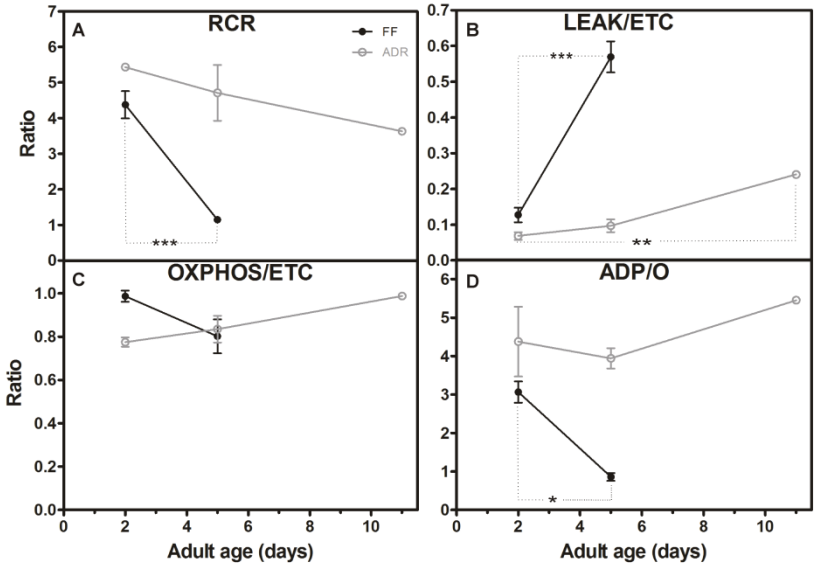

Figure 11: Age-related changes in control ratios under FF and ADR conditions. Mitochondria were fueled as described in Fig.10. A) Respiratory control ratio (RCR: state 3/state4). B) LEAK/ETC C) OXPHOS/ETC D) ADP/O ratio. Values represent mean of 3 biological replicates for samples taken at day 2 and 5 of adulthood ± S.E.M. One biological replicate was obtained for eleven-day old adults in ADR conditions.

*P<0.05, **P<0.01, ***P<0.001 (LMM).

3.3.8. ADR preserves supercomplex organization during lifespan.

As described in 3.3.4., supercomplex organization possibly contributes to the increased efficiency of the ADR mitochondria. Is this increased higher order organization responsible for the well-maintained function of aging ADR mitochondria as well? For CI in supercomplexes, individual $CIII_2$ and CV, there seems to be a trend towards an age dependent decrease in FF animals that is attenuated in ADR worms, but these differences did not reach significance (Fig. 12A-B, D). The CV monomer/dimer ratio (Suppl. Fig. 2), which has an impact on mitochondrial cristae formation (Davies et al., 2012), is not affected by age and dietary regimen. The distribution is approximately 60 % CV dimer to 40 % CV monomer. No significant age-dependent changes were observed for $CIII_2$ in supercomplexes or individual CIV, (Fig. 12C and F), in FF as well as in ADR conditions. Combining the respective data for CI and $CIII_2$, we quantified the supercomplexes b, consisting of I-III_2-IV, and c, composed of I-III_2-IV_2 (see 1.2.2.2) (Fig. 12E) but we could not find any significant age- or diet-dependent changes.

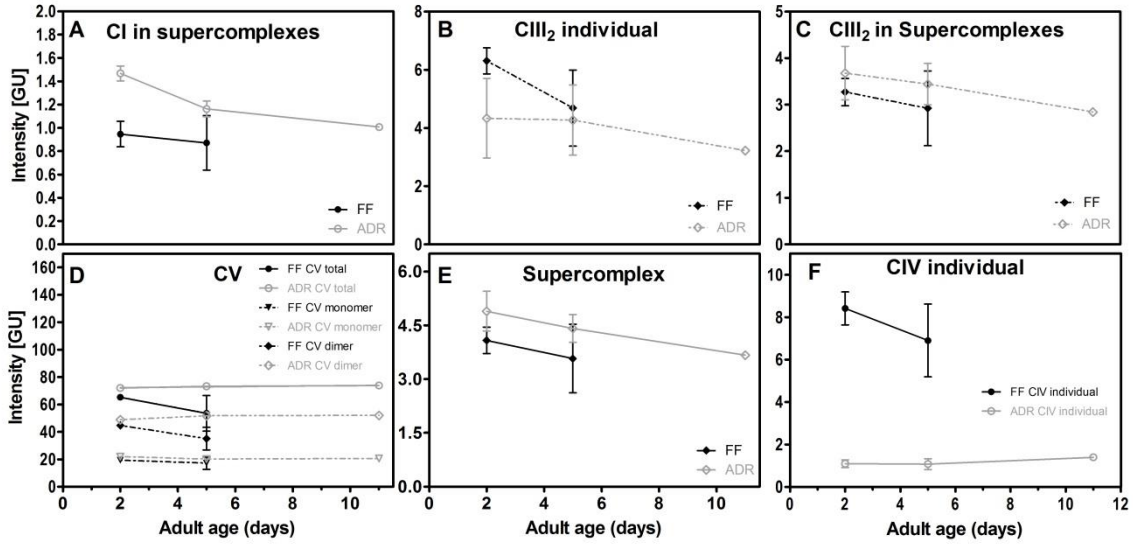

Figure 12: Changes in supercomplex organization with increasing age under FF and ADR conditions. A) Changes in CI abundance in SC with age. B) Changes in abundance of individual $CIII_2$ and $CIII_2$ in supercomplexes with age. C) Changes in abundance of individual CIV with age. D) Changes in abundance of CV monomers and dimers and total CV abundance. E) Changes in total supercomplex abundance. Total supercomplex is the sum of supercomplex b (I-III2-IV) and c (I-III2-IV2).

We believe that the individual CIV abundance may provide an explanation for the observed age-dependent changes in ETC capacity, RCR and LEAK/ETC. An age-dependent decrease in CIV enzymatic activity, as observed in other organisms (Petrosillo et al., 2013; Ren et al., 2010), could provide an explanation for the observed age-dependent decrease in ETC capacity under ADR. Mitochondria from FF worms contain much more individual CIV, possibly giving them the ability to compensate for the CIV activity loss and maintaining equally high ETC capacity while

aging. Since electron transport towards individual CIV lacks the benefit of substrate channeling as in supercomplexes, this compensation may come with the cost of age-reduced mitochondrial efficiency (Fig. 13). In all distantly related organisms studied so far, supercomplexes seem less affected by DR than the individual ETC components (Dani et al., 2010; van Diepeningen et al., 2010). This is also observed in the present study for *C. elegans*.

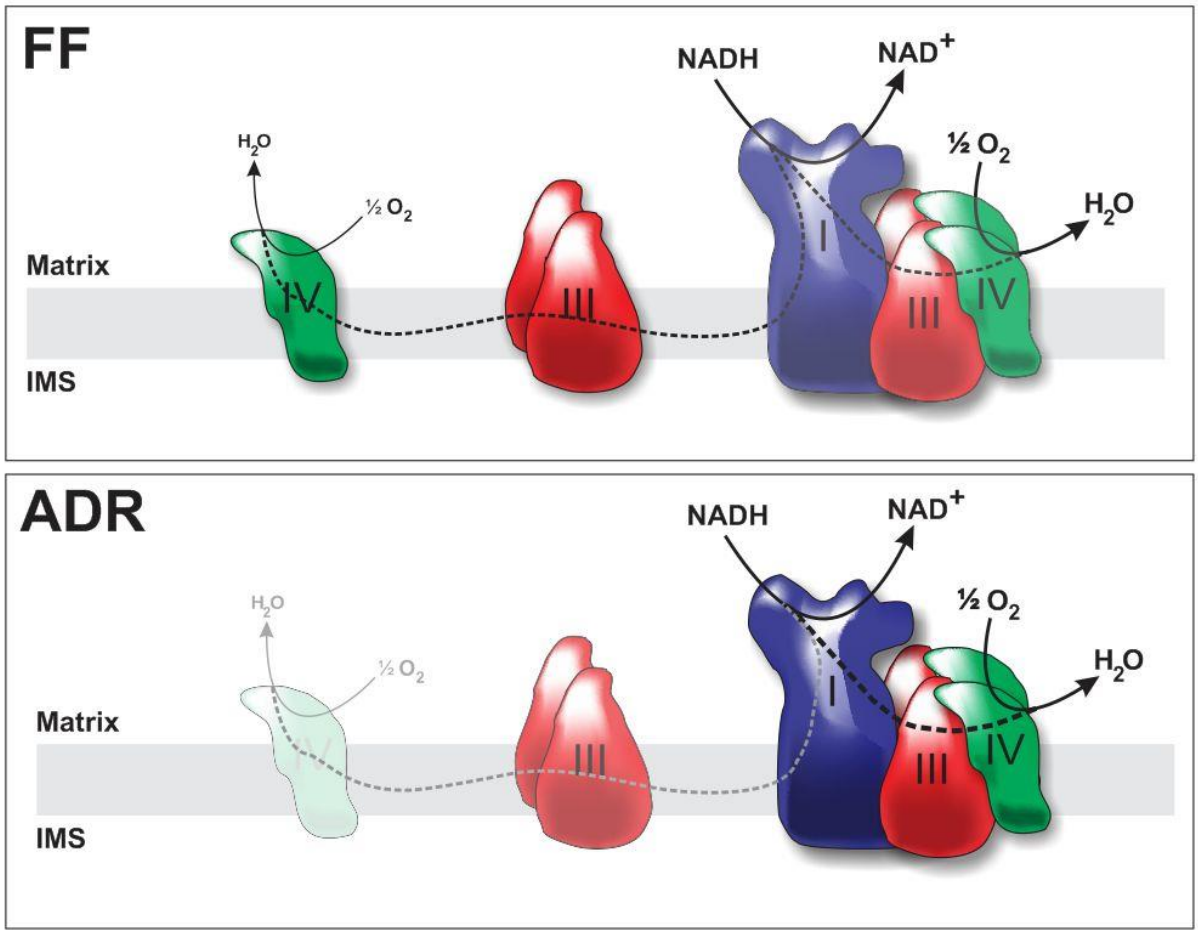

Figure 13: Schematic overview of supercomplex organization under FF and ADR conditions. Mitochondria from FF worms contain significantly more individual CIV besides CIV in supercomplexes, providing them an alternative pathway for electron transport besides the more efficient pathway through the supercomplexes. This may lead to less efficient electron transport and reduced coupling. Since ADR mitochondria contain less individual CIV, almost all electron transport has to follow the more efficient substrate channeling way of the supercomplexes rendering more efficient coupling.

3.4. Conclusion

In conclusion, ADR improves and preserves bioenergetic efficiency. We suggest that modest changes in supercomplex organization together with the decreased CIV abundance may play a role in establishing this efficiency by facilitating electron transport. ROS production capacity is not decreased, nor are *in* vivo H_2O_2 levels, questioning the importance of early ROS-induced damage for the lifespan effect of ADR.

3.5. Acknowledgements

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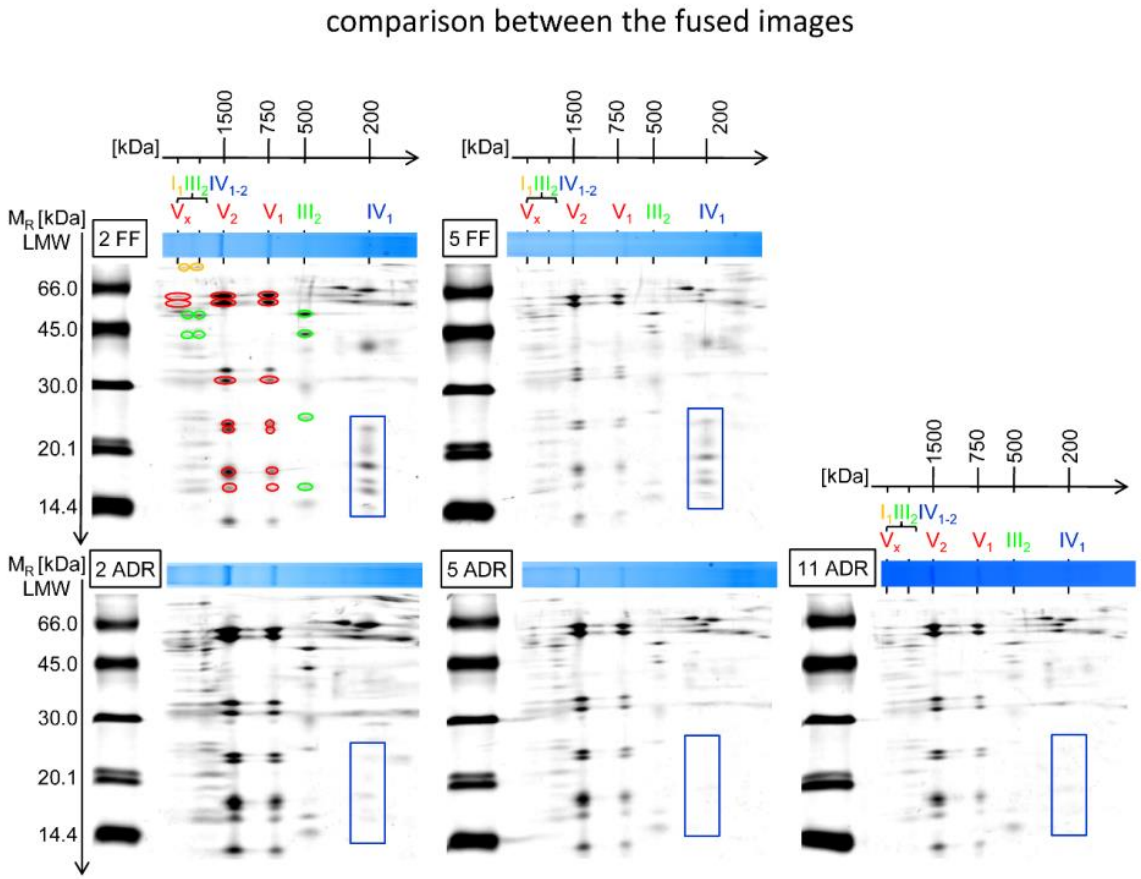
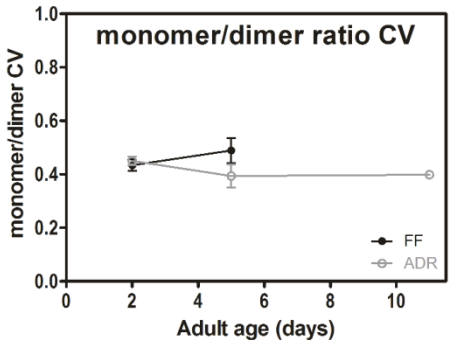


Suppl. Figure 1: Fused images of BN-PAGE gels. The gels present the results after 2D BN/SDS-PAGE for

each age and dietary condition and of three biological replicates. The spots that were picked for quantitative analysis are marked in color.



Suppl. Figure 2: . Age-related changes in CV monomer/dimer ratio under ADR and FF conditions.

Chapter 4. Lifespan regulation under axenic dietary restriction: a close look at the usual suspects.

Personal contribution: Experimental design

Data analysis

Writing the manuscript

Submitted as:

Lifespan regulation under axenic dietary restriction: a close look at the usual suspects.

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Abstract

In *C. elegans*, there are several ways to impose dietary restriction (DR) all of which extend lifespan to a different degree. Until recently, the molecular mechanisms underlying the DR-mediated lifespan extension were completely unknown but extensive efforts led to the identification of several key players in this process. Culture in sterile axenic medium is a method of DR (ADR), leading to an impressive doubling of lifespan. Earlier, we established that ADR-mediated longevity is independent of ins/IGF signaling and *eat-2*. The only gene reported to be indispensable for the ADR lifespan effect is *cbp-1* (Zhang et al., 2009) which was confirmed in this study. In an attempt to identify more genes involved in ADR-mediated longevity, we tested several candidate genes known to regulate lifespan extension in other DR regimens. We found that *cup-4* is equally important as *cbp-1* in ADR-mediated longevity and we identified some genes that may have a partial effect on ADR-induced longevity, but do not mediate the full lifespan effect.

Keywords: *Caenorhabditis elegans*; dietary restriction; axenic; molecular mechanism

4.1. Introduction

Dietary restriction (DR), the restriction of nutrients without malnutrition, has been shown to extend lifespan in a wide variety of species (Masoro, 2005). In *C. elegans*, several methods to impose DR were developed and they all extend lifespan to a different degree (reviewed in Greer and Brunet (2011)). The standard food source for *C. elegans* in laboratory cultures is the bacterium *Escherichia coli*, of which the OP50 strain is most often used (Brenner, 1974). The most straightforward DR method is reduction of the amount of bacteria, termed bacterial dilution, which can be conducted either in liquid (BDR) (Klass, 1977) or on solid agar culture (sDR) (Greer et al., 2007). Other plate methods are peptone dilution (PD) which leads to reduction of bacterial growth (Hosono et al., 1989), dietary deprivation (DD) or the total absence of bacteria (Kaeberlein et al., 2006) and intermittent fasting (IF), in which worms are fed only every other day (Honjoh et al., 2009). Mutation in the gene *eat-2* decreases food intake by reducing pharyngeal pumping rate and is often used as well (Avery, 1993; Lakowski and Hekimi, 1998). DR-like phenotypes can also be induced using chemically defined or undefined liquid media in the absence of bacteria, called axenic dietary restriction (ADR) (Vanfleteren, 1976).

Until recently, it was completely unknown which molecular mechanisms underlie the DR-mediated lifespan extension. Using BDR and the *eat-2* mutant, Bishop and Guarente (2007) showed that the transcription factor SKN-1 in the ASI neurons is indispensable and other transcriptional regulators, such as PHA-4, HIF-1, HSF-1 and CBP-1 were identified as important players mediating the DR effect (Chen et al., 2009; Panowski et al., 2007; Steinkraus et al., 2008; Zhang et al., 2009). Furthermore, other genes, including the energy sensor *aak-2* (Greer et al., 2007) and two downstream targets of SKN-1, *cup-4* and *nlp-7* (Park et al., 2010) are involved in the lifespan-extending effect of DR. However, the overview in Greer and Brunet (2011) indicates that none of these genes is equally important in all DR methods, suggesting that different DR methods may activate (partially) separate pathways, instead of a single universal DR pathway, to extend lifespan.

ADR worms share the typical characteristic traits of worms subjected to other DR methods: slowed development, reduced fecundity and prolonged lifespan (Houthoofd et al., 2002b; Vanfleteren, 1976). Since axenic medium is rich in peptides and amino acids, carbohydrate, vitamins and minerals, it is unclear why culturing worms in this medium leads to such an extensive lifespan extension. *C. elegans* is a filter-feeder, taking in fluids containing suspended particles and spitting out the fluid while retaining the particles (Avery, 1993). In axenic medium, there are no suspended particles of bacterial size and worms probably encounter difficulties taking up nutrients from this medium, leading to DR. Although unconfirmed, it has

been suggested that axenic culture induces a failure of endocytotic uptake of nutrients in the intestinal lumen (Vanfleteren, 1974; Vanfleteren, 1980). It is likely that ADR-cultured worms experience cues of a rich nutritious environment but are unable to take up these nutrients efficiently.

Previously, it was shown that ADR-induced longevity is independent of DAF-16, DAF-2 and EAT-2 (Houthoofd et al., 2003). Culturing *eat-2* mutants in axenic medium extends lifespan even further, suggesting that both DR methods act through separate molecular mechanisms. Recently, Zhang et al. (2009) established that ADR-mediated lifespan extension is almost completely abolished in the absence of CBP-1 and that this gene is indispensable for lifespan extension by other DR methods as well. This was the first report on a gene indispensable for ADR-mediated lifespan extension. With our study, we attempt to identify more genes that underlie the molecular mechanism of ADR-mediated longevity. As several genes have been shown to play a role in longevity mediated by other DR methods, we made a targeted selection of some of the usual suspects and extended these with candidate genes that interact with keyplayers or possibly play a role in down-stream processes.

We were able to confirm that CBP-1 is a major regulator of ADR-mediated lifespan extension and found that CUP-4 is equally important. In addition, we identified several other genes which may have a partial effect on ADR-mediated longevity.

4.2. Methods

4.2.1. C. elegans and RNAi strains.

The wild-type (WT) *C. elegans* used was Bristol N2 male stock (*Caenorhabditis* Genetics Center). The mutant strains were *aak-2(ok524), cup-4(ok837), ucp-4(ok195), crh-1(tz2), hif-1(ia4), hsf-1(sy441), trx-1(ok1449), cep-1(gk138), jnk-1(gk7)* and were provided by the CGC. *skn-1(zu135);nT1[qIS51]* was a kind gift from N. Bishop and *nlp-7(tm2984)* was provided by the National Bioresource project.

cbp-1 dsRNA expressing bacterial strains were from the genomic RNAi library (produced by J. Ahringer at the Wellcome/CRC Institute). As a control, the bacterial strain containing the empty vector L4440 was used.

4.2.2. Axenic culture conditions

Axenic basal medium consists of 3% soy peptone (Sigma-Aldrich, St. Louis, MO) and 3% yeast extract (Becton-Dickinson, Franklin Lake, NJ), final concentrations (f.c.). Since *C. elegans* is not capable of haem synthesis, after autoclaving, the basal medium was supplemented with 0.05%

haemoglobin f.c. (bovine; Serve, Heidelberg, Germany) diluted from a 100x stock in 0.1M KOH (autoclaved for 10 minutes). To obtain solid plates containing axenic medium, 2% agar N°1 (f.c.) (OXOID, Hampshire, United Kingdom) was added to the above described composition.
In the rich axenic medium bacteria can grow unrestrained. Hence, it is important to handle all equipment in a sterile manner and therefore all preparations were made in a laminar flow cabinet to ensure sterility. When using axenic solid plates for lifespan determination, the assay was set-up with a sufficient number of worms (double compared to fully fed or axenic liquid conditions), as they tend to crawl off the plates in search of food.
Before starting the lifespan assays, all WT and mutant strains, except for WT worms that are used for RNAi treatment, are cultured in liquid axenic medium from egg until adulthood. This medium needs supplementation with 20% skimmed milk (f.c.) to allow normal developmental rates (Houthoofd et al., 2002b).

4.2.3. Lifespan determination

For the lifespan assays, gravid WT and mutant adults were subjected to a microbleaching procedure. Approximately ten worms were brought in a drop of 10 µL sterile distilled water. Ten microliter of a concentrated bleach solution (stock concentration 13.5° hypochlorite and 1M NaOH) was added and left to incubate until all adults were dissolved or for maximally ten minutes. Then 5 mL of axenic medium containing 20% sterile skimmed milk was added. The eggs were allowed to hatch and were incubated at 20°C until adulthood. At L4 stage, 100 µM of FUdR was added to prevent progeny production. At adulthood, worms were transferred and exposed to the experimental conditions.

For RNAi experiments, N2 worms were first grown to adulthood on standard nutrient agar plates seeded with the *E. coli* K12 bacteria. RNAi was carried out following standard bacterial feeding protocols (Timmons et al., 2001) in these young adult worms for five days before they were transferred to the experimental conditions.

For each strain, ten worms were placed on small NGM plates seeded with *Escherichia coli* OP50 as fully fed (FF) control. For ADR, ten worms were placed on axenic plates, prepared as described by (Lenaerts et al., 2008) (ADR solid; ADRs) or three worms were transferred with sterile Pasteur pipettes to small screw-cap tubes (5 mL tube, 75x12 mm, PS, Sarstedt, Nümbrecht, Germany) containing 0.3 mL of liquid axenic medium (ADR liquid; ADRl). To prevent bacterial contamination, 0.1 g/L (final concentration) ampicillin was added to the axenic conditions containing RNAi treated worms. Progeny production was avoided by the addition of 200 µM and 100 µM FUdR to the FF and ADR cultures, respectively. Survival was scored at regular time intervals: daily for the FF conditions, every other day for the ADR conditions. In

solid conditions, worms were considered dead if they did not respond to gentle prodding with a platinum wire. In liquid conditions, worms were scored dead if no movement could be detected, even after gently tapping the tubes. Worms that died of protruding vulva or crawling off the plates were censored. All lifespan assays were conducted at 20°C.

4.2.4. Analysis

For statistical analysis of the obtained data, we used the online application for survival analysis (OASIS) as described by Yang et al. (2011). The relative importance of a certain gene to the lifespan-extending effect of ADR was calculated as:

$$Relative\ importance = 1 - \frac{\frac{Mut(ADR)}{Mut\ (FF)} - 1}{\frac{N2(ADR)}{N2(FF)} - 1}$$

In which N2(FF) is the mean lifespan of wildtype under fully-fed conditions and Mut(ADR) is the mean lifespan of the mutant under axenic dietary restriction. A gene that does not contribute to the lifespan extension under ADR will have a relative importance of 0, a gene that is fully responsible for this effect will have a relative importance of 1. Because this formula cannot take into account complex genetic interactions, the calculated relative importance values may exceed the [0,1] interval. To assess whether the relative importance differs significantly from 0, the one-sample *t*-test was used. We used a Student's *t*-test to assess whether the relative importance of two genes differs significantly.

4.3. Results and discussion

4.3.1. Transcriptional regulators: CRH-1, CBP-1, CEP-1, SKN-1, HSF-1 and HIF-1.

A large number of transcriptional regulators have been shown to play a role in lifespan extension. As described above, the histone acetyltransferase CREB-binding protein, or CBP-1, is the first gene shown to be almost completely indispensable for the ADR-mediated lifespan effect in *C. elegans*. Furthermore, this protein was found to be of importance in other DR regimens, such as *eat-2* and BDR, as well. Longevity of the Insulin/IGF mutant *daf-2* was also shown to be dependent on CBP-1, suggesting that the importance of CBP-1 is not strictly specific to DR (Zhang et al., 2009). We were able to confirm the pivotal role of *cbp-1* for the longevity effect of ADR (Fig.1A and 4). Worms submitted to *cbp-1* RNAi still displayed a minor lifespan extension under ADRl ($P < 0.0001$), the effect of culture in ADRl was much less pronounced than for worms treated with the empty vector, reflected in the high relative importance of *cbp-1* (0.72, $P = 0.067$; Fig.3). It could be argued that *cbp*-1 is of general importance for organismal health and therefore

has such an extensive effect on lifespan and lifespan extension can no longer be induced in these RNAi treated animals. However, Zhang et al. (2009) showed that *daf*-2 mutatns that were *cbp-1* RNAi treated still display lifespan extension. Although knock-down of *cpb-1* does not completely abolish lifespan extension in ADR conditions, it impacts lifespan to such an extent that *cbp-1* can be considered as a major regulator of ADR-induced longevity. CBP-1 was first identified as a nuclear protein important for co-activation of CREB, the cAMP response-element binding protein. In *C. elegans*, the CREB gene homologue, *crh-1*, was shown to be involved in the lifespan extension caused by AMPK activation and calcineurin deactivation (Mair et al., 2011). The fact that *cbp-1* is involved in ADR-mediated longevity and that it activates *crh-1* suggests that crh-*1* may be a key player in ADR lifespan extension as well. Our results show that, *crh-1* only slightly rescues the lifespan effect of ADR (ADRl $P < 0.05$, ADRs P = 0.0003). The relative importance of *crh-1* is low but significantly different from 0 for ADRl-mediated longevity (ADRl 0.25, $P = 0.034$; ADRs 0.37, $P = 0.21$; Fig. 3), indicating that it is unlikely that CBP-1 exerts its effect mainly through CRH-1 activation (Fig.1B and 4; Table 1). Although CRH-1 is not a major contributor, its partial effect may point out that it is one of the downstream effectors mediating ADRl-induced longevity.

CBP-1 was originally identified as CREB co-activator but it is involved in the transcriptional co-activation of many other transcription factors as well, among which *p53* (Grossman, 2001; Lee et al., 2010a). In *C. elegans*, it was shown that CBP-1 acts as a co-activator for CEP-1-induced germline apoptosis (Schumacher et al., 2001). Moreover, a link between *p53* and lifespan was established when it was found that a *p53* gain-of-function mutation in mice causes premature aging and reduced longevity (Tyner et al., 2002). In *C. elegans*, decreased expression of *cep-1*, the *C. elegans* p53 ortholog, was found to induce lifespan extension (Arum and Johnson, 2007) and Ventura et al. (2009) showed that the *cep-1*-mediated lifespan response to mitochondrial dysfunction depends on the level of mitochondrial stress. Therefore, we considered *cep-1* a good candidate for involvement in ADR-mediated longevity. We found that mutation in *cep-1* has no effect on lifespan in both FF and ADRl conditions, reflected in a low relative importance of 0.04 ($P = 0.25$, Fig. 3). Under ADRs, cep-*1* lifespan was extended beyond the dietary-matched control ($P = 0.0001$), indicating that under these conditions *cep-1* is dispensable for lifespan extension (relative importance -0.5, $P = 0.34$; Fig.3). These findings exclude CEP-1 as the main regulator of ADR-mediated lifespan extension (Fig.1C and 4; Table 1) and in ADRs, it seems that CEP-1 negatively influences lifespan.

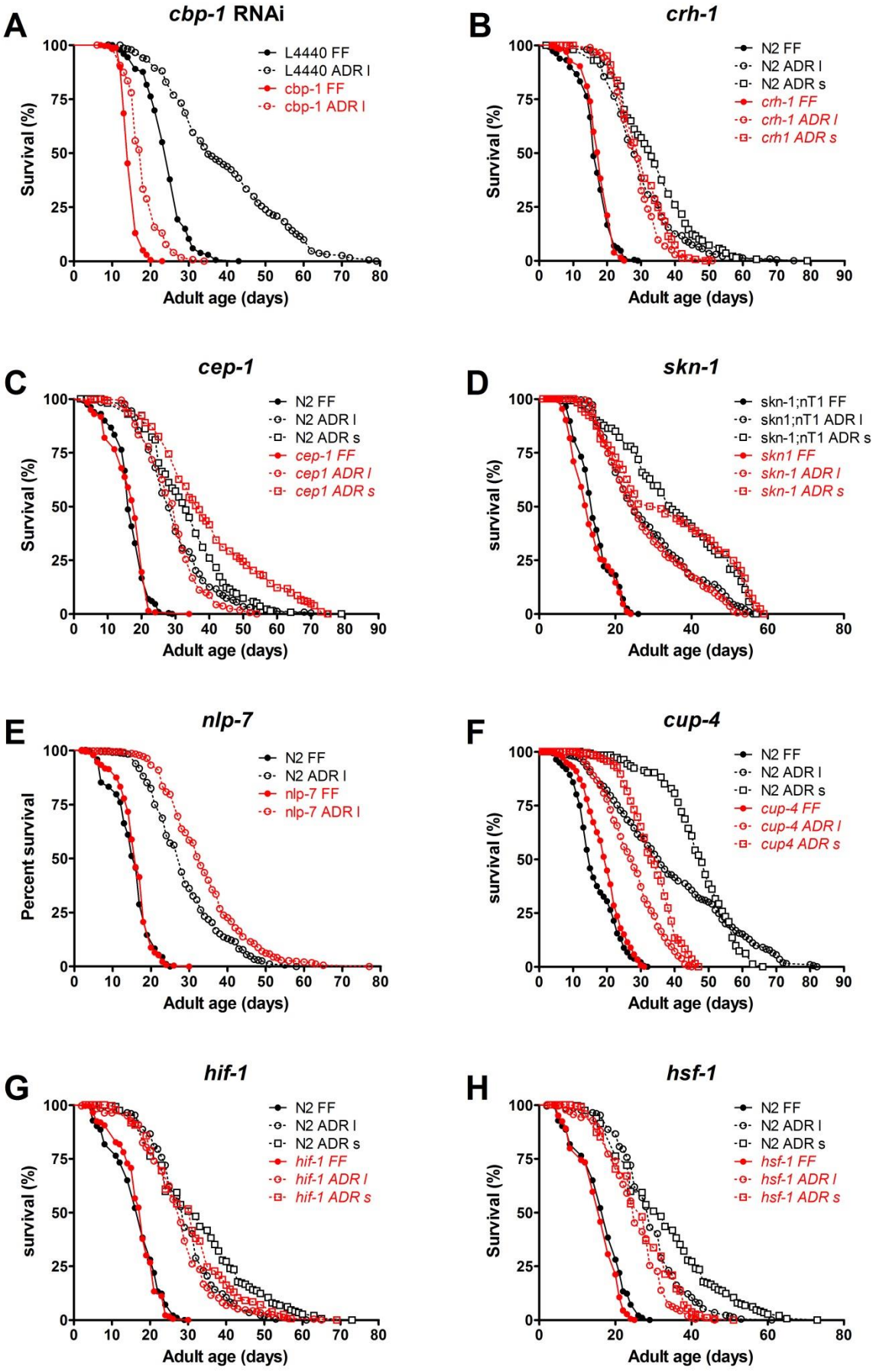
A
cbp-1 RNAi
L4440 FF
L4440 ADR I
cbp-1 FF
cbp-1 ADR I
Survival (%)
Adult age (days)
B
crh-1
N2 FF
N2 ADR I
N2 ADR s
crh-1 FF
crh-1 ADR I
crh1 ADR s
Survival (%)
Adult age (days)
C
cep-1
N2 FF
N2 ADR I
N2 ADR s
cep-1 FF
cep1 ADR I
cep1 ADR s
Survival (%)
Adult age (days)
D
skn-1
skn-1;nT1 FF
skn1;nT1 ADR I
skn-1;nT1 ADR s
skn1 FF
skn-1 ADR I
skn-1 ADR s
survival (%)
Adult age (days)
E
nlp-7
N2 FF
N2 ADR I
nlp-7 FF
nlp-7 ADR I
Percent survival
Adult age (days)
F
cup-4
N2 FF
N2 ADR I
N2 ADR s
cup-4 FF
cup-4 ADR I
cup4 ADR s
survival (%)
Adult age (days)
G
hif-1
N2 FF
N2 ADR I
N2 ADR s
hif-1 FF
hif-1 ADR I
hif-1 ADR s
survival (%)
Adult age (days)
H
hsf-1
N2 FF
N2 ADR I
N2 ADR s
hsf-1 FF
hsf-1 ADR I
hsf-1 ADR s
Survival (%)
Adult age (days)

Figure 1: The effect of gene knockdown on ADR-mediated longevity: A; *cbp-1*(RNAi), B; *crh-1(tz2)*, C; *cep-1(gk138)*, D; *skn-1(zu135)*, E; *nlp-7(tm2984)*, F; *cup-4(ok837)*, G; *hif-1(ia4)*, H; *hsf-1(sy441)*. FF is fully fed, ADRl is axenic dietary restriction in liquid medium, ADRs is axenic dietary restriction on solid medium. The graphs show the average results of at least 2 biological replicates. Numerical data is summarized in Table 1.

SKN-1 is an important transcription factor involved in the control of the oxidative stress response and because oxidative stress is considered a key cause of aging, it was proposed that SKN-1 may be a determining factor for aging (An and Blackwell, 2003). Indeed, loss of SKN-1 in the ASI neurons impaired the response to dietary restriction by BDR and *eat-2* mutation (Bishop and Guarente (2007)). However, SKN-1 is not necessary for sDR- or IF-induced longevity (Greer and Brunet, 2009; Honjoh et al., 2009). Park et al. (2009) identified two downstream targets of SKN-1, NLP-7 and CUP-4, that are required for normal lifespan and appear to be partially necessary for sDR- and *eat-2*-induced lifespan extension (Park et al., 2010). Our results show that both ADR conditions extend lifespan of *skn-1* mutants significantly ($P < 0.0001$) and the mutant does not display any lifespan shortening effects in these conditions, suggesting that SKN-1 is dispensable for ADR-mediated lifespan extension (Fig. 1D and 4, Table 1). This is reflected by the low and non-significant relative importance of *skn*-1 for the longevity effect of ADR (ADRl: -0.05, $P = 0.69$; ADRs: 0.17, $P = 0.058$; Fig.3). Against our expectations, one of the downstream targets of SKN-1, *nlp-7*, is slightly longer-lived than WT under ADRl ($P < 0.0001$) (Fig.1E and 4; Table 1) and is marginaly important for the ADR-mediated effect (-0.27, $P = 0.06$; Fig.3). On the other hand, *cup-4* seems to be involved in the ADR-mediated effect. Although the *cup-4* mutant shows lifespan extension under both ADR conditions ($P < 0.0001$ for both ADRl and ADRs; Fig.1F and 4; Table 1), *cup-4* is of high relative importance for ADR-mediated longevity (ADRl 0.65, $P = 0.027$; ADRs 0.59, $P = 0.03$; Fig. 3), indicating that this gene is as important as *cbp-1* for ADR-mediated lifespan extension (Student's *t*-test $P = 0.67$). CUP-4 is a coelomocyte-specific ion channel, involved in endocytosis, a basic function required for internalization of the extracellular fluid, nutrient uptake and recycling of membrane components. We suspect that *cup-4* is induced by signals other than those induced by the transcription factor SKN-1 and is one of the downstream players mediating lifespan extension. Contrary to the work of Park et al. (2009), we did not find that NLP-7 and CUP-4 are required for normal lifespan but instead the mutations lead to no change or even a significant lifespan extension under FF conditions ($P < 0.0001$ for *cup-4*), respectively. The fact that we do not find the expected lifespan shortening effect for *nlp*-7 in FF conditions may provide an explanation why we found ADRl extends *nlp*-7 lifespan to a larger degree compared to WT worms. Culturing in axenic medium during juvenile stages may induce a lifespan extending effect, explaining the absence of a lifespan shortening effect of *nlp-7* in FF conditions and maybe it is this effect we observe in ADRl conditions as well. Taken

together, these results indicate that it is unlikely that the complete SKN-1/CUP-4/NLP-7 pathway is activated by ADR in the same way as described by Park et al. (2010). However, CUP-4 is probably one of the more important downstream mediators of the ADR effect.

The hypoxia inducible transcription factor, HIF-1, is critical for the response to low oxygen concentrations. A loss-of-function mutation, *hif-1*, is reported to extend lifespan and it was shown that this lifespan extension is no longer present under sDR. The upstream negative regulator of *hif-1*, *egl-9*, is partially necessary for longevity by sDR (Chen et al., 2009). We could not confirm the lifespan extending effect of *hif-1* in FF conditions. In axenic conditions, *hif-1* lifespan is extended in a similar way as WT worms ($P < 0.0001$ for both ADRl and ADRs) and the mutation had no lifespan shortening effect, resulting in a low relative importance (ADRl 0.09, $P = 0.64$; ADRs 0.08, $P = 0.48$; Fig.3) . Contrary to the findings of Chen et al. (2009), our results indicate that HIF-1 does not play a major role in mediating the ADR effect (Fig.1G and 4, Table 1).

The heat shock transcription factor HSF-1 regulates the expression of many heat-inducible target genes, such as small heat shock proteins, and is involved in DR-induced lifespan extension by DD. It was suggested that it concerts its action by improving protein homeostasis (Steinkraus et al., 2008). Furthermore, it was found that *hsf-1* shortens lifespan in FF conditions. In agreement with this study, we found that *hsf-1* has a modest lifespan shortening effect in FF conditions ($P = 0.0047$). In both ADR conditions, *hsf-1* lifespan is significantly extended ($P < 0.0001$ for both ADRl and ADRs), although not as much as in WT worms (Fig.1H and 4, Table 1). This is reflected in the low relative importance of *hsf-1* for ADR-mediated longevity, which does not differ significantly from 0 (ADRl 0.22, $P = 0.11$; ADRs 0.23, $P = 0.21$; Fig.3), indicating that it is likely that the heat shock response is unimportant for the ADR effect.

4.3.2. The energy-sensing pathways: AAK-2

The energy-sensing AMP-activated protein kinase (AMPK) is activated by low energy levels and other stimuli increasing the AMP:ATP ratio. DR has been shown to increase this ratio (Greer et al., 2007) and *aak-2*, one of the catalytic subunits of AMPK, was found to be necessary for the lifespan extending effect of sDR (Greer and Brunet, 2009; Greer et al., 2007), but not for the longevity induced by *eat-2* (Greer and Brunet, 2009) or IF (Honjoh et al., 2009). Lifespan extension via bacterial dilution shows a partial dependence on AAK-2 (Greer and Brunet, 2009; Mair et al., 2009). Our results are in agreement with the latter studies as we found that mutation in *aak-2* partially suppresses the ADR-induced lifespan extension ($P < 0.0001$ for both ADRl and ADRs; Fig.2A and 4, Table 1), indicating that *aak-2* is relatively important for the ADR-induced effect, especially in ADRl conditions (ADRl: 0.39, $P = 0.002$; ADRs 0.22, $P = 0.13$; Fig. 3). These

results indicate a significant role for AAK-2 as one of the downstream mediators, at least for the ADRl-induced effect, but AAK-2 is not the central regulator of ADR-mediated longevity. However, it is possible that other AMPK family members, such as *aak-1*, compensate for the absence of *aak-2*.

4.3.3. Other genes involved in lifespan regulation: trx-1, sams-1, jnk-1 and ucp-4

Specific sensory cues may play an important role in mediating the ADR effect, indicating that genes involved in the aging process and expressed in sensory neurons, such as SKN-1, are good candidate effectors of ADR-induced longevity. *trx-1* encodes a thioredoxin expressed in the ASJ neurons, as well as in the proximal intestine (Jee et al., 2005). Thioredoxins act as anti-oxidants by reducing disulfide bonds in targeted proteins. Furthermore, they were shown to be involved in the aging process, since their overexpression extends lifespan in *C. elegans* and *trx-1* deletion was proven to shorten adult lifespan combined with an increased sensitivity to oxidative stress (Jee et al., 2005; Miranda-Vizuete et al., 2006). Fierro-Gonzalez et al. (2011) showed that TRX-1 is indispensable for lifespan extension induced by the *eat-2* mutation, as well as by DD. Although *trx-1* lifespan is significantly extended in both ADR conditions ($P < 0.0001$ for both ADRl and ADRs), we found that a mutation in the *trx-1* gene leads to a subtle decrease in longevity compared to the WT under ADR ($P < 0.0001$ for both ADRl and ADRs; Fig.2B and 4, Table 1) and its relative importance is rather low (ADRl 0.16, $P = 0.22$; ADRs 0.18, $P = 0.12$; Fig. 3). This questions the importance of *trx-1* for the lifespan-extending effect of ADR.

jnk-1 encodes a serine/threonine kinase and is the sole *C. elegans* member of the c-JUN N-terminal kinase (JNK) subgroup of the mitogen activated protein (MAP) kinases. It is activated in response to various environmental stress signals, including oxidative stress. Like TRX-1, JNK-1 was also shown to be a positive regulator of lifespan (Oh et al., 2005) and is mainly expressed in neuronal cells (Kawasaki et al., 1999). We found that *jnk-1* lifespan is extended by ADR ($P < 0.0001$ for both ADRl and ADRs; Fig. 2C and 4) and the relative importance of *jnk-1* for the ADR-mediated effect is low for both ADR conditions (ADRl 0.15, $P = 0.15$; ADRs -0.03, $P = 0.82$; Fig. 3). Contrary to the findings of Oh et al. (2005), FF *jnk-1* did not display reduced lifespan. From these results, it is clear that JNK-1 does not contribute to ADR-mediated longevity, nor is it a downstream effector.

ucp-4 encodes an uncoupling protein related to mammalian uncoupling protein 4, also known as solute carrier family 25. Consistent with the predicted uncoupling function, *ucp-4* animals display elevated ATP levels, but the mutation has no effect on lifespan (Iser et al., 2005). The Uncoupling-to-Survive hypothesis states that mild uncoupling contributes to DR-mediated

lifespan extension (Brand, 2000). Therefore, mutation in *ucp-4* may possibly reduce lifespan under ADR conditions. We found that *ucp-4* lifespan is still extended in both ADR conditions ($P < 0.0001$ for both ADRl and ADRs) and this extension is slightly but significantly reduced compared to the extension seen in WT worms, but only under ADRs conditions ($P < 0.0001$; Fig. 2D and 4). However, the relative importance of *ucp-4* for ADR-induced longevity indicates that UCP-4 is dispensable for this effect (ADRl -0.02, $P = 0.95$; ADRs 0.24; $P = 0.095$). These results allow us to conclude that UCP-4, and consequently uncoupling, does not play a major role in ADR-mediated longevity.

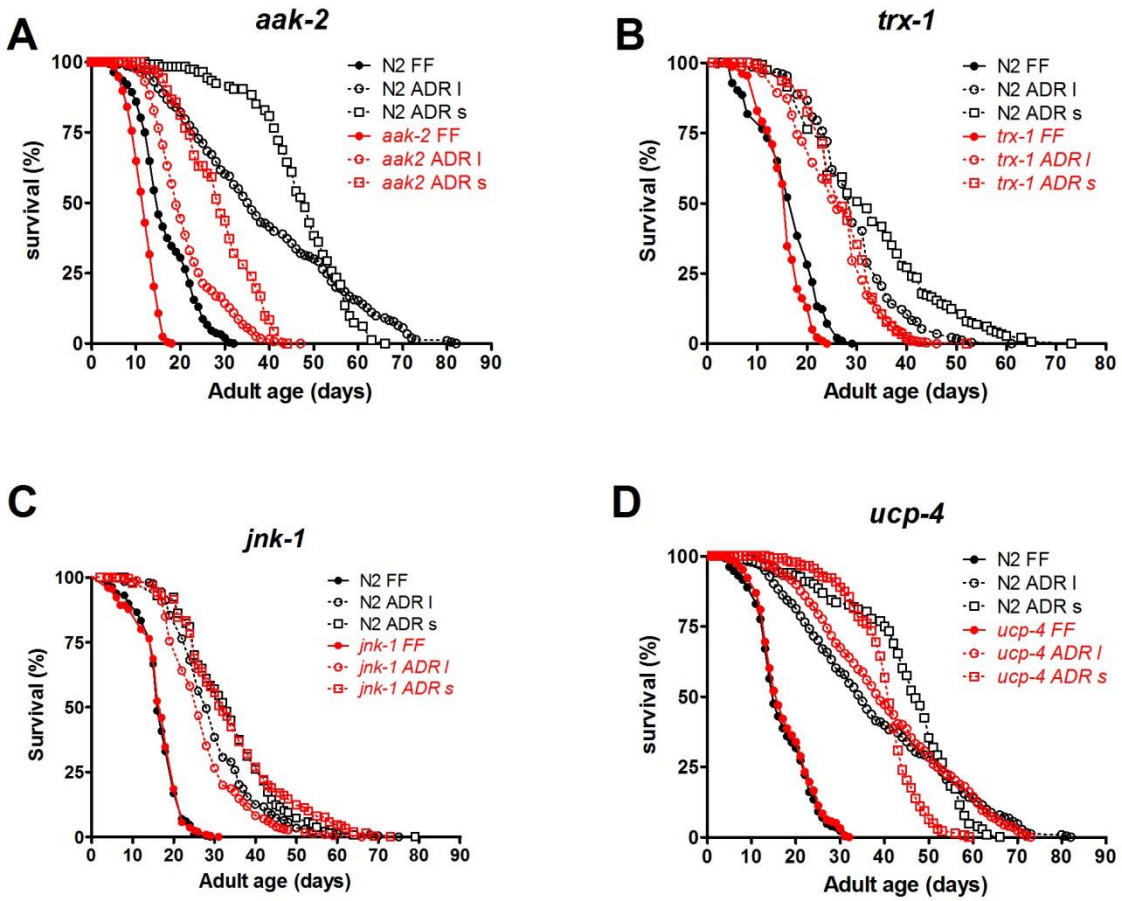

Figure 2: The effect of mutation in *aak-2(ok524)* (A), *trx-1(ok1449)* (B), *jnk-1(gk7)* (C) and *ucp-4(ok195)* (D) on ADR-mediated longevity. FF is fully fed, ADRl is axenic dietary restriction in liquid medium, ADRs is axenic dietary restriction on solid medium. The graphs show the average results of 3 biological replicates. Lifespan data is summarized in Table 1.

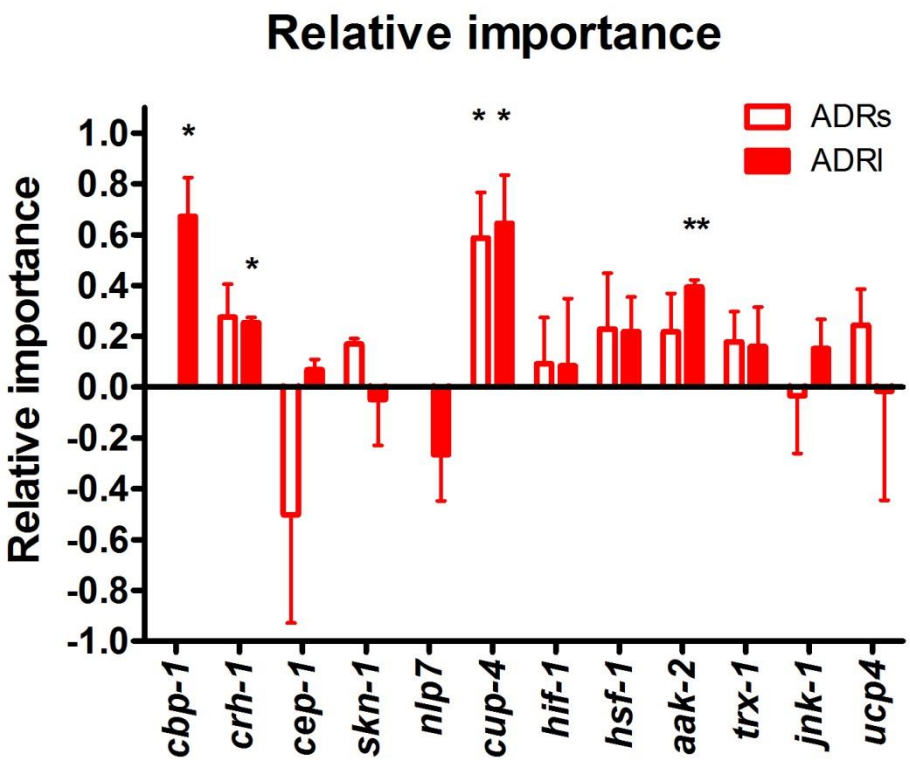

Figure 3: Relative importance of candidate genes for ADR-mediated longevity. A value of 0 indicates the gene is unimportant for the ADR-mediated effect. A value of 1 signifies that the gene is fully responsible for the ADR-mediated lifespan extension. A negative value suggests that the wild-type allele of the gene has a negative influence on ADR-induced longevity. The bars represent mean relative importance from at least 2 biological replicates ± SEM. * $P < 0.05$, ** $P < 0.01$.

4.3.4. Axenic culture during juvenile stages possible affects adult lifespan in certain mutants.

We noticed that for some genes (*nlp-7*, *cup-4*, *hif-1*, *cep-1* and *jnk-1*) literature reports a lifespan effect in FF culture conditions, which we could not reproduce in our experiments. An important difference and possible explanation is that, in our experimental setup, juveniles were raised in axenic medium until adulthood, before transfer to the experimental (FF and ADR) conditions. If worms were grown on *E. coli* plates and subsequently transferred to the ADR conditions, even the slightest trace of residual bacteria would easily overgrow the worms because of the nutritious environment and thereby compromising the long-term axenic culture. We cannot dismiss the possibility that this may have caused the discrepancies with earlier studies.

4.3.5. ADRs extends lifespan even further compared to ADRl.

ADRs conditions extend lifespan significantly further compared to ADRl conditions. We believe that ADRs is even a more stringent form of DR than the liquid condition because worms may have more problems extracting nutrients from the solid plates in which the nutrients are caught in the agar matrix than from the liquid medium which can be taken up easily.

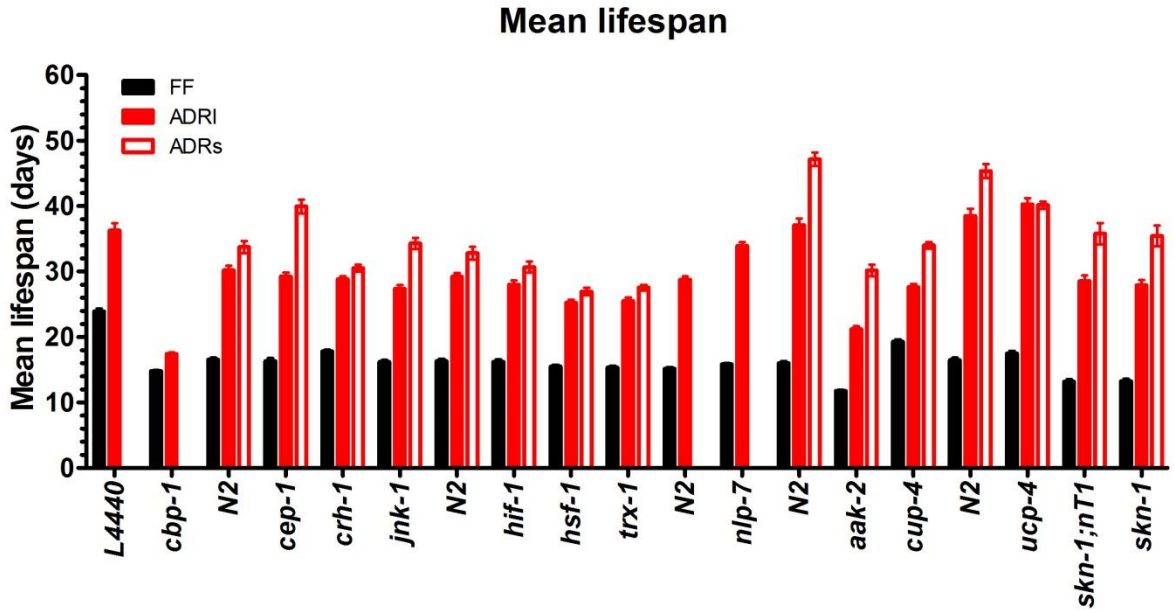

Figure 4: The effect of gene knockdown on mean lifespan in fully fed (FF), Axenic dietary restriction in liquid (ADRl) and solid (ADRs) conditions. The bars show the averages of at least 2 biological replicates ± SEM. Numerical data is summarized in Table 1.

Table 1: Mean Lifespan

Strain	Experiment	Mean Lifespan (±SD days)	#sample size/# trials	P-value[a]	P-value[b,c]
L4440 RNAi	FF	24.25±0.28	379/3		
	ADRl	39.27±1.00	239/3	$P < 0.0001$	
cbp-*1* RNAi	FF	14.81±0.14	278/3		$P < 0.0001$[c]
	ADRl	18.10±0.24	345/3	$P < 0.0001$	$P < 0.0001$[c]
N2	FF	16.55±0.31	268/3		
	ADRl	30.19±0.67	255/3	$P < 0.0001$	$P = 0.0130$[b]
	ADRs	33.73±0.92	324/3	$P < 0.0001$	
cep-1	FF	16.34±0.43	188/2		
	ADRl	29.23±0.62	182/2	$P < 0.0001$	$P < 0.0001$[b]
	ADRs	39.92±1.05	259/2	$P < 0.0001$	$P = 0.0001$[c]
crh-1	FF	17.81±0.28	199/2		
	ADRl	28.81±0.46	177/2	$P < 0.0001$	$P < 0.05$[b,c]
	ADRs	30.51±0.53	251/2	$P < 0.0001$	$P = 0.0003$[c]
jnk-1	FF	16.18±0.33	295/3		
	ADRl	27.36±0.57	255/3	$P < 0.0001$	$P < 0.0001$[b]
					$P = 0.0057$[c]
	ADRs	34.28±0.85	359/3	$P < 0.0001$	
N2	FF	16.31±0.37	290/3		
	ADRl	29.22±0.56	262/3	$P < 0.0001$	$P = 0.0004$[b]
	ADRs	32.79±0.96	395/3	$P < 0.0001$	
hif-1	FF	16.23±0.33	275/3		
	ADRl	27.9±0.62	249/3	$P < 0.0001$	$P = 0.014$[b]
	ADRs	30.66±0.84	362/3	$P < 0.0001$	
hsf-1	FF	15.46±0.3	298/3		$P = 0.0047$[c]
	ADRl	25.22±0.48	253/3	$P < 0.0001$	$P = 0.0269$[b]
					$P < 0.0001$[c]
	ADRs	26.95±0.55	398/3	$P < 0.0001$	$P < 0.0001$[c]
trx-1	FF	15.35±0.25	302/3		$P = 0.0002$[c]
	ADRl	25.51±0.51	256/3	$P < 0.0001$	$P < 0.0001$[c]
	ADRs	27.56±0.42	363/3	$P < 0.0001$	$P < 0.0001$[c]
N2	FF	15.15±0.25	463/4		

	ADRl	28.73±0.52	385/4	$P < 0.0001$	
nlp-7	FF	15.84±0.21	487/4		
	ADRl	33.91±0.56	409/4	$P < 0.0001$	$P < 0.0001$[c]
N2	FF	16±0.34	292/3		
	ADRl	37.16±1.04	295/3	$P < 0.0001$	$P = 0.006$[b]
	ADRs	47.15±1.03	287/3	$P < 0.0001$	
aak-2	FF	11.76±0.17	302/3		$P < 0.0001$[c]
	ADRl	21.2±0.44	300/3	$P < 0.0001$	$P < 0.0001$[b]
					$P < 0.0001$[c]
	ADRs	30.15±0.88	299/3	$P < 0.0001$	$P < 0.0001$[c]
cup-4	FF	19.28±0.36	301/3		$P < 0.0001$[c]
	ADRl	27.62±0.49	299/3	$P < 0.0001$	$P < 0.0001$[b]
					$P < 0.0001$[c]
	ADRs	34.01±0.49	301/3	$P < 0.0001$	$P < 0.0001$[c]
N2	FF	16.46±0.38	299/3		
	ADRl	38.50±1.10	298/3	$P < 0.0001$	
	ADRs	45.34±1.06	293/3	$P < 0.0001$	
ucp-4	FF	17.48±0.38	302/3		
	ADRl	40.27±0.94	299/3	$P < 0.0001$	$P = 0.0204$[b]
	ADRs	40.14±0.54	306/3	$P < 0.0001$	$P < 0.0001$[c]
skn-1;nT1	FF	13.20±0.33	223/3		
	ADRl	28.54±0.84	225/3	$P < 0.0001$	
	ADRs	35.77±1.61	224/3	$P < 0.0001$	
skn-1	FF	13.25±0.35	224/3		
	ADRl	27.98±0.78	225/3	$P < 0.0001$	$P < 0.0001$[b]
	ADRs	35.43±1.59	224/3	$P < 0.0001$	

Mean lifespan is represented as the average of at least 2 biological replicates. Notes: *P*-value compared to [a] own FF control, [b] ADRs conditions, [c] to N2 FF, ADRs or ADRl conditions.

4.4. Conclusion

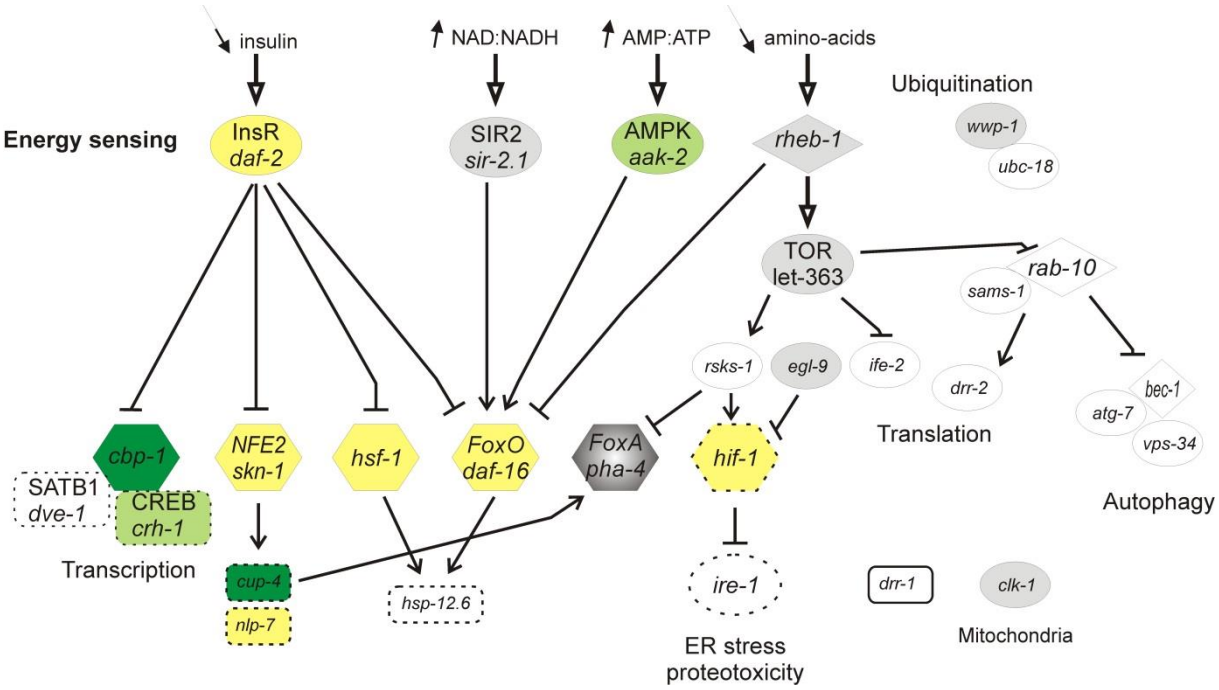

Figure 5: *C. elegans* DR network. Circles = enzymes; hexagons = transcriptional regulators; diamonds = small G proteins; rectangles = other proteins. Dark green: genes found to be important for ADR mediated longevity; Light green: genes found to be partially necessary for lifespan extension in ADRl conditions; yellow: genes found to be of little or no importance for ADR mediated longevity. Dark grey: genes tested in five to nine DR methods; light grey: genes tested in two to four DR methods; white genes: tested in one DR method. Dotted shapes represent genes partially necessary in all methods tested, or that have a similar effect on lifespan under DR and ad libitum conditions. In dark green are the genes that were found to be important for ADR mediated longevity. In light green are the genes that were found to be partially necessary in lifespan extension in ADRl conditions. Redrafted and adapted from Greer and Brunet (2011).

Both liquid and solid ADR significantly extend lifespan in all WT and mutant strains (Fig. 4, Table 1). Equally relevant effects on lifespan were found for *cbp-1* and *cup-4*, indicating that these genes play an important role in the ADR-mediated effect (dark green in Fig. 5). As suggested in Greer and Brunet (2011) (Fig. 5), it is very likely that the DR induced lifespan extension is not mediated by a single signaling pathway, but that several pathways act together as a DR network. Our results are in agreement with this statement since some genes involved in the lifespan extension of other DR regimens, such as *aak-2*, have only a partial effect on lifespan here (light green, Fig. 5), while still others such as *skn-1*, *nlp7*, *hsf-1* and *hif-1*, do not seem to mediate the ADR effect (yellow in Fig. 5). Since most of the genes assessed are knock-out mutants it is save to make clear conclusions about their role in ADR-mediated longevity but we also have to consider the possibility that other redundant genes limit their effect (e.g. *aak-1* is another AMPK family member that possibly compensates for *aak-2* absence). Moreover, some conditions used (*cbp-1* RNAi, *skn-1* hypomorphic mutation) are merely gene knock-down and a partial or absent

effect on lifespan does not mean that complete knock-down of these genes could not have a stronger effect. Currently, it remains unclear which molecular mechanism is at the foundation of the ADR-induced twofold lifespan extension. Although we screened for many candidate genes, Fig. 5 shows that the set was incomplete and we hope that further extensive screening will result in identification of the molecular mechanism downstream from CBP-1, clarification of the role of CUP-4 and possibly the tissue specificity of involved genes.

4.5. Acknowledgements

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PART III

Discussion

Chapter 5. General discussion and future perspectives

5.1. Role of energy metabolism in ADR-mediated longevity.

5.1.1. Rate of Living

Although many efforts were made recently to dissect the genetic pathways mediating the effects of dietary restriction in *C. elegans*, very little is known about the downstream biochemical processes. It was first suggested, in the light of the Rate-of-Living hypothesis, that the anti-aging effect of dietary restriction may be found in the reduction of metabolic rate and concomitant decrease in ROS production. However, the relationship between respiration and ROS production is not linear and ROS production depends upon the proton motive force, indicative of an inverse relationship: high proton motive force slows electron transport causing electron stalling and opportunities for electrons to escape to oxygen and thus promotes ROS production (Brand, 2000; Mookerjee et al., 2010). Furthermore, in *C. elegans*, it was shown that DR increases energy expenditure (Houthoofd et al., 2002b; Houthoofd et al., 2002c) and that increased respiration is a prerequisite for its lifespan extending effect (Bishop and Guarente, 2007; Schulz et al., 2007). These findings are supported by our results, since we found no evidence for a decrease of overall metabolic rate in the transcript profiles of axenically cultured *C. elegans*. The changes are rather discrete and point towards replenishing important carbohydrate compounds and glyceroneogenesis, which is in line with the low levels of lipids in axenic medium. Additionally, mitochondrial state 3 respiration and OXPHOS capacity were not or mildly decreased depending on the substrate used, in young adult worms. Considering the increased mitochondrial mass, this leads to the conclusion that ADR does not reduce respiratory rate presenting a further attenuation of the Rate-of-Living hypothesis.

5.1.1. Metabolic restructuring and longevity.

Although not always in agreement, several *C. elegans* studies pointed out that DR induces changes in the expression of genes involved in energy metabolism (Castelein et al., 2008; Mouchiroud et al., 2011; Szewczyk et al., 2006; Yuan et al., 2012). Measuring transcript abundance of genes involved in energy metabolism in axenically cultured worms, we observed the upregulation of two genes that have been linked to longevity before: PEPCK (*pck-1*) and ICL (*icl-1*). Overexpression of PEPCK is linked to increased lifespan and mitochondrial biogenesis in mouse skeletal muscle as well as in *C elegans* (Hakimi et al., 2007; Yuan et al., 2012) and *pck-1* RNAi decreases WT as well as *eat-2* lifespan. The increase in mitochondrial density found in axenically cultured *C. elegans* is in accordance with the upregulation of PEPCK levels. Together these data may suggest a role for this enzyme in the lifespan extending mechanism of DR (Yuan et al., 2012). The transcription of PEPCK is regulated by multiple transcription factors, such as CREB as well as nuclear factor I (NFI). Both transcription factors were found to be co-regulated

by CBP-1 and NFI has several isoforms that may act inhibitory as well as a stimulatory on PEPCK transcription depending on specific hormonal cues (Leahy et al., 1999). Since our results confirm the crucial role of CBP-1 in ADR-mediated longevity (Zhang et al., 2009) and PEPCK was upregulated in ADR conditions, it could be interesting to investigate whether *pck-1* and *nfi-1* possibly play a role in this effect. We already established that *crh-1*, the *C. elegans* CREB ortholog, does not have a major role in ADR-mediated longevity. Although *nfi-1* was found to be required for normal pharyngeal pumping rate and WT lifespan, it is not clear yet which genes are regulated by this transcription factor in *C. elegans* (Lazakovitch et al., 2008).

icl-1 encodes isocitrate lyase/malate synthase, an enzyme functioning in the glyoxylate pathway, previously known as *gei-7*. Activation of the glyoxylate pathway via the *icl-1* gene is required for the longevity phenotypes of Mit and *daf-2* mutants (Cristina et al., 2009; Gallo et al., 2011; Murphy et al., 2003). Moreover, *icl-1* RNAi reduced *eat-2* lifespan but ICL protein levels are lowered in *eat*-2 mutants and it was suggested that further decrease of already low protein levels is possibly detrimental explaining the decreased lifespan. The increased *icl-1* mRNA levels might hint towards a role for *icl-1* in ADR-mediated longevity. Nevertheless, such an involvement could not be confirmed (Huaihan Cai, personal communication).

As pointed out above, several studies in *C. elegans* show that DR induces metabolic changes (Castelein et al., 2008; Mouchiroud et al., 2011; Szewczyk et al., 2006; Yuan et al., 2012), but the results are not always in agreement. There are several possible reasons for this. First, the chosen methods of analyzing the metabolic profile differ between the studies. Proteomic versus transcriptomic analysis have been shown not to correlate entirely (Depuydt et al., 2013; Schwanhausser et al., 2011). Secondly, it is likely that worms subjected to different DR methods experience different cues, inducing complex regulatory networks leading to a precise and specific metabolic state. This is clearly reflected in the results of the lifespan assays to determine the molecular mechanism behind ADR-induced longevity as well. While most of the genes assessed were shown to play an important a role in one or multiple DR methods, except for *cbp-1* and *cup4*, not any of them is equally important for ADR-mediated longevity.

Interestingly, using metabolomic, proteomic and transcriptomic approaches, metabolic restructuring has been described for other longevity inducing mutants, such as *daf-2*, *ife-2* and Mit mutants (Cristina et al., 2009; Depuydt et al., 2013; Falk et al., 2008; Fuchs et al., 2010; Zuryn et al., 2010). Whether such metabolic restructuring is really required for longevity effects remains an important question. A possible clue comes from the involvement of the nuclear hormone receptor (NHR) NHR-49 in the induction of specific metabolic pathways (e.g. glyoxylate pathway) associated with the reduction of ETC subunit expression (Zuryn et al., 2010). A

deletion in the *nhr-49* gene attenuated this induction without affecting lifespan, thus uncoupling metabolic restructuring from lifespan effects. NHRs are critical regulators of animal metabolism and are well suited to link nutrient availability to transcriptional cascades. Recently, *nhr-62* was identified as a required factor for *eat-2* and BDR-induced longevity and transcriptional changes induced by the *eat-2* mutation were found to be regulated by *nhr-62* as well (Heestand et al., 2013). Interestingly, *nhr-49* displays functional similarity with mammalian peroxisome proliferator-activated receptors (PPARs) (Van Gilst et al., 2005a; Van Gilst et al., 2005b), which have characterized roles in fasting response and DR (Corton and Brown-Borg, 2005), and *nhr-49* was shown to be involved in nutrient responses (Van Gilst et al., 2005b). Furthermore, CBP is a known coactivator of several PPARs (Dowell et al., 1997), increasing the potency of NHRs, and especially NHR-49, to be involved in the (A)DR-mediated effect. Further research will have to point out, whether the DR-mediated metabolic changes and longevity will be able to be separated, similar as in Mit mutants, or whether these metabolic changes are a prerequisite for the DR-mediated longevity.

5.1.2. Mitochondrial efficiency and longevity

An increased and age-preserved bioenergetic competence was observed in axenically cultured worms. In aged worms that were cultured axenically, mitochondrial coupling was significantly increased and leakiness decreased compared to fully-fed controls. A similar age-preserving effect was found in *daf-2* mutants but these mutants did not display an increase in bioenergetic competence at young age. Although the effects of DR on mitochondria are ambiguous in other model organisms, such as rats and mice, often a common outcome of DR is either an increased or an age-preserved functionally efficient ETC (Bevilacqua et al., 2005; Hempenstall et al., 2012; Lal et al., 2001; Lanza et al., 2012; Lopez-Lluch et al., 2006; Sohal et al., 1994). However, this is not always the case (Lambert et al., 2004). It is clear that mitochondrial function changes with age and that DR has the potential to attenuate these changes but the question remains whether mitochondrial dysfunction is cause or consequence of aging. The fact that DR does not extend lifespan in the presence of ETC inhibitors in *C. elegans* (Bishop and Guarente, 2007) or absence of cytochrome c in yeast (Lin et al., 2002) suggest that mitochondria are critical factors for the DR-mediated effect. However, the requirement of respiration for DR-mediated lifespan extension may be species and DR-protocol dependent, since Kaeberlein et al. (2005) found that respiratory deficient yeast also displays DR-induced longevity depending on the DR protocol used.

Which factors could attribute to this DR-increased mitochondrial efficiency? There are several possible mechanism identified. As described in 1.2.3.2, DR has been suggested to increase mitochondrial biogenesis (Civitarese et al., 2007; Lopez-Lluch et al., 2006; Nisoli et al., 2005).

Along with an observed increase in mitophagy (Cuervo et al., 2005; Miwa et al., 2008), this possibly results in a higher turnover of the organelle and a quantitatively as well as qualitatively healthier mitochondrial pool (Cerqueira and Kowaltowski, 2013). These observations are in agreement with the emerging concept that a spare respiratory capacity of mitochondria forms a key feature ensuring survival under stressful conditions. Spare respiratory capacity is defined as the difference of basal respiration rate and the potential maximal respiratory capacity rate (Dranka et al., 2010) and was shown to be increased in the cerebella of calorie-restricted mice (Cerqueira et al., 2012). Since we found that ADR increased ETC capacity, beyond OXPHOS capacity, and also increased mitochondrial mass, our results are in line with this theory. Another factor described to contribute to high mitochondrial efficiency is the assembly and stability of supercomplexes, leading to facilitated electron transfer (described in 1.2.2.2). It has been suggested that supercomplexes are not required for maintaining basal respiratory capacity but for the support of spare respiratory capacity (Gomez and Hagen, 2012). Cardiolipin, a diphosphatidylglycerol lipid present in the IMM and important for the function of respiratory chain enzymes, seems to participate in the assembly and stabilization of the supercomplexes. Age-related alterations to the acyl chain composition of this lipid have been shown to contribute to destabilization of the supercomplexes (Paradies et al., 2010). Since, we found a slight upregulation of supercomplex abundance under ADR conditions, it might be interesting to investigate whether cardiolipin composition is changed under these conditions.

An interesting finding was the decrease of individual Complex IV abundance under ADR conditions. It is not clear what the specific reason of this decrease could be, but it has been proposed that Complex IV is a rate-limiting component of the respiratory chain *in vivo* (Kadenbach, 2003). Complex IV has been described as a source of proton slip, meaning that it can transfer electrons in the absence of proton pumping, at high proton motive force and this mechanism could attribute to a decreased coupling degree in the respiratory chain (Kadenbach, 2003). Furthermore, Complex IV activity is possibly regulated by the relative abundance of subunits II, Va and VIb (Weishaupt and Kadenbach, 1992). So, even though Complex IV abundance is decreased under ADR conditions, this does not mean that it will display reduced activity and it can even contribute to increased coupling efficiency. Determination of the specific subunit composition and enzymatic activity could help us to further elucidate the role of the observed changes.

The observed increase in coupling under ADR conditions is due to a decrease in LEAK. LEAK respiration is mainly a result of proton leak, a process in which protons diffuse across the IMM, in the absence of ATP synthesis and is the sum of a basal leak and an inducible leak. In contrast to the decreased proton leak and unchanged ROS production capacity under ADR conditions in

chapter 3, it has been suggested that increased proton leak reduces membrane potential and consequently reduces electron leak and ROS production (Mookerjee et al., 2010). Differences in proton conductance of the IMM have been correlated with the membrane phospholipid fatty acid composition (Porter et al., 1996). The more polyunsaturated fatty acids present, the higher the membrane proton conductivity. DR has been shown to induce a lower unsaturation/saturation index and probably this results in lower proton conductivity, which could explain the decreased LEAK respiration we observed. Additionally, the lower abundance of polyunsaturated fatty acids in DR conditions renders these fatty acids less prone to peroxidation (Merry, 2002). Whether similar changes occur under ADR conditions is unknown.

5.2. The role of ROS in ADR-mediated longevity

The implication of ROS in DR-mediated lifespan is controversial. The Uncoupling-to-Survive theory describes that mild uncoupling of mitochondria leads to decreased membrane potential and consequently lowered ROS production (Brand, 2000). On the other hand, mitohormesis has been suggested as an underlying mechanism of the DR effect, by increasing ROS production leading to upregulated ROS detoxification mechanisms (Ristow and Zarse, 2010). We found that ADR induces a slightly decreased membrane potential in accordance with the findings of Lemire et al. (2009) and at first sight, this supports the Uncoupling-to-Survive theory. However, Lemire et al. (2009) did not measure ROS production and we could not find decreased ROS production capacity of the mitochondria. Furthermore, H_2O_2 levels are unchanged in young adult worms and mitochondrial leakiness is significantly lower in ADR cultured *C. elegans*, resulting in increased coupling. Additionally, a deletion mutant of the only *C. elegans* uncoupling protein-like gene, *ucp-4*, contains elevated ATP levels but is not long-lived (Iser et al., 2005). In ADR solid conditions, *ucp-4*, and thus presumably uncoupling, displays as slightly decreased lifespan. Together, all these data question the importance of the Uncoupling-to-Survive theory for the ADR-mediated effect in *C. elegans*. However, we also have to mention that a DR-mediated attenuation of the age-dependent increase in H_2O_2 levels was observed in aging *C. elegans* (Back et al., 2012b). Since, we did not measure membrane potential and ROS production rates in mitochondria of aged axenically cultured worms and age-matched FF controls, we cannot completely dismiss the possibility that reduced ROS production at advanced age plays a role in ADR-mediated longevity.

Glucose-restriction has been shown to promote ROS production and increases catalase activity (Schulz et al., 2007). In ADR as well, ROS detoxification mechanisms were found upregulated (Houthoofd et al., 2002b). Furthermore, superoxide production capacity of isolated mitochondria from Mit mutants is increased (Yang and Hekimi, 2010a), but for ADR we did not find a similar result. However, this does not mean that mitohormesis can be completely dismissed as possible mechanism underlying the ADR effect. ROS production capacity of ADR

mitochondria is equally high as for mitochondria from FF controls. However, mitochondrial mass is increased and this could mean increased total ROS production. Together with the upregulated ROS detoxification systems this possibly results in equally high steady state H_2O_2 levels, as we found in accordance to Back et al. (2012b).

In a recent review, Walsh et al. (2013) surveyed available literature for trends in the effects of DR on oxidative stress in mammals. They found that, of all studies assessing mitochondrial ROS production, 63% reported no changes, while 37% reported a decrease in ROS production with DR. These results reflect the tendency of DR not to affect ROS production. However, it is also clear that the role of ROS is not straightforward and probably much more research will be devoted trying to elucidate its role in lifespan extension

5.3. The role of CBP-1 in ADR mediated longevity

Until now, CBP-1 and CUP-4 were the only identified factors capable of abolishing ADR mediated lifespan extension to a large extend ((Zhang et al., 2009) and chapter 4), which makes them interesting targets for further investigation. CBP-1 is a transcriptional regulator and although first identified as interaction partner for CREB, it is known to interact with many transcription factors, such as FoxO/DAF-16 and HIF-1. It would be interesting to link CBP-1 and its downstream effectors to the metabolic changes observed. Recently, Durieux et al. (2011) established that the mitochondrial unfolded protein response (UPR^{mt}) is required for ETC-mediated longevity. In response to mitochondrial perturbations, a signaling cascade is activated resulting in the upregulation of nuclear encoded mitochondrial chaperones, such as *hsp-6* and *hsp-60*. Members of this signaling cascade are UBL-5 and DVE-1. Interestingly, *dve-1* is the *C. elegans satb-1* ortholog and SATB-1 is a known interaction partner of CBP-1. Since the induction of the UPR^{mt} was observed under ADR conditions (Huaihan Cai, personal communication), it becomes very likely that CBP-1 plays a pivotal role in the initiation of this mechanism and its interaction partners may be successfully identified in the near future. The trigger for the induction of the UPR^{mt} could very well be the ADR-induced mitochondrial biogenesis, since it was shown that UPR^{mt} reporter gene activity is observed during the L3—L4 stage of larval development, when the germline begins to proliferate and induces a burst of mitochondrial biogenesis (Tsang and Lemire, 2002). How CUP-4 might be involved in ADR-mediated lifespan extension and whether its activation is the result of CBP-1 mediated signaling is unclear and is an interesting topic for future research.

5.4. General conclusions and perspectives for future research.

In conclusion, we found that culture in axenic medium led to a subtle metabolic restructuring and an increased and age-preserved bioenergetic efficiency. Two key players of the signaling pathway mediating the ADR effect were identified. In table 1, established findings concerning ADR-mediated longevity are completed with the new results described in this thesis and compared to other, often used DR methods, illustrating the complex phenomenon DR is. From the literature, in combination with our results, it is clear that dietary restriction induces metabolic changes that are species and even tissue-dependent and that differ according to the DR protocol applied. The signaling pathway leading to these changes requires more investigation. Using mutants disrupting the UPR^{mt}, it may be possible to reveal the importance of this response for ADR-mediated longevity. Furthermore, it would be interesting to find out which NHR might be involved in the ADR-mediated response. Using several *nhr* mutants, e.g. *nhr-49* or *nhr-62*, we may be able to identify one that is necessary for the longevity phenotype, or we might find that longevity and metabolic changes are not necessarily coupled. Whether the increased bioenergetic efficiency is a prerequisite for lifespan extension merits further attention as well. However, this is not easy to accomplish. The changes in mitochondrial bioenergetics are rather discrete and it may be problematic to find an intervention that undoes these changes in an equally subtle way. Further research into the formation of supercomplexes and their activity under ADR conditions may provide more insights in the role of mitochondria in the aging process, as would the elucidation of the effect of ADR on IMM composition. Also, analysis of the SU composition of CIV and the activity may yield a lot of information on how this complex possibly affects ETC efficiency.

Table 1: Comparing several DR methods: lifespan extension, genetics and biochemistry

	ADR	BDR	sDR	*eat-2*	IF	DD	GR
Lifespan % increase	50-150 %	32-101 %	18-35 %	0-57 %	30-57 %	42-50 %	
daf-16	I	I/PD	D	I	PD	I	
daf-2	I	I	I	D/I	PD	I	
eat-2	I		I			D	
aak-2	**PD (ADRl)**	I/PD	D	I	I		D
age-1		I					
cbp-1	D	PD		D			
wwp-1		PD		D			
pha-4		D	I	D/I	I		
hsf-1	**I**	D		I	PD	D	
hif-1	**I**			I		I	
clk-1			D	D	Pd		
skn-1	**I**		I	D	I		
cup-4	**D**		PD				
nlp-7	**I**		PD				
sir-2.1		I	I	D/I	I	I	I
rheb-1					D		
cep-1	**I**						
crh-1	**I**						
jnk-1	**I**						
trx-1	**I**						
Respiration	up	Up/no change		up			up
Heat dissipation	up			up			
ROS	**H_2O_2: No change**						Up
Mitochondrial bioenergetics	**Increased efficiency**						
References	[1-3]	[1, 3-8]	[7,9, 10]	[2,3,5,7,8, 10-18]	[19]	[16, 18, 20]	[21]

Table summarizing and comparing most studied DR methods with focus on genes that have been tested for their effect on DR-mediated longevity and that are also of interest in this thesis. The effects of different DR methods on lifespan and on biochemical changes are included as well. D, Dependent; PD, Partially dependent; I, Independent; The DR methods are ADR, axenic dietary restriction; BDR, bacterial dietary restriction (liquid); sDR, solid dietary restriction; *eat-2*; IF, intermittent fasting; DD, dietary deprivation; GR, glucose restriction. In green are the data that were collected during the research conducted fort his thesis. [1] Houthoofd et al., 2003 [2] Houthoofd et al., 2002 [3] Zhang et al., 2009 [4] Klass, 1977 [5] Panowski et al., 2007 [6] Mair et al., 2009 [7] Greer et al., 2009 [8] Carrano et al., 2009 [9] Greer et al., 2007 [10] Park et al., 2010 [11] Lakowski et al., 1998 [12] Wang et al., 2006 [13] Hansen et al., 2005 [14] Iser et al., 2007 [15] Hsu et al., 2003 [16] Kaeberlein et al., 2006 [17]Chen et al., 2009 [18] Lee et al., 2006 [19] Honjoh et al., 2009 [20] Steinkraus et al., 2008 [21] Schulz et al., 2007. Redrafted from (Greer and Brunet, 2011).

LIST OF ABBREVIATIONS

(A/G)TP	Adenosine/Guanosine triphsophate
Acetyl-CoA	Acetyl-Coenzyme A
ADR	Axenic dietary restriction
ADRl	Axenic dietary restriction liquid
ADRs	Axenic dietary restriction solid
ANT	Adenine nucleotide transferase
AMPK	AMP-activated protein kinase
BDR	Bacterial dilution dietary restriction
BN-PAGE	Blue Native-Polyacrylamide gelelektroforese
CBP	CREB binding protein
CeMM	*C. elegans* maintenance medium
CR	Calorie restriction
CREB	cAMP responsive bindin element
Cu/Zn	Cupper/Zinc
DD	Dietary deprivation
DR	Dietary restriction
drr	dietary restriction related
ETC	Electron transport chain
FA	Fatty acids
FAD	Flavin adenine dinucleotide
FF	Fully fed
FRTA	Free radical theory of aging
GFP	Green fluorescent protein
HRR	High resolution respirometry
IF	Intermittent fasting
IIS	Insulin/IGF
IMM	Inner mitochondrial membrane
ISE	Ion-sensitive electron

Mit	Mitochondrial
Mn	Manganese
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OMM	Outer mitochondrial membrane
Omy	Oligomycin
OxPhos	Oxidative phosphorylation
PD	Peptone dilution
PEEK	Polyetheretherketone
POS	Polarographic oxygen sensor
qRT-PCR	Quantitative reverse-transription polymerase chain reaction
RCR	Respiratory control ratio
RET	Reverse electron transfer
ROS	Reactive oxygen species
sDR	Solid dietary restriction
SOD	Superoxide dismutase
St 2	State 2
St 4	State 4
TCA	Tricarboxylic acid cycle
TPP	Tetraphenylphosphonium
UPR^{mt}	Mitochondrial unfolded protein response
VDAC	Voltage dependent anion channel

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Curriculum Vitae

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Education

2007-present	PhD in Biology, Lab of Aging Physiology and Molecular Evolution, Ghent University, Belgium
2004-2006	Master in Biomedical Sciences, with high distinction, Ghent University, Belgium
	Master thesis: Researching the therapeutic potential of glycolipids in animal models for rheumatoid arthritis and tumors.
	Department of Internal Medicine, Rheumatology-Molecular Immunology and Inflammation, Ghent University, Belgium
2001-2004	Bachelor in Biomedical Sciences, with distinction, Ghent University, Belgium

Publications

Castelein N, Hoogewijs D, De Vreese A, Braeckman BP, Vanfleteren JR. Dietary restriction by growth in axenic medium induces discrete changes in the transcriptional output of genes involved in energy metabolism in *Caenorhabditis elegans*. Biotechnol J 3 (2008), 803-812.

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Abstracts

Castelein N, Vanfleteren J. Poster presentation. "Effects of dietary restriction by axenic medium on mitochondrial function and metabolism in the nematode worm *C. elegans*" MARK-AGE/LINK-AGE Joint Summer School-From molecular mechanisms to biomarkers of human ageing. Fréjus, France (2009).

Castelein N, Vanfleteren J. Poster presentation. Poster presentation. "Effects of dietary restriction by axenic medium on mitochondrial function and metabolism in the nematode worm *C. elegans*" MiMage conference-Mitochondria in Ageing and Age-Related Disease, Les Diablerets, Switzerland (2009).

Castelein N, Berhanu K, Braeckman BP. Poster presentation. "Effects of dietary restriction by axenic medium on mitochondrial function in *Caenorhabditis elegans*" Aging, Metabolism, Stress, Pathogenesis, and Small RNAs in *C. elegans*. Topic Meeting, Madison WI, USA (2012). **Award winning poster:** one of the 10 best posters on a total of 118 posters presented on the meeting.

Attended workshops

50th International Course on High-Resolution Respirometry. April 18-22, 2009. Schröcken, Vorarlberg, Austria.

61st International Course on High-Resolution Respirometry. Advanced users group with focus on O2k-Multisensor applications of the TPP^+ electrode. April 26- May 01, 2011, Schröcken, Vorarlberg, Austria.

Teaching related activities

Practical courses

- Physiological Regulation in Animals (3rd Bachelor Biology), Prof. Dr. Bart P. Braeckman.

Bachelor projects

- 2008 Gregory Coucke "Onderzoek naar de mitochondriale deletiefrequentie in verouderende *C. elegans*."
- 2010 Lieselot Verduyn "Optimalisatie van een protocol voor het meten van *in vivo* ATP levels in *C. elegans"*

Master Projects

- 2009-2010 Haeike Josephy "Onderzoek naar de mitochondriale functie in verouderende en/of calorisch gerestricteerde *C. elegans*"
- 2010-2011 Berhanu Chekol Kassa "Identification of genes necessary for lifespan extension by different dietary restriction regimens in the nematode *C.elegans*"
- 2011-2012 Selamawit Araya Kidane "Quantification of reactive oxygen species (ROS) in dietary restricted *Caenorhabditis elegans."*