# Genetic modification of insulin/IGF-1 signalling promotes survival in a *C. elegans* model of mitochondrial dysfunction

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

**Sven Büttner**

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# Glossary of Abbreviations

| | |
|---|---|
| 2-NBDG | 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose |
| *aak-2* OE | *aak-2* overexpression |
| AMPK/AAK-2 | AMP-activated protein kinase / AMP activated kinase 2 |
| ABC | ATP-binding cassette |
| ACDH-1 | Acyl CoA dehydrogenase 1 |
| AD | Alzheimer's disease |
| ADP | Adenosindriphosphate |
| AGE-1/PI3K | Ageing alteration 1 / phosphoinositide 3-kinase |
| AICAR | 5-aminoimidazole-4-carboxamide riboside |
| AIF | Apoptosis Inducing Factor |
| AKT-1/2 | Akt kinase family 1/2 |
| ALS | Amyotrophic lateral sclerosis |
| AMP | Adenosinmonophosphate |
| ATFS-1 | Activating transcription factor associated with stress |
| ATP | Adenosintriphosphate |
| Aβ | Amyloid beta |
| BCAA | Branched chain amino acid(s) |
| bHLH | basic helix-loop-helix domain |
| C I | Complex I / NADH-ubiquinone oxidoreductase |
| C II | Complex II / succinate dehydrogenase |
| C III | Complex III / cytochrome c reductase |
| C IV | Complex IV / cytochrome c oxidase |
| C V | Complex V / ATP synthase (ATPase) |
| CaMKKβ | Calmodulin-dependent kinase kinase β |
| cDNA | Complementary DNA |
| CGC | Caenorhabditis Genetics Centre |
| CLPP-1 | Clp protein 1 |
| COPAS (Biosort) | Complex Object Parametric Analyzer and Sorter |
| CPEO | chronic progressive external ophthalmoplegia |

| | |
|---|---|
| CypA | Cyclophilin A |
| Cyt c | Cytochrome c |
| DAF-16/FOXO | Abnormal dauer formation 16 / forkhead-box-protein |
| DAF-18/PTEN | Abnormal dauer formation 18 / phosphatase and tensin |
| DAF-2 | Abnormal dauer formation 2 |
| DCF-DA | 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate |
| DDL-1/2 | daf-16-dependent longevity 1/2 |
| DVE-1 | defective proventriculus 1 |
| EGL-9/PHD | Egg laying defective 9 / prolyl hydroxylase domain protein |
| EndoG | Endonuclease G |
| ER | Endoplasmic reticulum |
| EtBr | Ethidium Bromide |
| ETC | Electron transport chain |
| ETF | Electron transfer flavoprotein |
| $FADH_2$ | Flavin adenine dinucleotide (reduced) |
| FIH-1 | Factor-inhibiting HIF-1 |
| G-6-P | Glucose-6-phosphate |
| G-6-PDH | Glucose-6-phosphate dehydrogenase |
| *gas-1* | General anaesthetic sensitivity abnormal 1 |
| GFP | Green fluorescent protein |
| *gpd-3* | Glyceraldehyde 3-phosphate dehydrogenase 3 |
| *gst-4* | Glutathione-S-transferase 4 |
| HAF-1 | Half-molecule ATP-binding cassette (ABC) transporter |
| HCF-1 | Host cell factor 1 |
| HD | Huntington's disease |
| HSF(-1) | Heat shock factor (1) |
| HSP(-6/-60/90) | Heat shock protein (6/60/90) |
| IGF-1 | Insulin-like growth factor 1 |
| IGF1R | Insulin-like growth factor 1 receptor |
| IIS | Insulin/IGF-1 signalling |
| IM | (Mitochondrial) inner membrane |
| IMS | (Mitochondrial) intermembrane space |

| | |
|---|---|
| IPTG | Isopropyl-β-D-1-thiogalactopyranoside |
| JNK-1 | c-Jun N-terminal kinase 1 |
| LB | Lysogeny Broth |
| LDH | Lactate dehydrogenase |
| LHON | Leber hereditary optic neuropathy |
| MELAS | myopathy, encephalopathy, lactic acidosis, and stroke |
| MEMSA | myoclonic epilepsy myopathy sensory ataxia |
| MERRF | myoclonic epilepsy with ragged red fiber |
| *mev-1* | Abnormal methyl viologen sensitivity |
| mtDNA | Mitochondrial DNA |
| mTOR | Mechanistic target of rapamycin |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| NARP | neuropathy, ataxia, retinopathia pigmentosa |
| NF-κB | Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| NGM | Nematode Growth Medium |
| NHR-57 | Nuclear hormone receptor 57 |
| O/N | Over night |
| OM | (Mitochondrial) outer membrane |
| OXPHOS | Oxidative phosphorylation |
| p53 | Tumor protein 53 |
| *pck-1*/PEPCK | Phosphoenolpyruvate carboxykinase |
| pDH | pyruvate dehydroxygenase |
| PDK-1 | Pyruvate dehydrogenase lipoamide kinase isozyme |
| PEO | Progressive external ophthalmoplegia |
| PINK1 | PTEN-induced putative kinase protein 1 |
| POLG | Mitochondrial DNA polymerase subunit gamma |
| PRMT-1 | Protein arginine methyltransferase 1 |
| $Q_{10}$ | Ubiquinone-10 |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| RACK1 | Receptor of activated protein kinase C |
| RLE-1 | Regulation of longevity by E3 ubiquitin ligase ring finger protein |
| RNAi | Ribonucleic acid interference |

| | |
|---|---|
| ROS | Reactive oxygen species |
| rRNA | ribosomal RNA |
| RT | Room temperature |
| SGK-1 | Serine/threonine-protein kinase |
| SIRT1 | Sirtuin 1 |
| SKN-1/Nrf2 | Skinhead 1 / nuclear factor (erythroid-derived 2)-like 2 |
| SMK-1/SMEK | Suppressor of MEK |
| tAIF | Truncated AIF |
| TAK-1 | Transforming growth factor-β-activated kinase |
| TCA | Tricarboxylic acid cycle |
| TFAM | Mitochondrial transcription factor A |
| TK2 | Thymidine kinase 2 |
| tRNA | transfer RNA |
| UBL-5 | ubiquitin-like protein 5 |
| UPR | Unfolded protein response |
| UPRmt | Mitochondrial unfolded protein response |
| VHL | von Hippel-Lindau protein |
| WAH-1 | Worm AIF homologue 1 |

# Species-specific nomenclature

*C. elegans* proteins are written as a three- to four-letter code in CAPITAL letters. Digits are separated by a hyphen. E.g.:
WAH-1

*C. elegans* genes are written as a three- to four-letter code in small letters in *italics*. Digits are separated by a hyphen. E.g.:
*wah-1*

Vertebrate proteins are written as a three- to four-letter code in CAPITAL letters. No separation between letters and digits. E.g.:
AIF

Vertebrate genes are written as a three- to four-letter code with the first letter CAPITAL and the other letters in lower case. No separation between letters and digits. E.g.:
Aif

Human proteins are written as a three- to four-letter code in CAPITAL letters. No separation between letters and digits. E.g.:
AIFM1

Human genes are written as a three- to four-letter code in CAPITAL letters in *italics*. No separation between letters and digits. E.g.:
*AIFM1*

# 1 Summary

Mitochondria are organelles in the cytoplasm of almost all eukaryotic cells. They supply large quantities of energy in the form of adenosine triphosphate (ATP) and are involved in cell signalling, differentiation and cell death. Mutations in mitochondrial genes cause organelle dysfunction and lead to a heterogeneous group of disorders. Apoptosis Inducing Factor (AIF) is a mitochondrial protein required for the maintenance of respiratory complex subunits and efficient oxidative phosphorylation (OXPHOS). Altered AIF function causes severe human pathologies and recapitulates the majority of features commonly observed in other mitochondrial disorders. There are no available treatments for mitochondrial disorders.

The present study demonstrates that AIF (or WAH-1 in nematodes) deficiency results in electron transport chain (ETC) defects in *Caenorhabditis elegans*. Consequently, impaired OXPHOS leads to defective mitochondrial function and shortens nematode lifespan. Conversely, reduced insulin/IGF-1 signalling elicits a compensatory response that results in lifespan extension independent of the downstream transcription factor DAF-16/FOXO. Compromised OXPHOS and decreased insulin/IGF-1 signalling promote stress response, AMPK/AAK-2 activation and autophagy. Importantly, enhancing AMPK/AAK-2 activity is sufficient to extend the survival of AIF/WAH-1 impaired animals and requires autophagy.

The identified cellular response mechanisms result in metabolic remodelling, protect from altered mitochondrial respiration and ultimately lead to increased survival of animals with compromised OXPHOS. These results may contribute to the development of novel strategies for the treatment of mitochondrial disorders.

# 2 Introduction

## 2.1 Mitochondria

Mitochondria are highly abundant organelles present in the vast majority of eukaryotic cells. Their number and morphology can differ greatly, depending on the cell type and its bioenergetic demands. The primary function of mitochondria is energy production. They provide the vast majority of cellular adenosine triphosphate (ATP). ATP is the fuel of any cell and often rhetorically referred to as the "cellular currency". The transfer of phosphate residues from ATP to proteins and enzymes is indispensable for all cellular processes that require energy. Mitochondria produce ATP through an intricate process termed oxidative phosphorylation (OXPHOS). This metabolic pathway produces ATP through a series of redox reactions catalysed by the mitochondrial electron transport chain (ETC). In addition to energy production, mitochondria are the central orchestrator of metabolism and execute crucial chemical reactions and functions for the cell, such as the tricarboxylic acid (TCA) cycle (Krebs, 1979), fatty acid β-oxidation (Houten & Wanders, 2010), $Ca^{2+}$ uptake (Rottenberg & Scarpa, 1974) as well as iron and metal homeostasis (Lill & Muhlenhoff, 2008). Furthermore, mitochondria take part in apoptotic cell death, a process which is essential for proper development and health (Green & Reed, 1998).

### 2.1.1 Morphology

Mitochondria are hypothesised to have evolved from an ancestral α-proteobacterium that infiltrated cells and was engulfed as an endosymbiont (Gray, 2012). Therefore, mitochondria still carry part of their own genome, termed mitochondrial DNA (mtDNA), which encodes for a subset of electron transport chain (ETC) proteins (Chapter 2.1.2 ). The remaining ETC proteins are encoded by nuclear DNA and imported into mitochondria. Due to their proteobacterial origin, mitochondria consist of an outer membrane (OM), an intermembrane space (IMS), an inner membrane (IM) and the mitochondrial matrix.

The OM contains membrane proteins that control mitochondrial import and export of proteins, molecules and ions (Gellerich *et al.*, 2000). These transport mechanisms are crucial for the import of the nuclear encoded ETC proteins and metabolites that are required for mitochondrial energy production.

The IMS is a reservoir for hydrogen ions that contains a variety of proteins and molecules that assist in the function of the respiratory chain and ensure efficient energy production (Saraste, 1999).

The IM is a highly folded membrane that shapes the mitochondrial matrix into cristae. This morphology ensures an efficient ratio between volume and surface area, as many of the crucial mitochondrial reactions occur along the IM. The homeostasis between components in the mitochondrial IMS and the matrix is regulated by transmembrane proteins in the IM. Importantly, many proteins of the ETC are integrated in the IM.

The mitochondrial matrix contains the mitochondrial genome. Proteins, such as ETC subunits, are produced and assembled in this compartment. Furthermore, important metabolic processes such as the tricarboxylic acid (TCA) cycle and fatty acid β-oxidation take place in the matrix.

Altogether, proteins within the different mitochondrial membrane spaces exert diverse and specialised functions. Since the single mitochondrial compartments serve as rather isolated reaction spaces, an interconnected network of proteins and signalling processes is indispensable for mitochondrial function.

### 2.1.2 Electron Transport Chain

The ETC is a functional series of proteins that produce ATP by OXPHOS. To this end, mitochondria shuttle electrons in the form of hydride protons ($H^+$) along the respiratory chain. NADH and $FADH_2$, which are produced during the TCA cycle, glycolysis, β-oxidation and other catabolic processes, serve as an electron source to fuel the OXPHOS process. The respiratory chain is comprised of 5 individual complexes anchored to the IM. Two mobile electron carriers in the IMS, ubiquinone-10 ($Q_{10}$) and cytochrome c (Cyt c), assist in the transport of electrons through the ETC. During this

process other hydrogen ions are transported into the IMS. This produces a H+ proton gradient between the IMS and the matrix across the IM. The proton gradient is ultimately utilised for the formation of ATP from ADP and free phosphate in the mitochondrial matrix.

For efficient OXPHOS the electron transfer follows regulated redox reactions through the ETC complexes:

- NADH-ubiquinone oxidoreductase (complex I or C I)

C I is an integral protein of the electron transport chain that receives hydride ions from NADH and passes the electrons on to ubiquinone.

- Succinate dehydrogenase (complex II or C II)

C II is a peripheral protein that receives electrons from succinate, which is an intermediate metabolite of the TCA cycle. C II uses these electrons to reduce the prosthetic group of FAD to $FADH_2$ to subsequently pass the electrons to ubiquinone.

- Ubiquinone ($Q_{10}$)

Ubiquinone is a mobile protein that can diffuse across the membrane. Upon reduction by C I and C II, ubiquinol (the reduced form of ubiquinone) shuttles electrons to C III.

- Cytochrome c reductase (complex III or C III)

C III then passes electrons on to Cyt c. C III receives electrons via $Q_{10}$ not only from C I and C II but also from glycerol-3-phosphate dehydrogenase and the electron transfer flavoprotein (ETF).

- Cytochrome c oxidase (complex IV or C IV)

C IV receives electrons from Cyt c. These electrons are then transferred to oxygen, and produce water within the mitochondrial matrix.

- ATP synthase (ATPase or complex V or C V)

C V is ultimately responsible for the generation of ATP. During electron transfer along C I, C III and C IV, $H^+$ protons are actively translocated across the IM into the mitochondrial IMS. This creates a proton gradient and an electrochemical potential across the membrane. ATPase utilises the reflux of protons into the matrix to drive a mechanical rotor. The energy from this rotor is used to phosphorylate ADP to ATP. This process is termed oxidative phosphorylation (OXPHOS) (Mitchell & Moyle, 1967)

### 2.1.3 ETC subunits

The membrane potential that is generated by the proton gradient is required for many mitochondrial processes. Apart from ATP generation it plays an important role for $Ca^{2+}$ homeostasis (Rottenberg & Scarpa, 1974), transport of metabolites across the IM (Eilers *et al.*, 1987) and apoptosis (Zamzami *et al.*, 1995). Therefore, efficiency of OXPHOS and ETC complex function is crucial for mitochondrial and cellular health.

Correct shuttling of electrons through the protein complexes and pumping of protons into the IMS ultimately depends on the stability of the ETC complexes. As the name already suggests, ETC complexes are comprised of several different subunits. These subunits are specific to each ETC complex. Nuclear genes encode for the majority of the 90 subunits that comprise the ETC complexes. However, 13 proteins are encoded by mtDNA and provide subunits for all complexes except C II. Furthermore, mtDNA encodes for other working units such as ribosomal RNA (rRNA) and transfer RNAs (tRNAs). They are required for the translation of mitochondrial genes and consequently for mitochondrial function. Therefore, the correct assembly of the subunits from different sources to functional complexes requires intricate regulation and strategy.

The stability of ETC complexes is promoted by two main mechanisms: The formation of supercomplexes and proteins that contribute to the stabilisation. Unlike the

classical textbook view of the ETC as a fluid-state model, ETC complexes are capable of forming supercomplexes. For instance, the supercomplex comprised of various stoichiometries of C I, C III and C IV builds a functional electron-channelling unit (Bianchi *et al.*, 2004; Schagger, 2001). This respirasome has been proposed to increase the efficiency of the respiratory chain by bringing the electron carriers in close proximity (Chance & Williams, 1955; Schafer *et al.*, 2006). Another supercomplex, comprised of dimeric ATPase, is crucial for the folding of the inner membrane and formation of cristae (Rabl *et al.*, 2009; Saddar *et al.*, 2008). Furthermore, dimers of ATPase form the mitochondrial permeability transition pore, a key effector of cell death (Giorgio *et al.*, 2013). Additional proteins and factors can also stabilise respiratory complexes and comply protective functions. For instance, the assembly of the biggest complex of the respiratory chain, C I, relies on a variety of factors such as C3orf1, TMEM126B and Ndufaf1 (McKenzie 2010).

## 2.2 Mitochondrial dysfunction, pathology and disease

Owing to the highly diverse mitochondrial function, impairment of the organelle can lead to drastic cellular consequences. Oxidative stress and subsequent damage of cellular components was hypothesised to substantially contribute to biological decline (Droge & Schipper, 2007; Romano *et al.*, 2010). In fact, aged mitochondria display reduced OXPHOS and ATP production but increased reactive oxygen species (ROS) levels (Gomez *et al.*, 2009; Gouspillou *et al.*, 2014; Sgarbi *et al.*, 2014). Impaired ETC function is the primary source for increased ROS production (Bratic & Trifunovic, 2010). The mitochondrial theory of ageing suggests a vicious cycle of the accumulation of oxidative damage to proteins, DNA and lipids by ROS (Gutierrez *et al.*, 2006; Pamplona & Barja, 2006; Radak *et al.*, 2011). As a result, damaged mitochondria generate even more ROS. Nevertheless, ROS are not just harmful by-products of (impaired) mitochondrial function. They are rather important cellular signalling molecules (D'Autreaux & Toledano, 2007).

### 2.2.1 Mitochondrial diseases

Dysfunction of mitochondrial activity, dynamics, trafficking and transport as well as altered morphology may cause or contribute to neuronal loss (Douglas & Dillin, 2010; Gkikas *et al.*, 2014; Parashos *et al.*, 2014; Sgarbi *et al.*, 2014). Consequently, mitochondria are implicated in the pathophysiology of late-onset, progressive neurodegenerative diseases such as Alzheimer`s, Parkinson`s and Huntington`s disease, amyotrophic lateral sclerosis (ALS) or Charcot-Marie-Tooth (Cassereau *et al.*, 2011; Lin & Beal, 2006). Furthermore, due to the complexity of mitochondria, impaired function of many mitochondrial proteins can lead to clinically heterogeneous syndromes, also referred to as "mitochondrial diseases". Many of the diseases arise as a result of ETC dysfunctions and may present at any age (Chinnery, 2014; Zeviani & Di Donato, 2004). Whereas acquired mitochondrial disorders manifest from accumulation of mitochondrial damage over time, inherited disorders are caused by mutations in nuclear or mtDNA genes. Furthermore, the unique character of mitochondrial genetics displays maternal and autosomal variations, making genetic counselling challenging. While some mitochondrial disorders only affect single organs, many involve multiple organ systems and often present with progressive neuronal decline and myopathic features (Venna, 2004) (Figure 2-1).

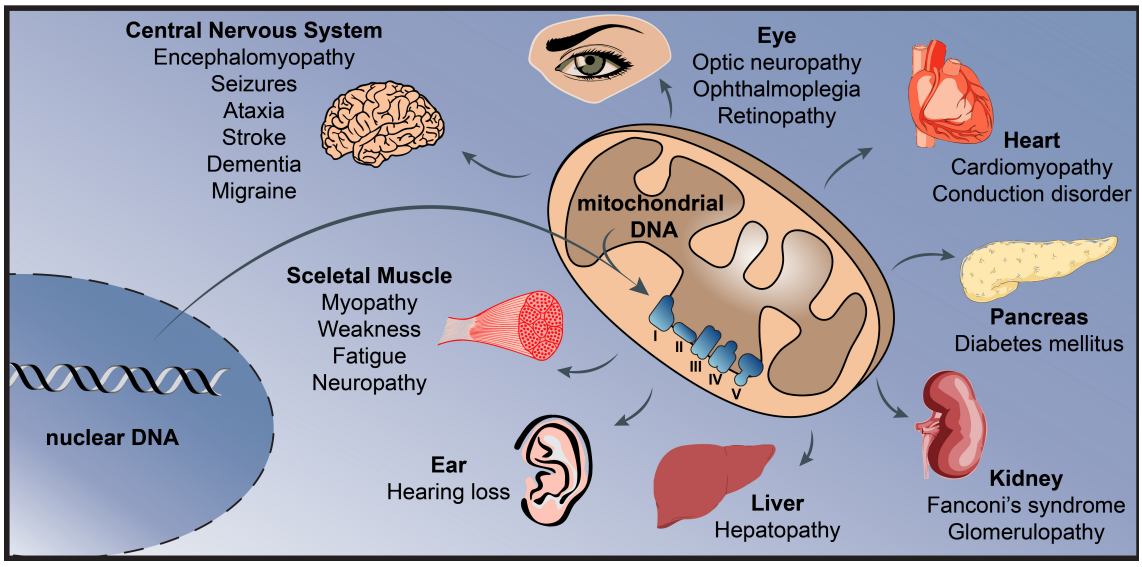


Figure 2-1: **Organ systems commonly affected by mitochondrial dysfunction.** Inherited and spontaneous mutations in mitochondrial genes encoded by nuclear or mitochondrial DNA can cause severe mitochondrial dysfunction. The resulting syndromes can be very heterogeneous and a variety of organs may be affected.

Accordingly, cells with high-energy expenditure such as neurons, myocytes and cardiomyocytes are most vulnerable to changes in mitochondrial function. Consequently, most syndromes, e.g. Leigh syndrome; neuropathy, ataxia, retinopathia pigmentosa (NARP); mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke (MELAS); myoclonic epilepsy myopathy sensory ataxia (MEMSA); Leber hereditary optic neuropathy (LHON); myoclonic epilepsy with ragged red fiber (MERRF) and chronic progressive external ophthalmoplegia (CPEO) display common pathological phenotypes such as neuronal degeneration, muscular atrophy and vision impairments (Schapira, 2012). However, even tissues with a relatively low metabolic demand can be affected, including peripheral nerves, sensory organs, kidneys and endocrine tissues (DiMauro, 2004).

The complexity of the mitochondrial system and variability of symptoms and genetic mutations make both diagnosis and treatment of mitochondrial disorders a challenge. No effective treatments have been developed so far and therapy is mainly limited to the amelioration of the specific symptoms (Benit *et al.*, 2008). Affected patients are commonly treated by dietary intervention. Furthermore, individuals with ETC deficiency may benefit from administration of riboflavin or coenzyme $Q_{10}$. However a cure is not available. In part, this can be attributed to the lack of suitable animal models for research.

### 2.2.2 Animal models of mitochondrial disease

Many mitochondrial diseases result from mutations in mtDNA. The development of mouse models that carry mutations in the mitochondrial genome has been challenging. Due to the non-Mendelian inheritance of mtDNA, mitochondrial mutations are transmitted maternally and therefore only inherited from the mother to her children (Dogan & Trifunovic, 2011). Furthermore, each cell contains hundreds of mtDNA copies. This results in mtDNA heteroplasmy, describing the presence of different mtDNA variants in a single cell. Dependent on the cellular energy demands, heteroplasmy leads to a threshold effect where a critical level of a pathogenic mutation needs to be present to cause a deleterious effect (Bourgeron *et al.*, 1993; Chomyn *et al.*, 1992; Hanna *et al.*, 1995). Consequently, mutations often affect only some mitochondria,

leaving others unimpaired. Whereas for patients suffering from a mitochondrial disease the threshold effect is an important factor for the severity of the symptoms, the inconsistency of affected mitochondria aggravates the research in mouse models of mtDNA dysfunction.

The first "mito-mouse" was generated by the introduction of exogenous mitochondria carrying an mtDNA deletion into mouse zygotes (Inoue *et al.*, 2000). Mito-mice exhibit some symptoms correlated with mitochondrial disease such as low body weight, lactic acidosis, systemic ischemia, hearing loss and male infertility (Inoue *et al.*, 2000; Nakada *et al.*, 2006). However, the overall phenotype mimics more specifically human early-onset Pearson syndrome that is caused by a single heteroplasmic mtDNA deletion. To overcome specificity of mutations introduced into mtDNA, the restriction endonuclease *PstI* was expressed in mitochondria of mouse skeletal muscle (Srivastava & Moraes, 2001). This leads to double-strand brakes and subsequent depletion of mtDNA, which ultimately results in mitochondrial myopathy. mtDNA maintenance and gene expression is dependent on proteins encoded by nuclear DNA. Therefore, disruption of nuclear-encoded genes can ultimately lead to mtDNA damage and mitochondrial dysfunction. Various models have been generated by disruption of these genes, including the mitochondrial transcription factor A (TFAM, (Larsson *et al.*, 1998), thymidine kinase 2 (TK2, (Akman *et al.*, 2008; Zhou *et al.*, 2008) and mitochondrial DNA polymerase subunit gamma (POLG, (Kujoth *et al.*, 2005; Trifunovic *et al.*, 2004). Although, the different models show specific phenotypes, they all suffer from myopathies and premature death.

Nevertheless, mitochondrial diseases are not restricted to mutations in mtDNA as only 13 protein subunits of the respiratory chain are encoded by the mitochondrial genome. Diseases caused by mutations in nuclear encoded genes are a very heterogeneous group. Nuclear DNA encodes for both structural proteins of the OXPHOS system and proteins required for their import and assembly in the mitochondria. Defects in any of these proteins can lead to functionally impaired OXPHOS and therefore to mitochondrial disease. Germ line and tissue-specific knockout mice have been generated for a number of mitochondrial proteins. These include the adenine nucleotide transporter (Ant1, (Graham *et al.*, 1997), the antioxidant genes glutathione peroxidase

(GPx1) and superoxide dismutase (Sod2, (de Haan *et al.*, 1998; Esposito *et al.*, 2000); (Li *et al.*, 1995; Melov *et al.*, 1999) and Frataxin (FRDA), involved in mitochondrial iron homeostasis (Puccio *et al.*, 2001). Again, these mice display features of myopathy and die early. Nevertheless, to date, no mouse models have been successfully created that recapitulate defects in the respiratory chain protein subunits (Hance & Larsson, 2005).

### 2.2.3 Mitochondrial dysfunction in the *harlequin* mouse

A mitochondrial disorder that primarily affects brain and muscle tissue and results in severe muscle atrophy and progressive neurodegeneration has been associated with the mitochondrial protein apoptosis-inducing factor (AIF; Chapter 2.3 ). Patients carrying a deletion of the *AIFM1* arginine residue R201 suffer from severe mitochondrial encephalomyopathy, characterised by delayed psychomotor development, muscle atrophy, sensory neuropathy, seizures and respiratory insufficiency (Ghezzi *et al.*, 2010). The pathology results from impaired ETC function and decreased levels of mitochondrial C I. Accordingly, a reduction of C I has also been demonstrated in *harlequin* mice (Benit *et al.*, 2008; Vahsen *et al.*, 2004). *Harlequin* mice show up to 80 % reduction in AIF protein levels due to a pro-viral insertion in the regulatory region of the gene and display features of progressive neurodegeneration, ataxia and muscle atrophy. Therefore, the *harlequin* mouse is one of the few models that show a defect in respiratory chain complex function. Consequently, the *harlequin* mouse might serve as a model to identify molecular mechanisms of impaired OXPHOS and provide valuable information of potential targets for the development of treatments for mitochondrial diseases (Benit *et al.*, 2008).

### 2.2.4 *C. elegans* as a model organism for mitochondrial dysfunction

Nevertheless, the mouse is a rather complex organism and entails some drawbacks as an *in vivo* model. Therefore, a simpler organism such as *C. elegans* might provide a suitable tool for the study of underlying pathways and mechanisms. In fact, various *C. elegans* mutant strains of ETC dysfunction display OXPHOS deficiency that ultimately affects *C. elegans* phenotype and lifespan. Important models of ETC dysfunction include mutations in the ETC subunit genes *gas-1*, *nuo-1*, *nuo-2* and *nuo-6* (C I),

*mev-1* (C II), *isp-1*, *cyc-1* and *ucr-2.3* (C III), *cco-1* (C IV) and *atp-3* (C V) (Dancy *et al.*, 2015; Tsang & Lemire, 2003). Furthermore, *clk-1* encodes for a demethyloxyubiquinone monoxygenase necessary for the synthesis of ubiquinone that serves as an electron carrier from CI/CII to CIII. Notably, although all these mutations affect ETC function and lead to impaired OXPHOS, the effect on the nematodes lifespan is heterogeneous. Whereas *gas-1* and *mev-1* mutations significantly decrease their lifespan, other mutations have no effect or even promote longevity, such as *nuo-6* and *isp-1* (Baruah *et al.*, 2014). Despite the different effect on lifespan, *C. elegans* ETC mutants serve as genetic models to study underlying pathways of impaired OXPHOS. Nevertheless, the lack of mouse model equivalents to these invertebrate mutants make translational research challenging. To this end, a *C. elegans* model of AIF deficiency might provide an invertebrate model of ETC deficiency that recapitulates the phenotype observed in *harlequin* mice to study underlying mechanisms of OXPHOS impairment.

## 2.3 Apoptosis Inducing Factor

The stability and function of the ETC depends on the correct assembly of the respiratory complexes subunits. Moreover, stabilisation of the maturated proteins is important for OXPHOS function and provides structural rigidity against adverse events. Mitochondrial C I assembly is an intricate dynamic process that involves various proteins and prosthetic groups (McKenzie & Ryan, 2010). A protein that has been associated with ETC stability and C I assembly is the mitochondrial flavoprotein AIF.

### 2.3.1 AIF in apoptosis

The name of AIF derives from its original discovery as a protein that plays a key role in triggering apoptotic cell death (Daugas *et al.*, 2000) (Figure 2-2). It was primarily found to be an integral protein located in the IMS, anchored to the IM (Susin *et al.*, 1999), and later to the OM (Yu *et al.*, 2009). Upon cytotoxic insult, mature AIF is cleaved and truncated AIF (tAIF) is released from mitochochondria to the cytosol (Polster *et al.*, 2005; Yuste *et al.*, 2005). Together with cyclophilin A (CypA) and CPS-6/endonuclease G (EndoG), tAIF is eventually translocated to the nucleus (Cande *et al.*, 2004). Whereas CypA promotes nuclear translocation of AIF, the heat shock protein Hsp70 inhibits re-

localisation (Gurbuxani *et al.*, 2003). Hsp70 is a chaperone that is expressed upon induced stress (Daugaard *et al.*, 2007). As a primary stress response, chaperones aid in the structural reassembly of proteins in affected organelles to prevent organellar and cellular damage. Therefore, Hsp70 inhibits nuclear tAIF translocation to prevent premature cell death (Matsumori *et al.*, 2005). However, when the protein enters the nucleus, tAIF recruits CypA and associates with histone H2AX (Artus *et al.*, 2010). This interaction induces chromatinolysis and DNA degradation (Artus *et al.*, 2010; Baritaud *et al.*, 2010). In *C. elegans*, tAIF and EndoG trigger chromatin condensation and DNA fragmentation as an apoptotic event (Joza *et al.*, 2001; Susin *et al.*, 1999; Zamzami *et al.*, 1996).

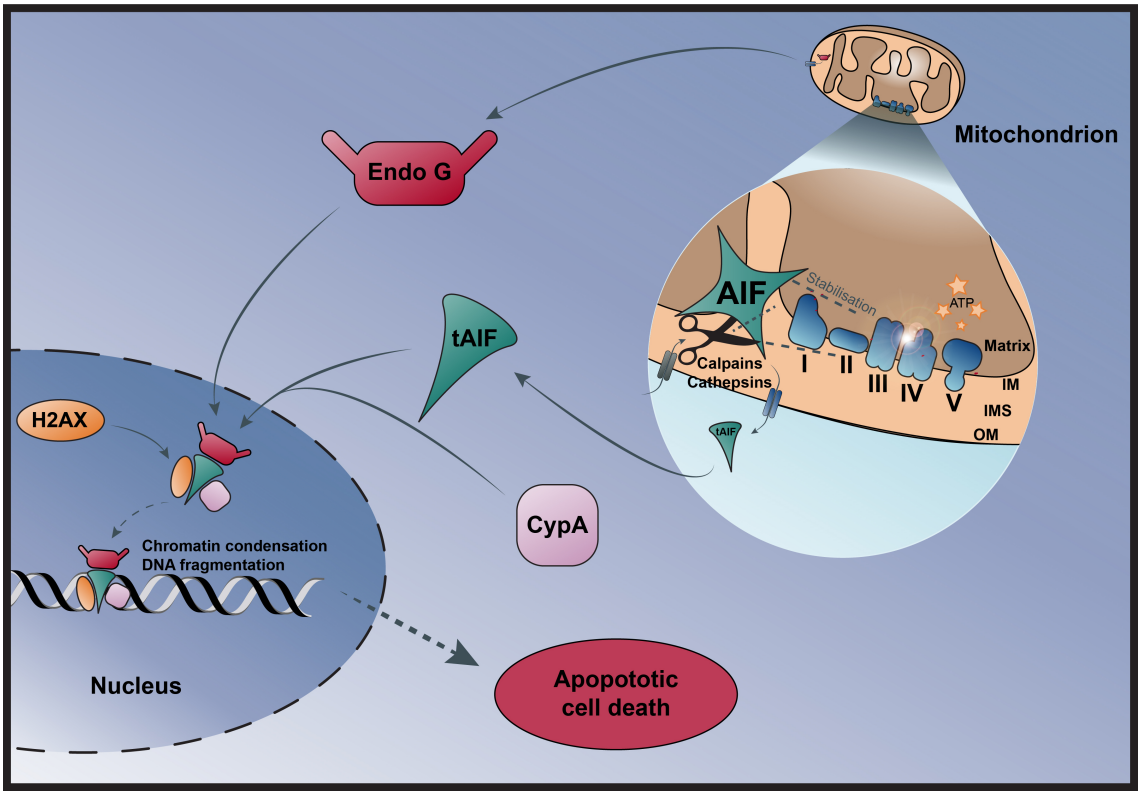


Figure 2-2: **Functions of AIF in cell survival and apoptosis.** Mature AIF is located in the intermembrane space (IMS) of mitochondria and important for stabilising the electron transport chain (ETC) in non-apoptotic conditions. Upon induced apoptosis, AIF is cleaved by calpains and cathepsins. Truncated AIF (tAIF) is released from the mitochondria, recruits cyclophilin A (CypA) and translocates to the nucleus. In the nucleus, tAIF forms a complex with CypA, endonuclease G (EndoG) and Histone H2AX and induces chromatin condensation and DNA fragmentation.

### 2.3.2 AIF in ETC stabilisation

Importantly, AIF is involved in the assembly of C I and indispensible for cell survival. For instance, C I subunit levels (NDUFA9, NDUFS7 and NDUFB6 in mammals) are reduced in cells lacking AIF and the *harlequin* mouse brain (Vahsen *et al.*, 2004). AIF exhibits NADH and NADPH oxidase activity (Miramar *et al.*, 2001) and can form stable NADH-associated dimers (Churbanova & Sevrioukova, 2008), suggesting a possible role in redox-dependent substrate delivery to C I (McKenzie & Ryan, 2010). Furthermore, AIF interacts with the dynamin-related GTPase optic atrophy 1 (Opal), located in the IM (Cipolat *et al.*, 2004; Meeusen *et al.*, 2006). Opal is implicated in the fusion of the mitochondrial IM and critical during apoptosis signalling. Nevertheless, the role of AIF in C I biogenesis remains elusive as no direct association with any structural subunits, assembly intermediates, or mature C I has been found.

## 2.4 Stress response mechanisms and ageing

Pathophysiological conditions such as impaired ETC function, for instance by C I deficiency, may induce strong mitochondrial stress. However, also under physiological conditions temporal cellular and mitochondrial stress can occur. For example, physical activity increases energy expenditure, temperature and the generation of ROS (Di Meo & Venditti, 2001). To prevent cellular damage, cells respond to a wide variety of stresses through the transcriptional activation of protective genes. Organelle-specific stress can induce retrograde signalling specific to the subcellular compartments. The mitochondria-specific stress response is termed mitochondrial unfolded protein response (UPRmt).

### 2.4.1 Mitochondrial Unfolded Protein Response (UPRmt)

Mitochondrial stress commonly leads to loss of membrane potential and results in disturbed organelle homeostasis (Satoh *et al.*, 1997). This homeostatic imbalance affects the equilibrium of newly synthesised mitochondrial proteins and chaperones that assist in correct protein folding (Haynes & Ron, 2010). As a consequence, proteins in the mitochondrial matrix become misfolded or even unfolded. These non-functional

proteins are recognised by the cell and cleaved by the mammalian mitochondrial matrix protease ClpP orthologue CLPP-1 in *C. elegans* (Benedetti *et al.*, 2006; Haynes *et al.*, 2007) (Figure 2-3). The peptide products are then exported from the matrix to the cytosol by the mitochondrial half-molecule ATP-binding cassette (ABC) transporter (HAF-1). In the cytosol, the peptide products originating from mitochondrial proteins induce formation of a protein complex between the homeodomain-containing transcription factor defective proventriculus (in drosophila) homolog (SatB2/DVE-1) and the small ubiquitin-like protein Ubl5/UBL-5 (Benedetti *et al.*, 2006). This complex is then redistributed to the nucleus where it binds to the promoters of mitochondrial chaperone genes and enhances their expression (Haynes *et al.*, 2007). These chaperones (e.g. HSP-6 and HSP-60) are transported back to mitochondria and assist in the folding of proteins to restore mitochondrial homeostasis (Martinus *et al.*, 1996; Ryan & Hoogenraad, 2007; Zhao *et al.*, 2002). This reaction to mitochondrial stress was identified as the classical pathway of UPRmt.

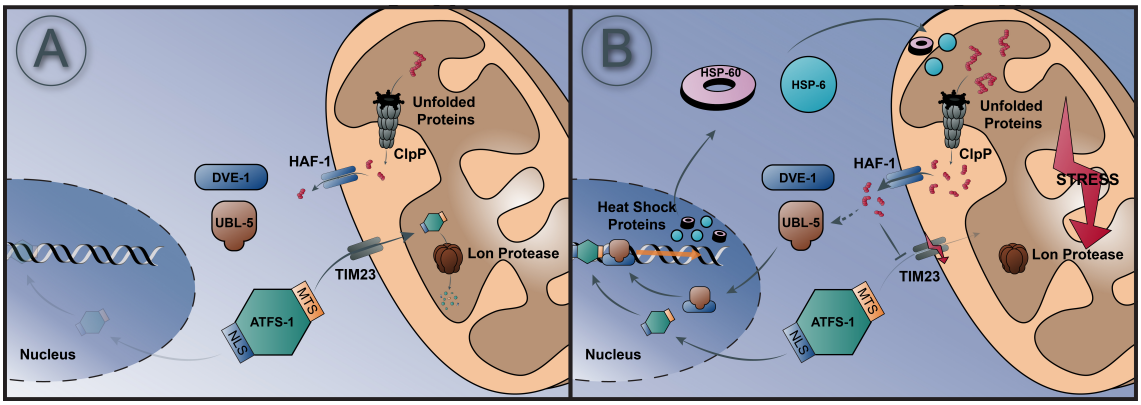


Figure 2-3: **Schematic overview of the mitochondrial unfolded protein response (UPRmt). (A)** The transcription factor ATFS-1 is constantly translated in the cytosol of the cell and contains a nuclear localisation sequence (NLS) and a mitochondrial targeting sequence (MTS). Under normal conditions ATFS-1 is translocated into the mitochondrial matrix and degraded by Lon protease. **(B)** When mitochondria are stressed, newly synthesised mitochondrial proteins become misfolded or unfolded and are cleaved by Clp protein (ClpP) into small peptides. These peptides are then exported through the HAF-1 transporter and activate UBL-5 and the transcription factor DVE-1. DVE-1/UBL-5 translocate to the nucleus and induce expression of heat-shock proteins (HSP-6 and HSP-60) that are transported to mitochondria and aid in protein folding. Furthermore, mitochondrial stress affects the membrane potential and impairs mitochondrial import proteins. Hence, ATFS-1 translocates into the nucleus and induces expression of HSPs.

However, a second potent mediator of the UPRmt that operates in parallel to DVE-1/UBL-5 was identified thereafter. Activating transcription factor associated with stress (ATFS-1) is a bZip transcription factor that acts downstream of HAF-1 and mediates the expression of the heat shock proteins HSP-6 and HSP-60 (Haynes *et al.*, 2010). However, later it was demonstrated that ATFS-1 is not activated by exported mitochondrial peptides but rather depends on the import efficiency of mitochondrial transporters (Nargund *et al.*, 2012). ATFS-1 is permanently produced in the cytosol and contains both a mitochondrial targeting sequence (MTS) and a nuclear localisation sequence (NLS). Under normal conditions, ATFS-1 is transported into the mitochondria where it is immediately degraded by Lon protease (Nargund *et al.*, 2012). Both mitochondrial stress and altered mitochondrial membrane potential lead to impairment of mitochondrial transporters, which finally fail to channel ATFS-1 into the matrix. Hence, ATFS-1 accumulates in the cytosol and is imported into the nucleus where it induces the expression of chaperones that restore mitochondrial protein folding capacity (Rauthan *et al.*, 2013). Therefore, the UPRmt is an important mechanism in the quality control and stress response of mitochondria and contributes to the health of a cell. Furthermore, UPRmt has been implicated in ageing and plays an important role for the lifespan of long-lived *C. elegans* mutants (Durieux *et al.*, 2011).

### 2.4.2 Ageing and pathology

Ageing is an enigmatic fate of all organisms. Many pathological syndromes from cardiovascular disease, cancer, atherosclerosis, impaired vision, diabetes (type 2), osteoporosis and sarcopenia to neurodegenerative diseases manifest at an advanced age. For instance, the relentless neuronal degeneration associated with Alzheimer's disease (AD) and related forms of dementia affects an estimated 44 million people worldwide, set to almost double by 2030 (Huttin, 2014). With the overall increase in the age of the world's population, numbers of age-related disease patients are expected to grow exponentially. The tremendous number of individuals affected holds an enormous challenge for health care and physicians and has an enormous impact on societies. The group of ageing-associated diseases can affect almost any organ and encompasses a variety of harmful and in some cases fatal syndromes. This is due to increased vulner-

ability of the patients caused by decline in fitness, decrease of protective mechanisms and low regenerative capacities. Although the mechanisms that lead to some of the age-related disorders like atherosclerosis are rather well characterised, very little is known about the causes of others, such as AD and ALS. For a long time, ageing has been characterised as a passive process. However, whereas the processes of deterioration may partially be the result of the persistent exposure to extrinsic environmental and physiological cues, ageing itself is a risk factor for the development of disease.

Studies on ageing have demonstrated that a variety of different cellular processes affect the senescence of an organism. Hallmarks that have been implicated with ageing include telomere length, epigenetic alterations, development, growth factors, hormones, stress response and diet. Importantly, also mitochondria, metabolism and insulin/IGF-1 signalling are important factors in the ageing process (Lopez-Otin *et al.*, 2013; Tosato *et al.*, 2007). The number of factors involved in senescent decline and their heterogeneous functions display the complexity of ageing. Nevertheless, many of these processes underlie classic cell signalling. More precisely, like any other biological process, classical cellular signalling pathways and transcription factors can influence ageing. The regulation of these pathways is subject to environmental and physiological signals. This finding reveals that in fact, ageing is not just the coincident and indispensable result of environmental stress but also a genetically inherited trait of active regulation and programmed cellular death. Consequently, genes and proteins involved can be specifically targeted and manipulated by genetic or pharmacological intervention. This finding is important for the understanding of the ageing process and the development of strategies to counteract senescent decline.

## 2.5 Insulin/IGF-1 signalling

### 2.5.1 Insulin/IGF-1 signalling and lifespan

Many pathways that extend lifespan have been discovered through studies in nematodes. These include the longevity effect of caloric restriction and mechanisms involving genes that affect the rate of development and behaviour in *C. elegans* (Lakowski & Hekimi, 1998). Insulin/insulin-like growth factor (IGF-1) signalling (IIS)

has been identified to be the single most important pathway to regulate lifespan. Mutations that decrease the activity of DAF-2 or other proteins involved in the IIS can double the lifespan of the animals (Kenyon *et al.*, 1993; Kimura *et al.*, 1997). Importantly, the role of the IIS is conserved across species and downregulation of the signalling pathway or activation of downstream targets such as abnormal dauer formation-16/forkhead-box-protein (DAF-16/FOXO, Chapter 2.5.3 ) has been demonstrated to extend the lifespan in various organisms (Bartke, 2008). Thus, IIS reduction hyperactivates downstream transcription factors that create stress response and long-lived worms (Kenyon, 2005). The transcription factors cooperatively regulate pathways that adapt metabolism and growth to environmental conditions (Jasper, 2008).

In the fruitfly *Drosophila melanogaster*, inhibiting IIS results in similar consequences for the organism as observed in nematodes (Giannakou & Partridge, 2007). Both, systemic and tissue-specific downregulation leads to increased lifespan, providing evidence for cell non-autonomous signalling. In mice, low levels of IGF-1 correlate with increased stress resistance (Holzenberger *et al.*, 2003). Mutations in genes encoding for the insulin and IGF-1 receptor as well as inhibition or activation of upstream regulators and downstream targets can extend lifespan (Bartke, 2008; Holzenberger *et al.*, 2003; Kappeler *et al.*, 2008; Selman *et al.*, 2008; Yuan *et al.*, 2009). Even in dogs it has been demonstrated that a mutation that decreases IGF-1 levels results in an extension of lifespan (Greer *et al.*, 2011; Kraus *et al.*, 2013). The finding that mutations in IIS lead to a long life is intriguing, given that it is such an evolutionarily conserved pathway that exerts critical cellular functions. Predictably, the idea that any given IIS mutation would increase stress resistance and lead to an extension of lifespan does not apply. For example, the knockout of the gene encoding for insulin/IGF-1 in the liver of mice causes diabetes (Yakar *et al.*, 2001). Nevertheless, the consistent effect of a variety of mutations on longevity, consistent across species, makes IIS an attractive target for the development of potential treatment in humans. In fact, even in humans IIS seems to be an important mechanism of lifespan regulation. The genetic analysis of various human populations around the globe has unveiled common functions of IIS as well as upstream and downstream effectors (van Heemst *et al.*, 2005). Strikingly, the comparison of human cohort subpopulations displayed an overrepresentation of mutations known

to impair IGF-1 receptor function in long-lived individuals (Kojima *et al.*, 2004; Pawlikowska *et al.*, 2009; Suh *et al.*, 2008). Noteworthy, many other human cohort studies also underline the important role of downstream targets. Throughout the world, variants of IGF-1 downstream target genes have been linked to longevity (Anselmi *et al.*, 2009; Flachsbart *et al.*, 2009; Li *et al.*, 2009; Lunetta *et al.*, 2007; Willcox *et al.*, 2008). It is worth mentioning that these populations also show a significantly lower risk of developing common age-related diseases.

Hence, there is emerging evidence that manipulating the ageing process is a promising strategy aimed at postponing the manifestation of late-onset neurodegenerative disorders and to slow their progression once emerged (Morimoto, 2006). Reducing the activity of IIS protects worms from proteotoxicity of various aggregative proteins associated with neurodegenerative diseases. These include the AD-associated amyloid beta (Aβ) peptide (Link, 1995) and the Huntington's disease (HD)-linked polyQ40 peptide (Morley *et al.*, 2002). Similarly, reduction of IGF-1 signalling protects mice from AD-like diseases (Cohen *et al.*, 2009). Furthermore, pharmacological IIS inhibitors have been reported to mediate efficient reduction of the signalling cascade and protect nematodes from AD- and HD-associated proteotoxicity (El-Ami *et al.*, 2014). Moreover, they promote stress resistance and are used in clinical trials for cancer treatment (Weroha & Haluska, 2008). Results like these prove that IIS inhibition has a therapeutic potential as a treatment for neurodegenerative maladies (Cohen, 2011). Furthermore, manipulation of ageing-related pathways might have beneficial effects for other pathologies.

### 2.5.2 The insulin/IGF-1 signalling pathway

In mammals, the insulin-like growth factor 1 receptor (IGF1R) is present on various cell types in many tissues (van Heemst, 2010). Ligand binding initiates intracellular IIS. The activated receptor phosphorylates several intracellular substrates, inducing two major signalling pathways: The PI-3K- PKB/AKT pathway is a central regulator of cell metabolism, whereas the Ras-MAPK pathway has been shown primarily to regulate cell proliferation (Taniguchi *et al.*, 2006). The *C. elegans* receptor abnormal dauer formation-2 (DAF-2) is the sole IGF1R homolog in nematodes (Lin *et al.*, 2001). Interest-

ingly, nematodes express 40 insulin/IGF-1-like peptides, agonists and antagonists that affect IIS function (Pierce *et al.*, 2001). Mediated by a receptor tyrosine kinase, the binding of agonist peptides activates several downstream kinases via a cascade of sequential phosphorylation events (Figure 2-4):

- phosphoinositide 3-kinase (AGE-1/PI3K)
- pyruvate dehydrogenase lipoamide kinase isozyme (PDK-1)
- Akt kinase family 1 and 2 (AKT-1 and AKT-2)
- serine/threonine-protein kinase (SGK-1)

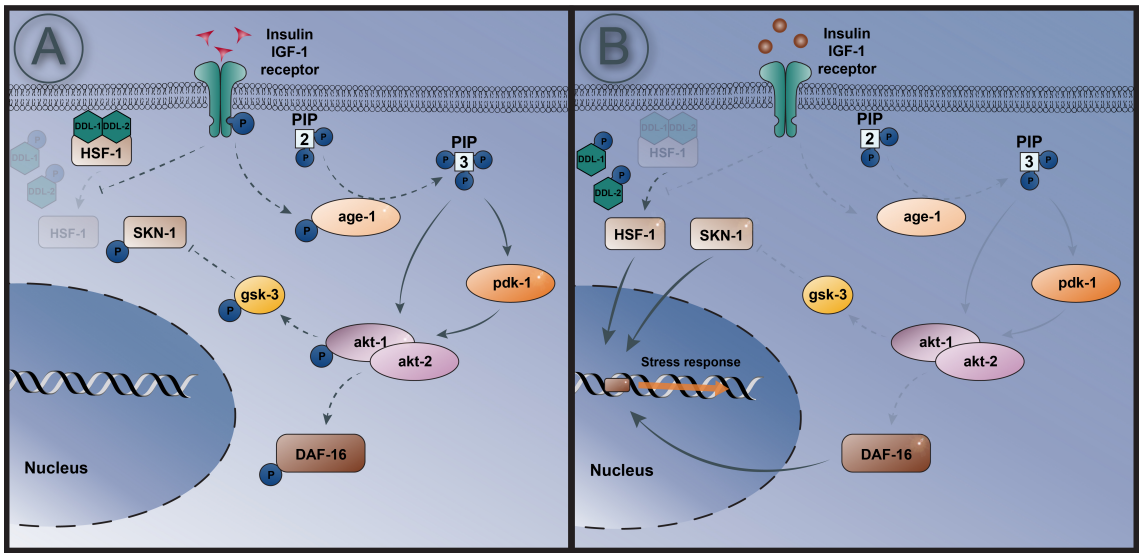


Figure 2-4: **Schematic overview of insulin/IGF-1 signalling. (A)** Active insulin/IGF-1 signalling (IIS) induces a PI3K-dependent phosphorylation cascade that ultimately phosphorylates the transcription factors DAF-16 and SKN-1 and keeps them in the cytosol. Furthermore, heat-shock factor 1 (HSF-1) is inactivated by the incorporation in a complex with DDL-1 and DDL-2. Activation of HSF-1 requires phosphorylation of DDL-1/2, which is inhibited by active IIS. **(B)** Upon impairment of IIS, DDL-1/2 become phosphorylated and release HSF-1, which translocates into the nucleus and induces expression of stress response genes. Furthermore, DAF-16 and SKN-1 are no longer phosphorylated by AKT-1/2 and GSK-3, respectively, and also drive the expression of stress response genes.

Ultimately, active PDK-1, AKT and SGK-1 phosphorylate the FOXO transcription factors DAF-16 and nuclear factor (erythroid-derived)-like / skinhead 1 (NRF/SKN-1) (Lee *et al.*, 2001; Paradis & Ruvkun, 1998; Tullet *et al.*, 2008). The phosphorylation prevents the translocation of the transcription factors to the nucleus and the proteins are kept in the cytosol. Thereby transcription of target genes is blocked (Wolff & Dillin, 2006). Similarly, IIS negatively regulates heat shock factor 1 (HSF-1) by preventing the phosphorylation of 'DAF-16-dependent longevity' DDL-1 and DDL-2 (Chiang *et al.*,

2012). DDL-1/2 proteins interact with HSF-1 and collaboratively form a complex. Upon phosphorylation DDL-1/2 detach from HSF-1, allowing the protein to enter the nucleus. There, the transcription factor promotes expression of chaperones that assist in protein folding, targeting damaged proteins for degradation and inducing other responses associated with the protection from cellular damage (Hartl, 1996; Jolly & Morimoto, 2000).

### 2.5.3 DAF-16

IIS regulates a variety of transcription factors and co-regulators that induce the expression of a diverse set of genes that act in a cumulative way to promote resistance to various stresses and ultimately increase lifespan (Hsu *et al.*, 2003; Murphy *et al.*, 2003; Wolff *et al.*, 2006). The forkhead-box transcription factor DAF-16/FOXO plays a key role in this pathway and can translocate from the cytosol to the nucleus. There, DAF-16/FOXO induces the expression of a diverse set of antioxidant, metabolic, chaperone and antimicrobial target genes that are functionally important to promote stress resistance (Wang *et al.*, 2008).

However, DAF-16/FOXO is not only regulated by the insulin/IGF-1 receptor-DAF-2. Several other mechanisms promote DAF-16/FOXO nuclear localisation, increasing the regulatory capacities and complexity of the pathway. Phosphatase and tensin homolog (DAF-18/PTEN) dephosphorylates and inhibits AGE-1/PI3K, preventing DAF-16/FOXO phosphorylation (Ogg & Ruvkun, 1998); c-Jun N-terminal kinase (JNK-1) stimulates DAF-16 nuclear translocation by alternate phosphorylation (Oh *et al.*, 2005); and type I protein arginine methyltransferase (PRMT-1) promotes nuclear maintenance of DAF-16 (Takahashi *et al.*, 2011). Furthermore, the transcription factor SKN-1 can act together with DAF-16 and regulate stress resistance and detoxification genes downstream of DAF-2 (Tullet *et al.*, 2008). Among others, these factors modulate DAF-16/FOXO activity and regulate the transcriptional response to specific environmental and physiological signals. The extensive regulatory opportunities of DAF-16/FOXO point to a diverse cellular effect when the transcription factor is activated. In fact, gene expression profiling of *daf-2* mutants has identified hundreds of DAF-16-regulated genes (Halaschek-Wiener *et al.*, 2005; McElwee *et al.*, 2003; Murphy, 2006). In

*C. elegans* these genes are responsible for the twofold lifespan extension in the long-lived *daf-2* mutants (Kenyon *et al.*, 1993).

## 2.6 HIF-1

Another factor that is co-regulated by IIS is the hypoxia-inducible factor (HIF-1). Oxygen plays a central role for the survival of multicellular organisms since they rely on oxidative respiration (Lane & Martin, 2010). Low $O_2$ levels or the generation of reactive oxygen species (ROS) that react with cellular macromolecules can lead to oxidative stress and result in cell dysfunction or death. As a consequence, metazoan organisms have developed elaborate cellular systems that maintain oxygen homeostasis (Semenza, 2012). HIF-1 activates glycolytic enzymes and is critical for the metabolic adaptation to hypoxic condition (Loenarz *et al.*, 2011). It acts like a metabolic switch that shunts glucose metabolites from the mitochondria to the cytoplasm in order to maintain ATP production by glycolysis and promote longevity (Kim *et al.*, 2006).

HIF-1 consists of two subunits: HIF-1$\alpha$ and HIF-1$\beta$. Both subunits contain basic helix-loop-helix domains (bHLH) (Ema *et al.*, 1997; Wang *et al.*, 1995) and work only as a heterodimer. While ubiquitous HIF-1$\beta$ is present in excess and heterodimerises with other bHLH proteins, HIF-1$\alpha$ protein levels determine HIF-1 transcriptional activity (Semenza *et al.*, 1996). Under normoxic conditions, HIF-1$\alpha$ is bound to the von Hippel-Lindau (VHL) protein (Figure 2-5). VHL recruits an ubiquitin ligase that targets HIF-1$\alpha$ for proteasomal degradation (Kaelin & Ratcliffe, 2008). VHL binding depends on hydroxylation, mediated by prolyl hydroxylase domain proteins (PHD1-3, homolog in *C. elegans*: EGL-9). The hydroxylation of specific proline residues in HIF-1$\alpha$ by EGL-9/PHD requires molecular $O_2$ as a substrate, and thus, this process is inhibited under hypoxic conditions (Epstein *et al.*, 2001). As a consequence, HIF-1$\alpha$ and HIF-1$\beta$ translocate into the nucleus and heterodimerise. Subsequently, the complex binds to DNA and induces the transcription of more than 100 genes crucial for adaptation to hypoxia (Jiang *et al.*, 1996; Semenza & Wang, 1992). In addition to the enzymatic inhibition of EGL-9/PHD, increasing levels of cytoplasmic ROS can control HIF-1. ROS are generated by perturbed mitochondrial ETC and alter the oxidation state of $Fe^{2+}$ (a co-factor for

PHD activity) to $Fe^{3+}$. Such oxidised ions cannot be utilised, inhibiting EGL-9/PHD activity and promoting HIF-1$\alpha$ stabilisation (Simon, 2006). Nevertheless, the role of ROS in hypoxia and HIF-1 regulation remains controversial owing to discrepancies in different model systems as well as the debate over tools for accurate detection of ROS (Yee Koh *et al.*, 2008). Furthermore, various other factors have been implicated in HIF-1 regulation independent of VHL-1 or PHD (Berchner-Pfannschmidt *et al.*, 2004; Epstein *et al.*, 2001; Lando *et al.*, 2002; Mahon *et al.*, 2001; Nakayama & Ronai, 2004). In summary, the regulation of HIF-1$\alpha$ protein levels is complex. It involves multiple pathways and regulatory factors that are oxygen-independent and rely on posttranslational regulation and other signalling pathways, some yet to be fully characterised (Arnesen *et al.*, 2005; Arsham *et al.*, 2002; Bae *et al.*, 2004; Baek *et al.*, 2007; Carbia-Nagashima *et al.*, 2007; Cheng *et al.*, 2007; Jung *et al.*, 2006; Koritzinsky *et al.*, 2006; Li *et al.*, 2005; Liu *et al.*, 2007a; Liu *et al.*, 2007b; Mottet *et al.*, 2003; Shao *et al.*, 2004; Sonenberg & Hinnebusch, 2007; Stiehl *et al.*, 2002; Toker & Newton, 2000; van den Beucken *et al.*, 2006).

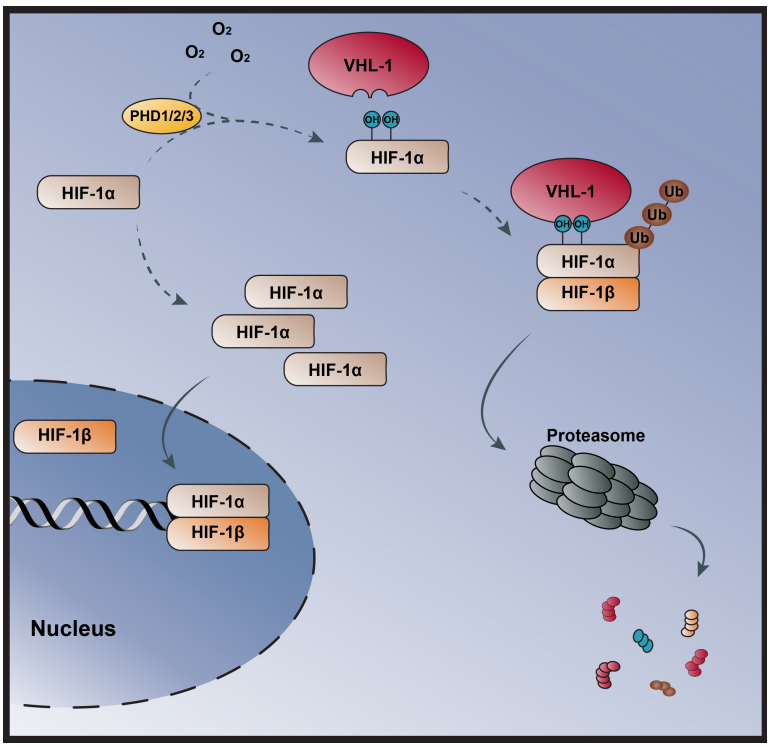


Figure 2-5: **Schematic overview of HIF-1 signalling.** Under normoxic conditions, HIF-1$\alpha$ is bound to the von Hippel-Lindau (VHL) protein mediated by prolyl hydroxylase domain proteins (PHD1-3). The complex recruits an ubiquitin ligase and targets HIF-1$\alpha$ for proteasomal degradation. VHL-1 binding is inhibited under hypoxic conditions and HIF-1$\alpha$ translocates into the nucleus along with ubiquitously expressed HIF-1$\beta$. Subsequently, the two subunits dimerise and induce gene expression.

## 2.7 AMP-activated Kinase (AMPK)

Similar to HIF-1, AMP-activated Kinase (AMPK, AAK-2 in *C. elegans*) is a metabolic sensor that efficiently controls energy metabolism, cellular housekeeping and stress resistance. AMPK/AAK-2 promotes food intake and influences energy metabolism on the organism level. The metabolic sensor is activated upon low energy conditions and primarily promotes catabolism over anabolic reactions (Ferrer *et al.*, 1985; Yeh & Kim, 1980; Yeh *et al.*, 1980). Reduced energy levels result in decreased ATP and increased AMP levels. This high AMP/ATP ratio activates AMPK/AAK-2, which consequently switches off anabolic pathways (Lee *et al.*, 2008). For example, energy production from glucose and fatty acids is stimulated, whereas energy consumption for protein, cholesterol, triglyceride and glycogen synthesis is inhibited (Hardie, 2011b; Steinberg & Kemp, 2009). Furthermore, transcription processes that consume ATP are downregulated (Shin *et al.*, 2011). In contrast, catabolic pathways that generate ATP, such as fatty acid oxidation and glycolysis are switched on. Consequently, caloric restriction stimulates AMPK/AAK-2 activity whereas nutritional overload impairs AMPK/AAK-2 function (Salminen & Kaarniranta, 2012).

Although the name AMP-activated protein kinase implicates AMP as the main and potentially exclusive activator of AMPK/AAK-2, other kinases have been discovered that can activate AMPK/AAK-2 independently from cellular AMP levels (Witters *et al.*, 2006). AMPK/AAK-2 is a serine/threonine protein kinase, comprised of a catalytic $\alpha$-subunit and regulatory $\beta$- and $\gamma$-subunits. Whereas the functional role of $\beta$- and $\gamma$-subunit phosphorylation is relatively poor understood, the phosphorylation of the $\alpha$-subunit regulates AMPK/AAK-2 activity. The phosphorylation of Thr-172 in the activation loop of the $\alpha$- subunit catalytic domain (Hawley *et al.*, 1996) is induced by the serine/threonine kinase LKB-1 (Hawley *et al.*, 2003; Shaw *et al.*, 2004), Calmodulin-dependent kinase kinase $\beta$ (CaMKK$\beta$) (Hawley *et al.*, 2005; Hawley *et al.*, 1995; Hurley *et al.*, 2005) and transforming growth factor-$\beta$-activated kinase (TAK-1) (Momcilovic *et al.*, 2006; Xie *et al.*, 2006). The circumstances under which AMPK/AAK-2 is activated is dependent on the developmental and environmental situation (Mantovani & Roy, 2011) as well as physiological and pathological conditions such as exercise (Richter & Ruderman, 2009) and several diseases (Steinberg & Kemp, 2009). Activation of the

metabolic sensor has been identified to extend lifespan via an integrated signalling network. For example, DAF-16/FOXO (Chapter 2.5.3 ) is regulated by AMPK/AAK-2. Moreover, AMPK/AAK-2 promotes autophagy via mechanistic target of rapamycin (mTOR) inhibition and serine/threonine-protein kinase ULK1 activation (Chapter 2.8 ).

In contrast, AMPK/AAK-2 can be inactivated by protein phosphatases (Gimeno-Alcaniz & Sanz, 2003; Marley *et al.*, 1996; Voss *et al.*, 2011). However, chronic AMPK/AAK-2 inactivation induces insulin/IGF-1 resistance in various tissues (Ruderman *et al.*, 2013). As an effect, the incidence of metabolic syndromes such as obesity, diabetes and cardiovascular diseases is promoted (Lage *et al.*, 2008).

Since AMPK/AAK-2 plays a diverse role in the response to stressful insults and its activation has beneficial consequences for the cell, it is believed that novel AMPK/AAK-2 activators might contribute as an important milestone to the therapy of metabolic and neurodegenerative diseases (Hardie, 2011c; Zhou *et al.*, 2009). The 5-aminoimidazole-4-carboxamide riboside (AICAR) is an AMP analogue and a potent activator of AMPK/AAK-2. AICAR has been used clinically to treat and protect against ischemic injury (Corton *et al.*, 1995; Idrovo *et al.*, 2015; Lempiainen *et al.*, 2012). Similarly, metformin, statins, thiazolidinediones and many phytochemicals, e.g. berberine, quercetin and resveratrol, are clinically used drugs that have been reported to activate AMPK/AAK-2 signalling (Hwang *et al.*, 2009; Steinberg & Kemp, 2009; Zhou *et al.*, 2009).

## 2.8 Autophagy

### 2.8.1 Autophagy in cell homeostasis, disease and lifespan

AMPK/AAK-2 regulates a versatile response at low energy conditions and upon mitochondrial damage that is beneficial for the function and survival of a cell. One process that has been implicated with disease, ageing and the response to AMPK activation is macroautophagy, or simply autophagy. Autophagy merely means self-eating and has originally been defined as a form of programmed cell death. In fact, autophagy primarily has a pro-survival function and promotes cellular and organismal health

(Levine & Kroemer, 2009). AMPK/AAK-2 activates autophagy, which sequesters cytosolic materials and damaged organelles and targets them to lysosomes for degradation (Egan *et al.*, 2011; Kim *et al.*, 2011). In this way, autophagy generates macromolecular building blocks, increases cellular energy levels and clears the cell from damaged organelles (Yen & Klionsky, 2008).

Notably, autophagy plays an important role for lifespan extension under various conditions and is also linked to changes in metabolism (Hansen *et al.*, 2008; Hars *et al.*, 2007; Jia & Levine, 2007; Lim *et al.*, 2014; Melendez *et al.*, 2003; Toth *et al.*, 2008). For example, upon mammalian starvation autophagy generates fatty acids and amino acids, which are catabolized to yield energy and drive gluconeogenesis and ketogenesis (Rabinowitz & White, 2010). Genetic manipulations that increase lifespan in model organisms often stimulate autophagy (Juhasz *et al.*, 2007; Pyo *et al.*, 2013). In contrast, its inhibition compromises the longevity-promoting effects of caloric restriction, inhibition of insulin/IGF-1 and Sirtuin 1 activation, or by administration of survival-promoting drugs (Hansen *et al.*, 2008). Furthermore, genetic inhibition of autophagy induces degenerative changes in mammalian tissues (Cecconi & Levine, 2008). These resemble pathologies commonly associated with ageing.

### 2.8.2 The autophagic process

When initiated, autophagy is primed by the formation of an isolation membrane, or phagophore, that begins with the engulfment of cytoplasmic material. The phagophore membrane is then further elongated until its edges fuse to close the autophagosome. The cytoplasmic material is now sequestered within double-membrane vesicles and delivered to lysosomes for bulk degradation. The outer membrane of the autophagosome fuses with the lysosome and forms the autolysosome. The material in the lumen, including the internal membrane, is then degraded (Rubinsztein *et al.*, 2011). Subsequently, the digested products are released through permeases and recycled in the cytosol (Ravikumar *et al.*, 2010). Thus, autophagy is the mechanism through which the cytoplasmic parts of the cell can be recycled.

Four enzymatic complexes are required for autophagosome formation:

- A serine/threonine protein kinase complex (LET-363 (target of rapamycin – TOR), UNC-51 (in neurons)) that induces autophagic activity,
- a class III phosphatidylinositol 3-kinase (PI3K) complex (LET-512, BEC-1) that acts in vesicle nucleation,
- ubiquitin-like conjugation pathways (ATG-3, ATG-4, ATG-5, ATG-7, ATG-16, LGG-1 and LGG-3) that lead to vesicle expansion and completion and
- a protein retrieval system (ATG-9 from the phagophore assembly site (PAS) involves ATG-2 and ATG-18).

Key signalling mechanisms for regulating autophagy include AMPK/AAK-2, TOR, class I and class III PI3Ks, the insulin/IGF-1 receptor, and the Ras/Raf/mitogen-activated protein kinase kinase 1/2 (MEK1/2) (Codogno & Meijer, 2005; Meijer & Codogno, 2004). When nutrients and growth factors are abundant, the TOR and Class I PI3Ks pathways inhibit autophagic function (Chang *et al.*, 2009). Class III PI3K and MEK1/2 positively regulate autophagy under impaired cellular conditions (Chen & Karantza-Wadsworth, 2009). Both TOR and AMPK/AAK-2 regulate autophagy by controlling the activity of essential components of the autophagic machinery, such as UNC-51 or BEC-1 (Lapierre & Hansen, 2012).

### 2.8.3 Autophagy and mitochondria

Mitochondria play a central role in ageing-related pathologies and autophagy is a critical regulator of organelle homeostasis. Autophagy is required for the clearance of mitochondrial debris such as damaged proteins and apparently also for the selective removal of whole damaged mitochondria, specifically referred to as mitophagy. Under normal conditions, the PTEN-induced putative kinase protein 1 (PINK1) is imported to the inner mitochondrial membrane (Youle & Narendra, 2011). Subsequently, the protein is cleaved and proteolytically degraded (Deas *et al.*, 2011; Meissner *et al.*, 2011). Dysfunctional mitochondria often lose their membrane potential, leading to an impaired mitochondrial membrane transport and increased probability to generate toxic

ROS and release pro-apoptotic mediators. This loss of membrane potential prevents the import of PINK1, leading to the accumulation of unprocessed PINK1 on the outer membrane surface. This induces recruitment of the cytosolic E3 ligase Parkin (Narendra *et al.*, 2010). Subsequently, Parkin mediates ubiquitination of mitochondrial membrane proteins which recruits the damaged mitochondria to autophagosomes (Greene *et al.*, 2012; Kim *et al.*, 2008; Lazarou *et al.*, 2012; Rubinsztein *et al.*, 2011; Shiba *et al.*, 2009). Such tagged mitochondria can be cleared from the cell while healthy mitochondria are spared. Thus, the delivery function of autophagy is an important mechanism by which the cell rids itself of potentially harmful constituents. This adaptive cellular housekeeping mechanism helps to maintain normal functioning of the cell and protects against ageing, neurodegenerative diseases as well as infections and other cellular insults (Arroyo *et al.*, 2014).

### 2.8.4 Autophagy and metabolism

Autophagy digests large-scale protein structures and even whole organelles as a rapid mobilisation of endogenous reserves. The subsequent degradation of macromolecules supplies the cell with energy and basic modules for the initiation of essential anabolic reactions and generation of new macromolecules (Singh & Cuervo, 2011). Therefore, autophagy ultimately affects cellular and mitochondrial metabolism and results in metabolic changes that influence biologic function and determine cellular fate (Guo *et al.*, 2011; Houtkooper *et al.*, 2012; Kim & Lee, 2014; Settembre *et al.*, 2013; Shimobayashi & Hall, 2014).

Vice versa, metabolism also affects the process of autophagy. The signal transduction cascades are mainly regulated by upstream stress and energy sensors, which are intricately coordinated to adapt to environmental changes. The diversity of sensors that affect autophagy also reflect the variety of regulatory signals, such as AMPK (AMP/ATP, glucose and NADH/$NAD^+$), TOR (amino acids), sirtuins (acetyl-CoA) and eukaryotic translation initiation factor 2a (eIF2a) kinases (lipids) (Galluzzi *et al.*, 2014). However, some molecules may directly favour the formation of autophagosomes without the signalling of additional factors, such as the accumulation of lipids or the depletion of iron.

## 2.9 Metabolism

Despite the many functions of mitochondria, energy production and the control of metabolism remain their pivotal role. For example, dietary restriction results in metabolic rewiring and has an extensive impact on the organism. IIS, HIF-1, AMPK and autophagy control cellular metabolism and are potent mediators of cellular health. Cooperatively, these factors promote survival of the organism and ultimately regulate lifespan.

Mutations in genes that are involved in metabolism can cause a variety of developmental symptoms, seizures, mental retardation, liver and kidney failure, visual loss and other pathologies (Saudubray *et al.*, 2006). Affected patients are commonly treated by dietary intervention. Despite the fact that supplementation of specific metabolites or changes in diet might not cure the disease, the simplicity of the treatment provides a strategy with great potential for the therapy of other syndromes that effect metabolism, such as mitochondrial diseases.

However, metabolism is complex. For instance, changes in the TCA cycle, glucose metabolism, amino acids and lipids are only some, although potentially the most important key players. Nevertheless, the networks that control dietary responses *in vivo* remain largely elusive and transcription factors and proteins involved are still to be further characterised. However, Acyl CoA dehydrogenase 1 (ACDH-1), a dietary sensor that has recently been linked to global changes in *C. elegans* metabolism could help in the identification of signals that alter the metabolic footprint in nematodes (Watson *et al.*, 2013). *acdh-1* encodes for a mitochondrial acetyl CoA dehydrogenase enzyme. The protein is predicted to catalyse the first step of fatty acid β-oxidation. Furthermore, it is implicated in the breakdown of branched-chain amino acids (BCAAs). However, apart from its enzymatic function, ACDH-1 has been characterised as a potent sensor for broad shifts in metabolism (Watson *et al.*, 2013).

In summary, metabolism plays a central role in ageing, health and disease. The understanding of the intricate signalling pathways that underlie the control of metabol-

ic networks could ultimately aid in the development of strategies to treat mitochondrial impairments.

## 2.10 Aim of the study

Mitochondria are essential organelles for cellular function and health of the organism. Consequently, mitochondrial dysfunction is associated with common diseases such as neurodegeneration and metabolic syndrome, as well as ageing. Mitochondrial complex I deficiency is causally linked to over thirty per cent of human mitochondrial diseases (Hangen *et al.*, 2010). Yet, the underlying mechanisms and cellular consequences of the diseases are not fully investigated and no treatment is available.

A fundamental problem for understanding common mechanisms of mitochondrial disorders is the lack of good model organisms that resemble pathologies and symptoms of patients affected by mitochondrial disease (Wallace, 2002). Desirably, the model organism is closely related to humans and the protein of interest associated with disease is conserved across species. However, at close relation, organism complexity and ethical boundaries increase. Furthermore, the number of research techniques available decreases while time and costs increase. The nematode *C. elegans* has a short life cycle, fast development and allows for easy genetic modification. Importantly, an estimated 60–80 % of human genes have a counterpart in the worm (Harris *et al.*, 2004; Sonnhammer & Durbin, 1997). Therefore, *C. elegans* serves as a valuable research organism.

In this study, I present a nematode model of mitochondrial disorder that is characterised by ETC dysfunction and impaired OXPHOS. For this purpose, I downregulated the mitochondrial protein AIF/WAH-1 in the nematode *C. elegans.* Low levels of the protein lead to a decrease in C I levels and progressive mitochondrial encephalomyopathies in humans and the *harlequin* mouse model. Consequently, AIF/WAH-1 deficiency allows for the study of impaired ETC function and the pathways that ultimately lead to disease. Furthermore, this study aimed to identify signalling cascades that protect from the detrimental consequences of OXPHOS deficiency. Impaired insulin/IGF-1 signalling protects from cellular stress and promotes survival in various organisms. Therefore, I reduced IIS to promote survival in AIF/WAH-1 deficient nematodes. I identified that AMPK and autophagy play a central role in the protection from

detrimental effects by *wah-1* downregulation. Finally, the present study points to a central role of metabolism in the adaption to cellular stress induced by ETC deficiency.

# 3 Results

## 3.1 *wah-1* downregulation results in mitochondrial deficiency and reduced lifespan in *C. elegans*

Alteration of ETC activity and OXPHOS determine mitochondrial function and lifespan in various organisms. AIF/WAH-1 has been demonstrated to play an important role in the stabilisation, maintenance and function of the ETC. To test whether downregulation of *wah-1* affects mitochondrial activity in nematodes and might serve as a model for mitochondrial disease, I tested the impact of AIF/WAH-1 deficiency on mitochondria in *C. elegans*.

To this end, *wah-1* was downregulated by targeted RNAi knockdown and the lifespan of the nematodes was observed. The reduction of WAH-1 caused a decrease in the mean survival by about 20 % (Figure 3-1 A). However, it was not clear whether the reduction in lifespan was caused by impaired ETC function or other factors downstream of *wah-1* deficiency. Therefore, I performed an epistasis analysis using the mitochondrial mutant *gas-1(fc21)*. *gas-1* encodes for a 49 kDa subunit of C I and a mutation in the gene results in a short lifespan (Kayser *et al.*, 1999). Additional knockdown of *wah-1* did not alter the lifespan of *gas-1* animals, indicating that the survival of *wah-1* deficient animals was dependent on ETC function (Figure 3-1 B).

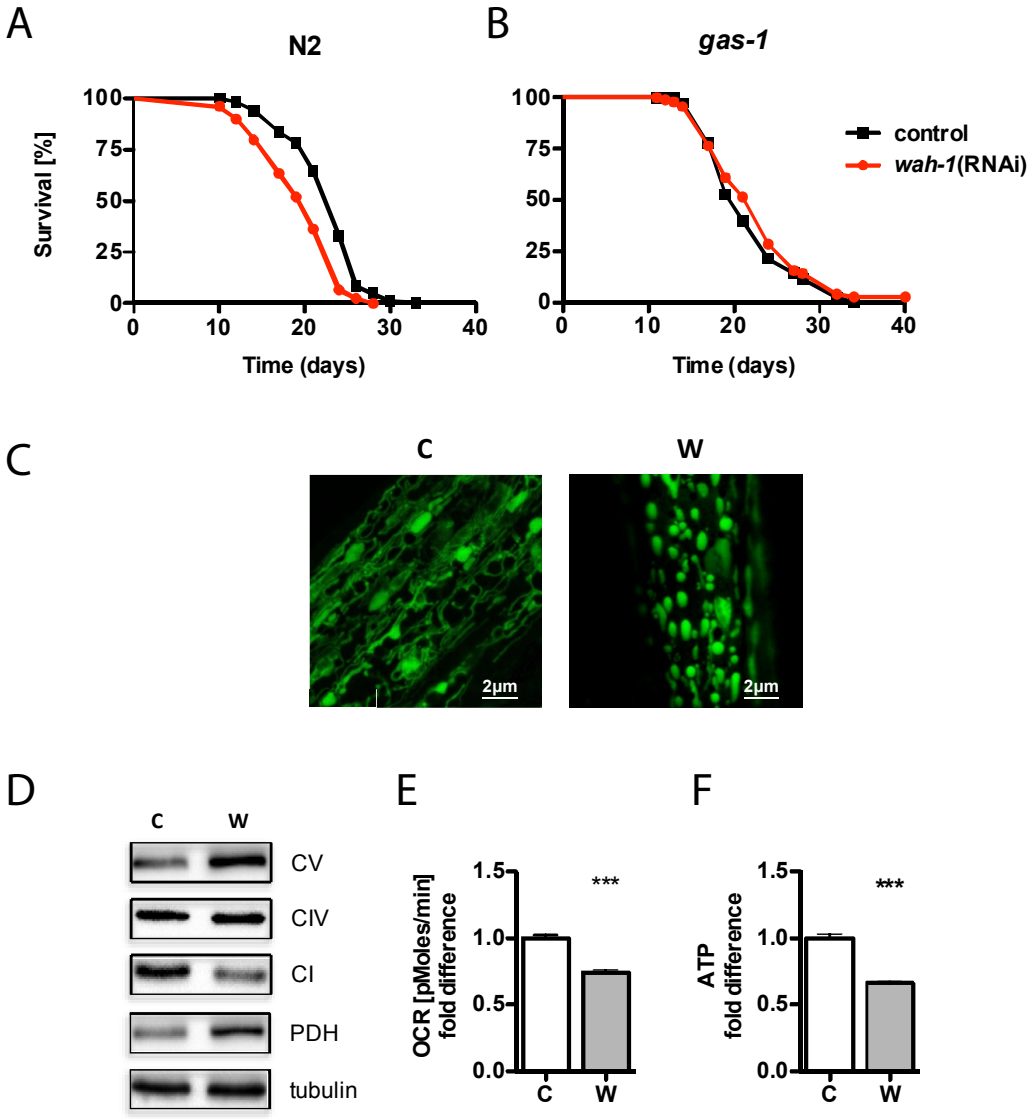


Figure 3-1: **Downregulation of WAH-1 shortens *C. elegans* lifespan and affects mitochondrial morphology and function.** Survival curves represent the lifespan of a nematode population. y-axis shows the percentage of animals remaining alive plotted over time. **black**: control, **red**: *wah-1* (RNAi). **(A)** Depletion of WAH-1 shortens the lifespan of wild type N2 animals. **(B)** Knockdown of *wah-1* does not affect the lifespan of the C I mutant *gas-1(fc21)*. **(C)** STED microscopy on worms expressing mitochondrial GFP in animals grown on *wah-1*(RNAi). **(D)** Immunoblot of ETC complexes C I, C IV, C V and pDH in control and *wah-1* (RNAi) treated wild type animals. **(E)** Oxygen consumption rates (OCR) analysed by SeaHorse XF24 and **(F)** ATP levels measured with the Luminescent ATP Detection Assay Kit (abcam) in control and *wah-1* (RNAi) animals. (C: control, W: *wah-1* (RNAi) treatment. Results are means ± S.E.M., ***p-value <0.001, two-tailed unpaired Student's t-test).

Since AIF is an important protein for mitochondrial function, mitochondrial morphology was examined in transgenic lines carrying a mitochondria-targeted GFP reporter driven by the muscle-specific *myo-3* promoter. This allowed for non-invasive analysis of mitochondrial morphology by STED microscopy. Adult, WAH-1 deficient *C. elegans* exhibited altered mitochondrial morphology with irregular, swollen appearance (Figure 3-1 C). To further investigate whether low *wah-1* levels affected OXPHOS components, I performed western blot analysis of ETC subunits. Similar to the observations in AIF deficient mice, *wah-1* RNAi strongly reduced CI protein levels (Figure 3-1 D) (Bouaita *et al.*, 2012; Vahsen *et al.*, 2004). Furthermore, *wah-1* downregulation increased ATPase protein levels, whereas C IV was not affected. Pyruvate dehydrogenase (pDH) was upregulated. To confirm OXPHOS impairment at the functional level, I assessed oxygen consumption rates (OCR) in living animals. *wah-1* (RNAi) treatment significantly decreased *C. elegans* respiration (Figure 3-1 E). Consequently, ATP production was decreased as demonstrated by the measurement of ATP levels (Figure 3-1 F).

Mitochondria play an important role for many cellular functions and are crucial during nematode development. Consequently, dysfunctions in OXPHOS result in common *C. elegans* phenotypes (Dillin *et al.*, 2002). Similarly to other mitochondrial mutants, low respiration rates in WAH-1 deficient nematodes resulted in altered growth rate, body size, body thrashing in liquid as a marker of motility, pharyngeal pumping, fertility and a slow defecation rhythm (Figure 3-2).

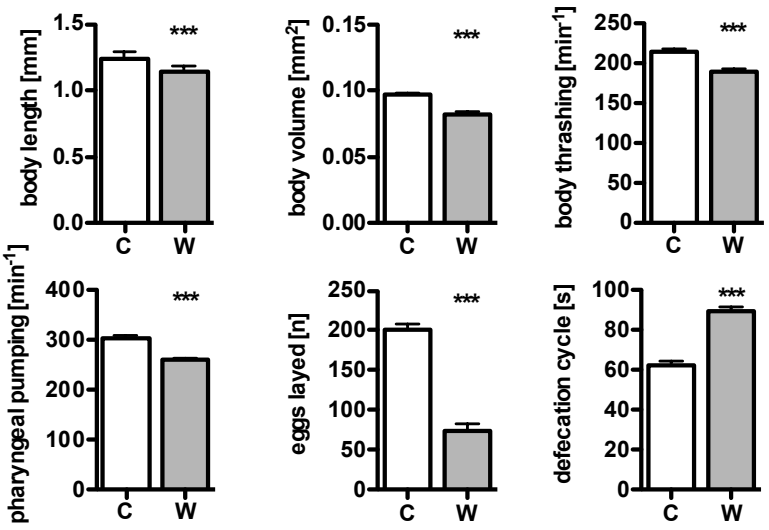


Figure 3-2: **Downregulation of WAH-1 affects *C. elegans* phenotype.** Phenotypic analysis of body length, body volume, body thrashing (body movements in M9 buffer per minute), pharyngeal pumping, number of laid eggs and the defecation cycle in *wah-1* (RNAi) treated *C. elegans*. (C: control, W: *wah-1* (RNAi) treatment. Results are means ± S.E.M., ***p-value <0.001, two-tailed unpaired Student's t-test).

In conclusion, these data demonstrate the evolutionarily conserved role of AIF/WAH-1 in the assembly and/or stabilisation of the mitochondrial ETC. Its loss severely compromised mitochondrial C I and impaired OXPHOS, which led to mitochondrial dysfunction that ultimately shortened the lifespan of *C. elegans*. Importantly, this impairment of the ETC by low *wah-1* levels and the resulting mitochondrial defects in nematodes resemble the mitochondrial consequences observed in AIF deficient mice and even patients suffering from mitochondrial encephalomyopathies. Therefore, *wah-1* (RNAi) in *C. elegans* is a suitable model for studying mechanisms that underlie disease related ETC deficiencies.

## 3.2 Mitochondrial deficiency leads to stress response activation and reduced ROS

Eukaryotic cells respond to impaired mitochondrial function by relaying signals to the nucleus and other organelles and robustly induce expression of detoxifying systems. Therefore, I measured the expression levels of genes that play a role in the innate response to mitochondrial stress. To this end, I measured the fluorescence intensity of

GFP controlled by the promoter of the respective stress response gene with the COPAS Biosorter. *wah-1* downregulation activated gene expression of *gst-4* (glutathione-S-transferase), an enzyme involved in the cellular detoxification of xenobiotic and endobiotic compounds and *sod-3* (superoxide dismutase), a mitochondrial superoxide dismutase that might defend against oxidative stress (Figure 3-3 A), indicating induced mitochondrial stress and activated stress response.

Altered respiration and induced stress response has been linked to increased oxidative stress in some mitochondrial mutant nematodes (Hekimi *et al.*, 2011). I hypothesized that *wah-1* downregulation could alter ROS homeostasis as a consequence of mitochondrial dysfunction. To asses ROS levels *in vivo*, I incubated RNAi treated nematodes with the cell permeate reagent 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), which becomes fluorogenic when oxidised. Surprisingly, WAH-1 loss resulted in a decrease of fluorescent signals (Figure 3-3 B). Although, DCF-DA is one of the most widely used techniques for directly measuring the redox state of cells and nematodes (Eruslanov & Kusmartsev, 2010), detection of ROS and oxidative stress *in vivo* remains a strongly debated challenge. Given that *wah-1* downregulation might lead to potential differences in cellular uptake of the drug, an alternative genetic approach for monitoring ROS was used. To this end, two strains expressing the redox sensitive biosensors Hyper and roGFP were subjected to *wah-1* RNAi and fluorescence was measured by spinning disc microscopy (Knoefler *et al.*, 2012). These strains change their fluorescent spectrum when mitochondria are oxidised. As demonstrated by DCF-DA staining, *wah-1* (RNAi) treatment of nematodes resulted in decreased $H_2O_2$ (Hyper) and GSH (roGFP) signals (Figure 3-3 C). As a positive control, the ETC mutant strain *mev-1* was used since it exhibits increased ROS levels (Dingley *et al.*, 2010; Senoo-Matsuda *et al.*, 2001). The *mev-1* mutation increased the oxidation of Hyper but did not show an effect on roGFP fluorescence compared to control animals. As an additional control, ROS levels were measured at different age of the nematodes as oxidative stress increases with age (Back *et al.*, 2012). Supportingly, the measurement of control animals by Hyper and roGFP at day 4 and day 7 showed an increase in ROS levels. However, animals subjected to *wah-1 (RNAi)* showed a decrease in ROS levels compared to control animals at both time points (Figure 3-3 D).

Taken together, *wah-1* (RNAi) alters mitochondrial function and results in expression of stress response genes. However, mitochondrial stress response is not induced by excessive ROS production, as their levels are decreased in *wah-1* (RNAi) animals.

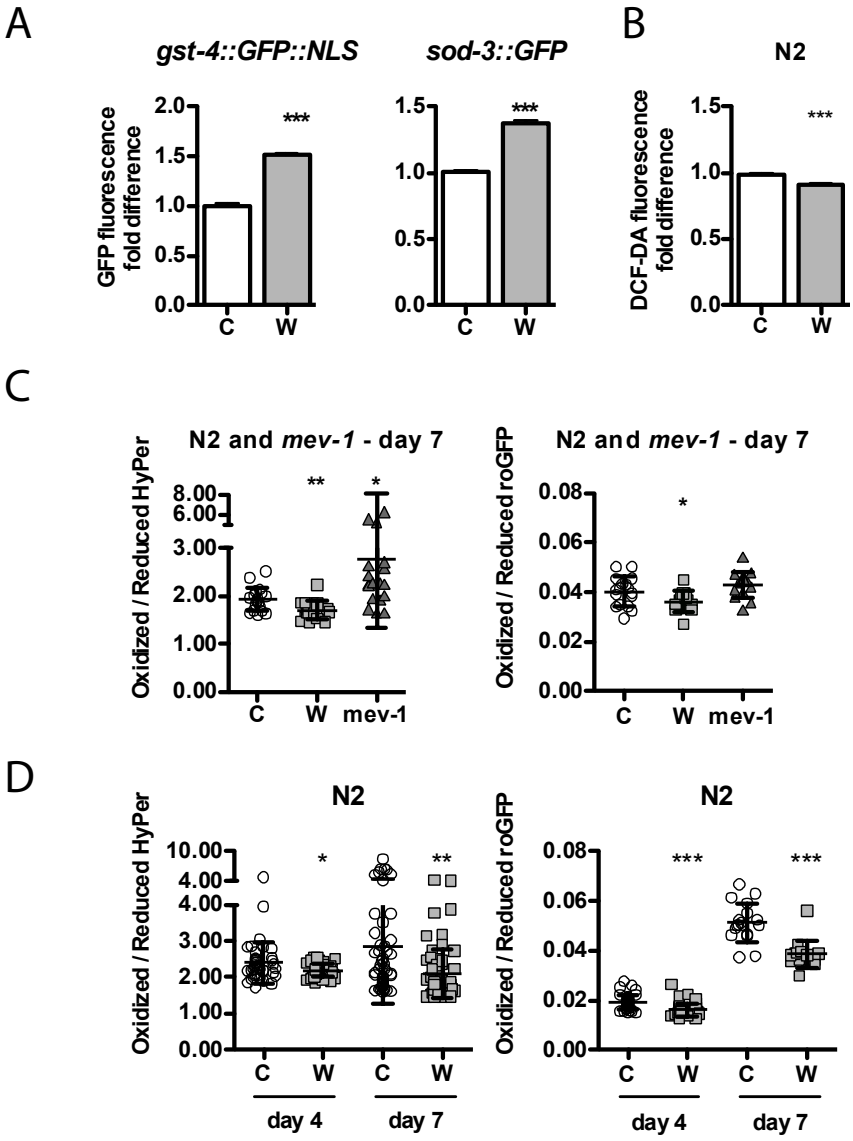


Figure 3-3: ***wah-1* (RNAi) induces stress response and decreases ROS production**. **(A)** Fluorescence analysis with the COPAS Biosorter of genes encoding for the detoxifying enzymes GST-4 and SOD-3 fused to GFP. **(B)** Measurement of ROS by DCF-DA staining. **(C)** Spinning disc microscopy of worms expressing *jrIs1 [Prpl-17::Hyper]* and *jrIs2 [Prpl-17::Grx-1-roGFP2]* in life *C. elegans* on day 7 in control, *wah-1* (RNAi) and *mev-1(kn-1)* animals. **(D)** Microscopy of *jrIs1 [Prpl-17::Hyper]* and *jrIs2 [Prpl-17::Grx-1-roGFP2]* in live *C. elegans* on day 4 and day 7 in control and *wah-1* (RNAi) animals. (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, **p< 0.01, *p< 0.05, two-tailed Student's t test)

## 3.3 Reduced WAH-1 levels induce the mitochondrial Unfolded Protein Response (UPRmt)

Organelle stress can induce the activation of protective mechanisms that restore organelle function and cell homeostasis. In mitochondria, the UPRmt can counteract disturbed mitochondrial function. Upon mitochondrial stress, the expression of small interfering heat shock proteins and chaperones is induced and molecules are transported to the mitochondria. A marker for the induction of the UPRmt is the expression of the heat shock protein HSP-6. To analyse whether *wah-1* deficiency in nematodes also induced the UPRmt, I analysed mRNA expression levels of *hsp-6* by qRT-PCR. *hsp-6* levels were increased by 2 fold in *wah-1* deficient conditions (Figure 3-4 A). To confirm the induction of the UPRmt by *wah-1* (RNAi), I assessed the expression of GFP under control of the *hsp-6* promoter with the COPAS Biosort system. Furthermore, I tested *hsp-60* expression levels, another chaperone activated by the UPRmt. GFP signals for both HSPs increased when nematodes were treated with *wah-1* (RNAi) (Figure 3-4 B). To assess weather the induced stress response was mitochondria-specific or also affected other cellular organelles, I tested the potential influence of mitochondrial dysfunction on the ER stress response (UPR) by *hsp-4* mRNA levels. HSP-4 is an ER-specific heat shock protein in *C. elegans* and the homologue of mammalian binding immunoglobulin protein (BiP) that is important for folding and oligomerisation of newly synthesised proteins. Fluorescence analysis with the COPAS Biosort system of an *hsp-4p::GFP* strain displayed no difference in GFP signal when treated with *wah-1* (RNAi), indicating no increase in ER stress (Figure 3-4 C). However, mitochondria play an important role for the general health of an organism. Their dysfunction can lead to severe consequences for the whole cell. Therefore, I measured the expression levels of the cytosolic heat shock protein *hsp-16.2 (*Figure 3-4 D*)*. However, decreased *wah-1* levels did not affect unfolded protein response mechanisms other than the mitochondria-specific UPRmt.

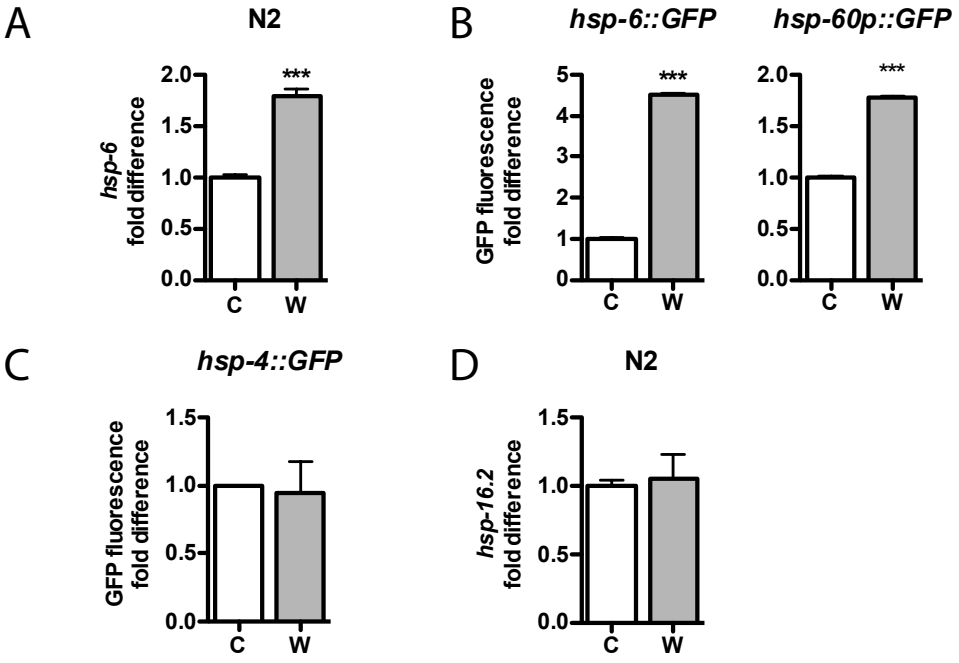


Figure 3-4: ***wah-1* (RNAi) induces UPRmt. (A)** Quantitative real-time analysis of *hsp-6* levels after *wah-1*(RNAi) treatment. **(B)** COPAS Biosort measurement of *hsp-6*::*GFP* and *hsp-60*::*GFP* strains after *wah-1* (RNAi) treatment. **(C)** COPAS Biosort measurement of the endoplasmic reticulum UPR in *hsp-4*::*GFP* animals. **(D)** qRT-PCR for the cytosolic UPR gene *hsp-16.2*. (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, two-tailed Student's t test)

In *C. elegans*, the original discovery of the UPRmt suggested that disturbed mitochondrial function results in the increase of un- or misfolded proteins in the mitochondrial matrix due to insufficient chaperone activity. Misfolded proteins are then digested into small peptides, exported through the HAF-1 transporter into the cytosol and activate UBL-5 and the transcription factor DVE-1 (Haynes & Ron, 2010). In turn, UBL-5/DVE-1 translocates into the nucleus and induces the expression of heat shock proteins. To test whether *wah-1* also activated the UPRmt via this classic pathway, I measured *hsp-6* expression levels in *haf-1* mutants, which lack the mitochondrial transporter that is important for the export of peptides. Surprisingly, *haf-1* mutants also displayed increased *hsp-6* levels (Figure 3-5 A). To test, whether a reduction of *wah-1* actually induced UPRmt via UBL-5/DVE-1, I analysed UBL-5 levels in a transgenic worm strain expressing UBL-5 fused to GFP, driven by the *ubl-5* promoter. Indeed, *wah-1* downregulation resulted in increased *ubl-5* expression compared to control animals, whereas the inhibition of UBL-5 by RNAi blocked GFP expression (Figure 3-5 B).

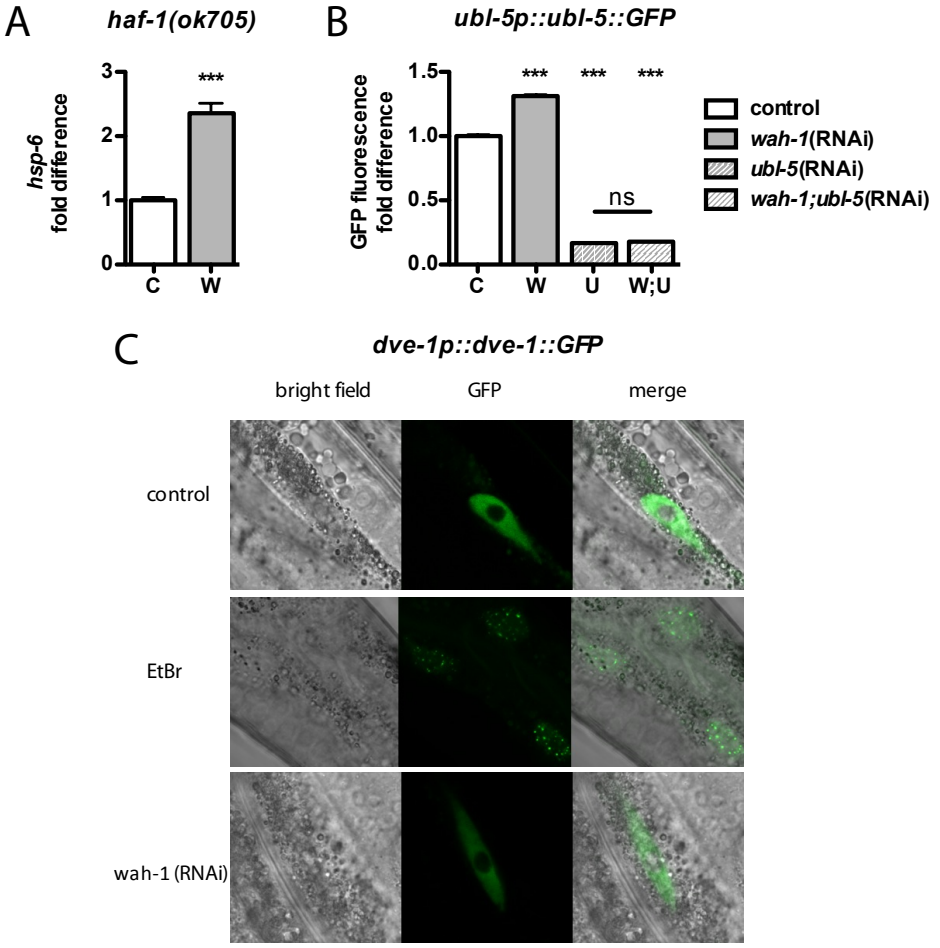


Figure 3-5: **UPRmt in WAH-1 deficient animals is not activated through the classical DVE-1/UBL-5 route. (A)** qRT-PCR of the UPRmt marker *hsp-6* in mutant *C. elegans* for the peptide exporter HAF-1. **(B)** COPAS Biosort analysis of the UPRmt marker strain *ubl-5p::ubl-5::GFP*. **(C)** Spinning disc microscopy of the cellular localisation of dve-1p::dve-1::GFP signal. (C: control, W: *wah-1* (RNAi), U: *ubl-5* (RNAi), W;U: *wah-1;ubl-5* (RNAi) treatment. Means ± S.E.M., ***p< 0.001, two-tailed Student's t test)

Nevertheless, even low expression levels of the protein might be sufficient to activate chaperone expression, as the determining factor for stress response activation is the nuclear translocation of the transcription factor DVE-1 from the cytosol. In order to assess the nuclear translocation of DVE-1, I studied its localisation by microscopy using the *dve-1p::dve-1::gfp* reporter strain. In untreated animals, DVE-1::GFP signal is observed in the cytosol of *C. elegans* cells (Figure 3-5 C). As a positive control for the translocation of DVE-1, worms were treated with the mtDNA intercalating drug ethidium bromide (EtBr). EtBr is a well-known mitochondrial stressor and is widely used to induce UPRmt (Addo *et al.*, 2010). The treatment of *dve-1p::dve-1::gfp* animals with 50 µg/ml EtBr resulted in pronounced nuclear translocation. However, *wah-1 (RNAi)*

treated animals did not display nuclear GFP foci, indicating that UBL-5/DVE-1 might not be involved in *wah-1* (RNAi) dependent UPRmt induction.

Additionally, fluorescent signals of *hsp-60p::*GFP animals were measured after *wah-1* downregulation and simultaneous block of UBL-5 or DVE-1 by RNAi. Interestingly, increased HSP expression in *wah-1* (RNAi) treated animals was not abrogated by *ubl-5* knockdown (Figure 3-6 A). Remarkably, also DVE-1 downregulation did not affect the *wah-1* (RNAi)-induced mitochondrial stress response. A second route of UPRmt induction is the activation of ATFS-1. ATFS-1 is a transcription factor that was identified to work independently from DVE-1/UBL-5. Therefore, I tested the involvement of ATFS-1 in the *wah-1* (RNAi) dependent induction of UPRmt by the measurement of HSP expression levels. In fact, *atfs-1* mutant animals neither showed induction of *hsp-60p::gfp* protein expression levels (Figure 3-6 B), nor increased *hsp-6* mRNA levels after knockdown of *wah-1* (Figure 3-6 C).

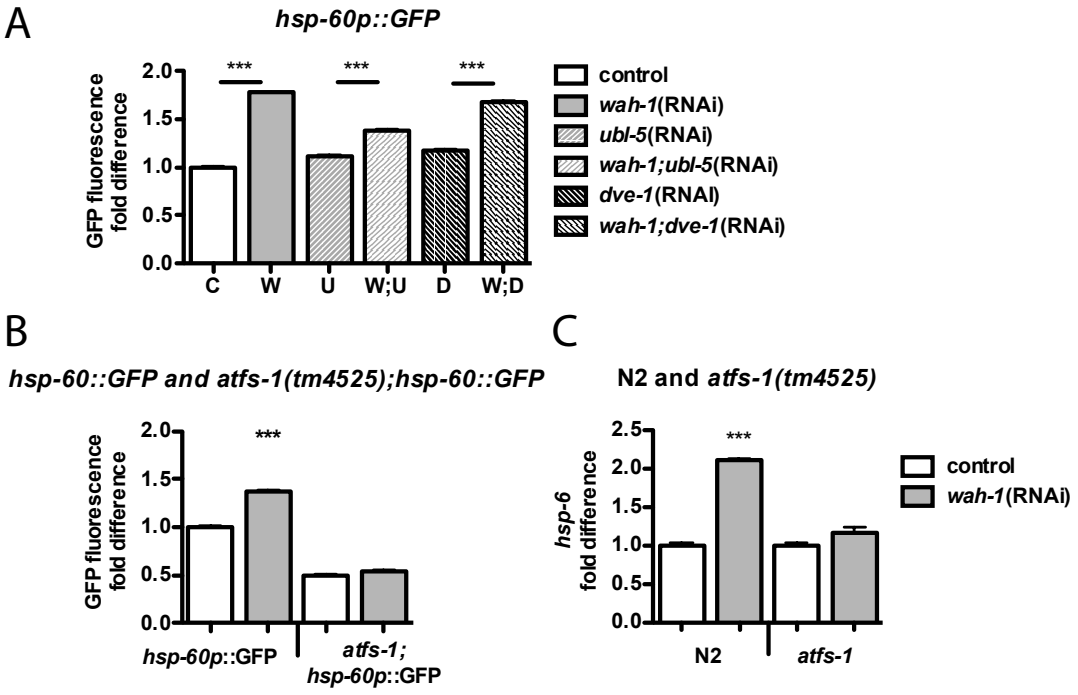


Figure 3-6: **UPRmt is activated by the transcription factor *atfs-1*. (A)** Analysis of *hsp-60p::GFP* levels by the COPAS Biosorter after downregulation of *wah-1* (W), *ubl-5* (U), *wah-1;ubl-5* (W;U), *dve-1* (D) and *wah-1;dve-1* (W;D). **(B)** Biosorter fluorescence measurement of *hsp-60::GFP* and *atfs-1(tm4525);hsp-60p::GFP* animals. **(C)** qRT-PCR for *hsp-6* in wild type N2 and mutant *atfs-1(tm4525)* nematodes. (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, two-tailed Student's t test)

Together, these data demonstrate the activation of the mitochondria-specific UPRmt by *wah-1* (RNAi). Furthermore, the experiments unravel that the UPRmt activation requires ATFS-1, while the DVE-1/UBL-5 axis is dispensable.

## 3.4 Impaired insulin/IGF-1 signalling extends the lifespan of *wah-1* deficient nematodes

ETC deficiency and impaired mitochondrial function is observed in many mitochondrial disorders. However, to date, no effective treatment is available to restore OXPHOS function caused by inherited or acquired genetic mutations. Therefore, I used *wah-1* downregulation in *C. elegans* as a model to study human pathology caused by mutations in the *AIFM1* gene. The aim was to investigate genetic ways to protect from the detrimental effects of mitochondrial deficiency caused by WAH-1 loss. Insulin/IGF-1 signalling is a fundamental and conserved regulator of survival and metabolism. In *C. elegans*, DAF-2 is the sole insulin/IGF-1 like receptor that negatively regulates the forkhead transcription factor DAF-16/FOXO through a phosphorylation cascade involving AGE-1/PI3K and AKT-1/2 kinases (Kenyon *et al.*, 1993; Ogg *et al.*, 1997). A reduction of IIS in nematodes dramatically extends the lifespan of the organism. To assess whether IIS deficiency could counteract *wah-1* (RNAi)-induced mitochondrial defects, I analysed the effect of *wah-1* (RNAi) on the lifespan of insulin/IGF-1/DAF-2 deficient nematodes.

In contrast to wild type animals, loss of DAF-2 did not only abolish the reduction of lifespan caused by *wah-1* (RNAi) but rather extended it significantly by ~15 % (Figure 3-7 A). Interestingly, the extension of the mean lifespan of insulin/IGF-1 mutant animals was independent of the downstream DAF-16/FOXO transcription factor (Figure 3-7 B). This suggests, that the protective effect of reduced IIS was not only mediated by global effects of lifespan extension, but elicited its capabilities through distinctive alterations in cellular homeostasis. Activated DAF-16 induces the expression of diverse stress response genes that influence cellular function and homeostasis. As the IIS pathway is complex and the transcription factor DAF-16 was not responsible for the lifespan extending effect in *wah-1* deficient animals, further experiments were conduct-

ed in *daf-16;daf-2* double mutant animals that lack both the insulin/IGF-1/DAF-2 receptor and the transcription factor DAF-16/FOXO. This allowed investigating the pathways that were responsible for the lifespan extension of *wah-1* (RNAi) nematodes excluding DAF-16-dependent influences.

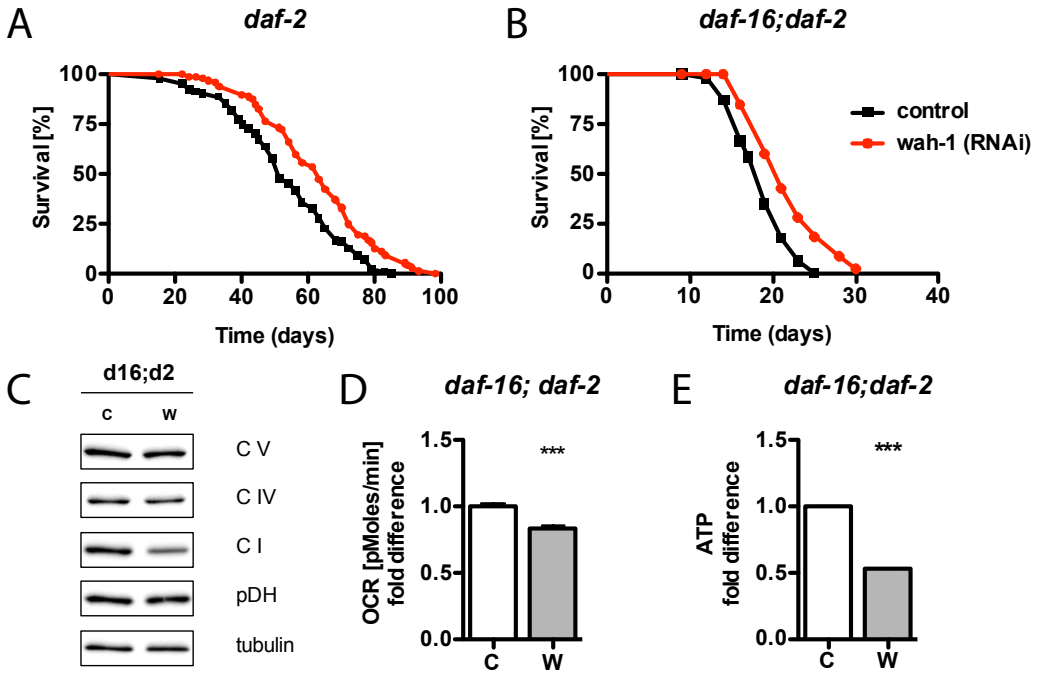


Figure 3-7: **Reduced insulin/IGF-1 signaling extends the lifespan of WAH-1 deficient animals.** Downregulation of WAH-1 extends the lifespan of **(A)** daf-2 mutants, **(B)** independently of the transcription factor DAF-16/FOXO. **black**: control, **red**: *wah-1* (RNAi). **(C)** Immunoblot of mitochondrial ETC complexes C I, C IV, C V and pDH in *daf-16(mu86);daf-2(e1370)* animals. **(D)** Oxygen consumption rates and **(E)** ATP levels in *daf-16(mu86);daf-2(e1370)* mutants. (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p value <0.001, two-tailed unpaired Student's t-test).

To test whether impaired IIS protected nematodes from the adverse effects of low *wah-1* levels through a stabilisation of OXPHOS, I analysed ETC complexes in *daf-16;daf-2* mutants by western blot analysis. Similar to wild type animals, *wah-1* silencing significantly reduced C I protein levels (Figure 3-7 C). ATP synthase, C IV and pDH levels were not significantly affected. In line with the changes in the ETC subunits of wild type and mutant nematodes, animals displayed similarly reduced mitochondrial respiration (Figure 3-7 D). Furthermore, ATP levels were also decreased (Figure 3-7 E). As observed in wild type N2 animals, the downregulation of *wah-1* also decreased ROS levels in the *daf-16;daf-2* mutant background (Figure 3-8 A), indicating that ROS signal-

ling does not play a role in the opposing effect on lifespan. Since loss of *wah-1* caused ETC impairment, I performed epistasis analysis in *daf-16;daf-2;gas-1* triple mutants that already carry a mutation impairing CI (Figure 3-8 B). Notably, *wah-1 (RNAi)* did not affect the survival of the triple mutant. However, the *daf-16;daf-2* mutation significantly extended the lifespan of nematodes with a loss-of-function mutation in the *gas-1* gene in control conditions, similarly to *wah-1* silencing.

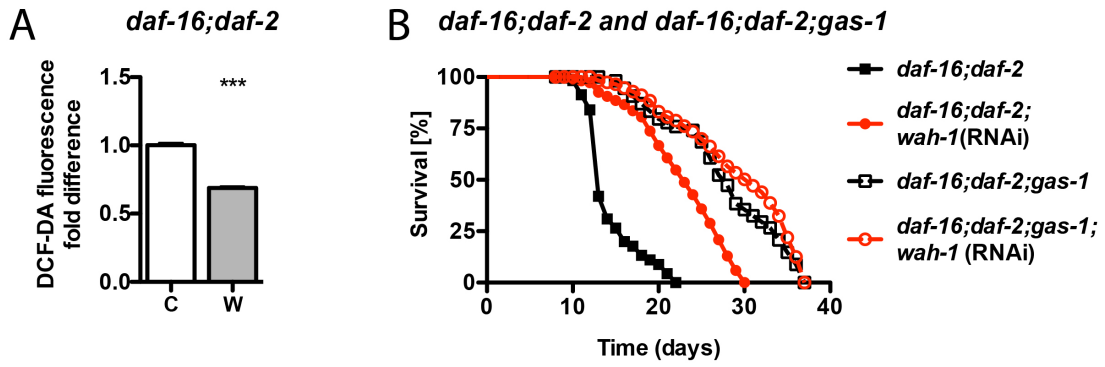


Figure 3-8: ***wah-1* (RNAi) reduces ROS in insulin/IGF-1 mutants and does not affect the lifespan of *daf-16;daf-2;gas-1* triple mutants. (A)** Measurement of ROS in *daf-16(mu86);daf-2(e1370)* animals by DCF-DA staining. **(B)** Lifespan analysis of *daf-16(mu86);daf-2(e1370)* and *daf-16(mu86);daf-2(e1370);gas-1(fc21)* mutants on control and *wah-1* (RNAi) bacteria. **Black**: control, **red**: *wah-1* (RNAi). (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, two-tailed Student's t test)

In conclusion, impaired IIS signalling significantly extends the lifespan of *wah-1* deficient animals in a *daf-16* independent manner. Importantly, low IIS increases survival also in other C I mutants such as *gas-1*. This observation is important for the evaluation of reduced IIS as a protective mechanism for a broader spectrum of OXPHOS dysfunction and mitochondrial diseases that are independent of WAH-1.

## 3.5 Impaired insulin/IGF-1 signalling leads to metabolic rewiring in *wah-1* treated nematodes

OXPHOS deficiency can dramatically affect cellular metabolism. However, IIS plays a central role in the metabolic homeostasis of the cell. Consequently, impaired IIS could no longer operate as an orchestrator of metabolism when mitochondrial function is affected. Therefore, the metabolism of *wah-1* deficient wild type and *daf-16;daf-2* mutant *C. elegans* could be different and cause an opposing effect on nematode lifespan.

It has been demonstrated that inhibition of the ETC can stimulate the switch from cellular respiration to glucose metabolism (Koopman, 2013; Koopman, 2012). Therefore, I tested whether loss of AIF/WAH-1 could lead to a metabolic shift in favour of alternative pathways that do not rely on mitochondrial energy production. To this end, I measured mRNA expression of different metabolic markers by qRT-PCR. Interestingly, qRT-PCR analysis of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (*gpd-3*) revealed a significant upregulation in both wild type and *daf-16;daf-2* mutants treated with *wah-1 (RNAi)* (Figure 3-9 A). In comparison, qRT-PCR analysis of phosphoenolpyruvate carboxykinase (PEPCK), which catalyses the conversion of oxaloacetate to phosphoenolpyruvate during gluconeogenesis (McElwee *et al.*, 2006), was not affected when animals were treated with *wah-1* (RNAi) (Figure 3-9 B).

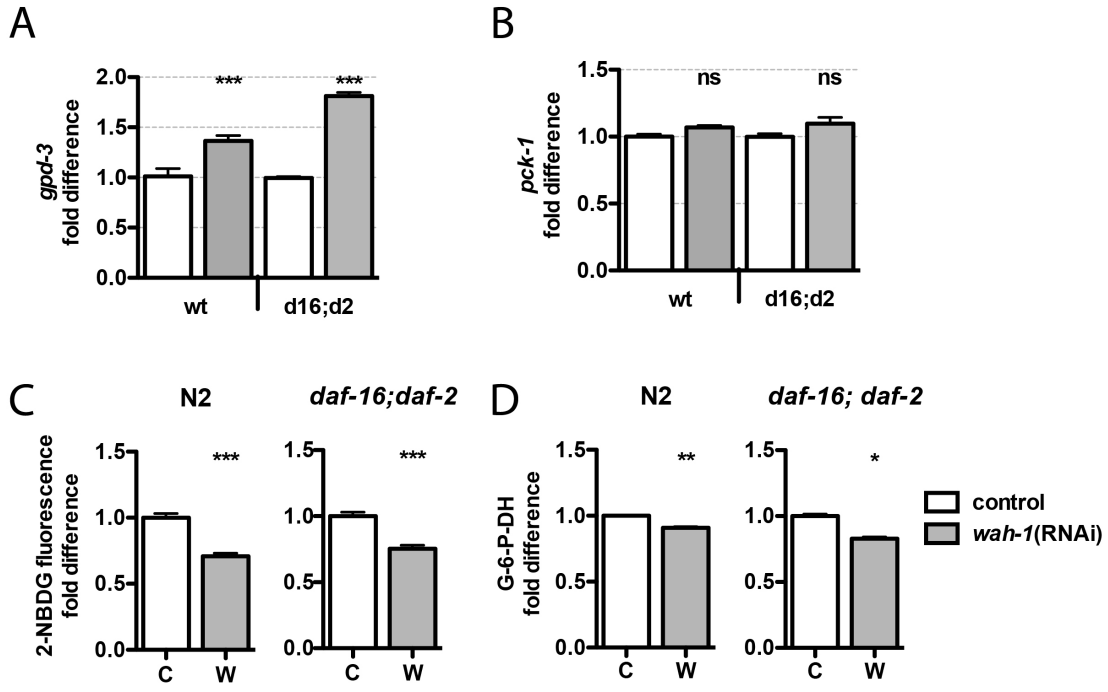


Figure 3-9: **WAH-1 deficiency affects glucose metabolism.** mRNA expression levels of **(A)** glyceraldehyde 3-phosphate dehydrogenase (*gpd-3*) and **(B)** phosphoenolpyruvate carboxykinase *(pck-1)* in wild type animals and *daf-16(mu86);daf-2(e1370)* mutants. **(C)** Fluorescence analysis of 2-NBDG stained wild type and *daf-16;daf-2* animals. **(D)** Calorimetric measurement of glucose-6-phosphate dehydrogenase (G-6-PDH) activity after *wah-1* downregulation by RNAi. (Mean ± S.E.M., ***p< 0.001, **p< 0.01, *p< 0.05, ns: not significant, two-tailed Student's t test)

Although, both wild type and mutant animals display increased glycolytic activity, glucose uptake of the cells could be different. To this end, I measured glucose uptake of *C. elegans* cells *in vivo.* Surprisingly, upon *wah-1* downregulation, wild type and *daf-16;daf-2* cells displayed decreased glucose uptake as demonstrated by incubation with the fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Figure 3-9 C). Furthermore, also glucose-6-phosphate dehydrogenase (G-6-P-DH) levels were decreased in both backgrounds, as measured by qRT-PCR (Figure 3-9 D). Thus, altered glucose metabolism does not explain the different lifespan of *wah-1* (RNAi) treated wild type and IIS mutant animals as neither glycolysis and gluconeogenesis nor glucose uptake were differentially affected in the genetic backgrounds. Hence, cellular metabolism might have switched to glucose independent energy sources.

Therefore, I measured other metabolite levels in whole body extracts from wild type and *daf-16;daf-2* mutant nematodes treated with *wah-1* (RNAi). Lactate dehydrogenase (LDH) activity, fumarate and branched chain amino acids (BCAA) levels were analysed with specific calorimetric test kits. However, decreased WAH-1 levels did not affect fumarate levels (Figure 3-10 A), whereas BCAA levels (Figure 3-10 B) and LDH activity (Figure 3-10 C) were slightly decreased in wild type and *daf-16;daf-2* nematodes without significant difference between the strains.

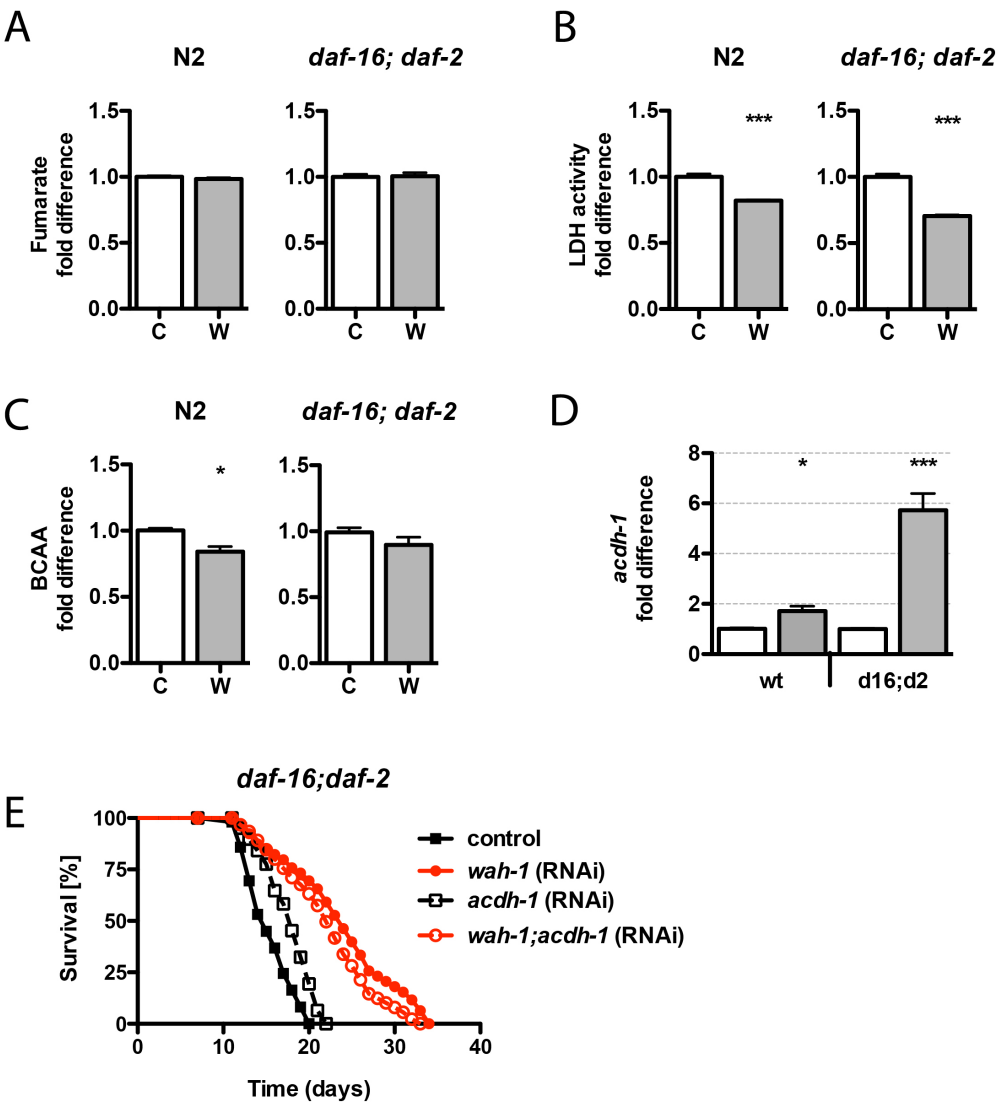


Figure 3-10: **Decreased insulin/IGF-1 signalling shifts expression of the metabolic marker *acdh-1*. (A)** Calorimetric metabolite analysis of **(A)** fumarate, **(B)** lactate dehydrogenase (LDH) activity and **(C)** branched chain amino acids (BCAA) in *C. elegans* full body lysates. **(D)** mRNA expression levels of **(C)** acyl-CoA dehydrogenase (*acdh-1*) in wild type animals and *daf-16(mu86);daf-2(e1370)* mutants by qRT-PCR. **(D)** Lifespan analysis of *daf-16(mu86);daf-2(e1370)* mutants after *wah-1* (RNAi) and additional downregulation of *acdh-1* by RNAi. (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, *p< 0.05, two-tailed Student's t test)

In *C. elegans*, *acdh-1* encodes a mitochondrial short-chain acyl-CoA dehydrogenase that catalyses fatty acid β-oxidation and thus plays a key role in energy production (Taubert *et al.*, 2006). Moreover, ACDH-1 has also been identified as a marker of global metabolic network perturbation (Watson *et al.*, 2013). Therefore, I tested *acdh-1* expression levels in *wah-1* deficient wild type and *daf-16;daf-2* mutant animals. Although, the expression of this gene was slightly increased in wild type animals, WAH-1-loss had a strong impact on *acdh-1* levels in conditions of low IIS and increased the expression up to 6-fold (Figure 3-10 D). Despite ACDH-1 being an important metabolic sensor, the increase in its activity was not causative for the lifespan extension as knockdown of *acdh-1* in *wah-1 (RNAi)* treated *daf-16;daf-2* mutants did not affect the nematodes survival (Figure 3-10 E).

In summary, ETC deficiency induced by *wah-1* downregulation reduced the energy production through OXPHOS. Consequently, cells adapted metabolism accordingly and induced oxygen-independent glucose metabolism. However, differences in glucose metabolism could not explain the opposing effect of *wah-1* downregulation in *wah-1* (RNAi) treated wild type and IIS mutant animals. In both backgrounds, glucose metabolism was similarly affected. Nevertheless, expression of the metabolic marker *acdh-1* was highly increased in *daf-16;daf-2* mutants but not in wild type animals. These data indicate a strong involvement of metabolism upon reduction of WAH-1 levels. However, it was not clear how the cells would control the changes in metabolism.

## 3.6 HIF-1, AMPK and autophagy mediate metabolic rewiring

Upon cellular stress and energy deprivation, nutrient sensors and metabolic processes are activated that tune metabolism and protect the cell from detrimental consequences. HIF-1, AMPK and autophagy are important factors in this cellular stress response. I demonstrated that impaired IIS extended the lifespan of OXPHOS deficient animals and induced the expression of *acdh-1*. Therefore, I first asked whether this expression was dependent on HIF-1 and AMPK. To this end, I downregulated *wah-1* by RNAi in triple mutant IIS strains, i.e. *daf-16;daf-2;hif-1* (HIF-1 loss-of-function) and *daf-16;daf-2;aak-2* (AMPK loss-of-function) and measured *acdh-1* mRNA levels by qRT-PCR.

As already demonstrated, *acdh-1* expression increased in *wah-1* (RNAi) treated *daf-16;daf-2* animals. Interestingly, the additional loss of *hif-1* or *aak-2* function suppressed the *acdh-1* mRNA increase (Figure 3-11 A) and reduced expression to wild type levels. Therefore, *hif-1* and *aak-2* seem to play an important role for the metabolic changes in WAH-1 deficient IIS mutants. To elucidate the role of autophagy in IIS-mediated *acdh-1* expression, I decreased autophagic function by *bec-1* (RNAi) in the *daf-16;daf-2* mutant background and measured *acdh-1* by qRT-PCR. RNAi solely against *bec-1* strongly reduced *acdh-1* expression levels in *daf-16;daf-2* mutants compared to control levels (Figure 3-11 B). Further, double RNAi against *wah-1* and *bec-1* reduced the strong *acdh-1* expression by *wah-1* alone. Hence, autophagy seems to play an important role in the metabolic adaption to mitochondrial stress along with HIF-1 and AMPK.

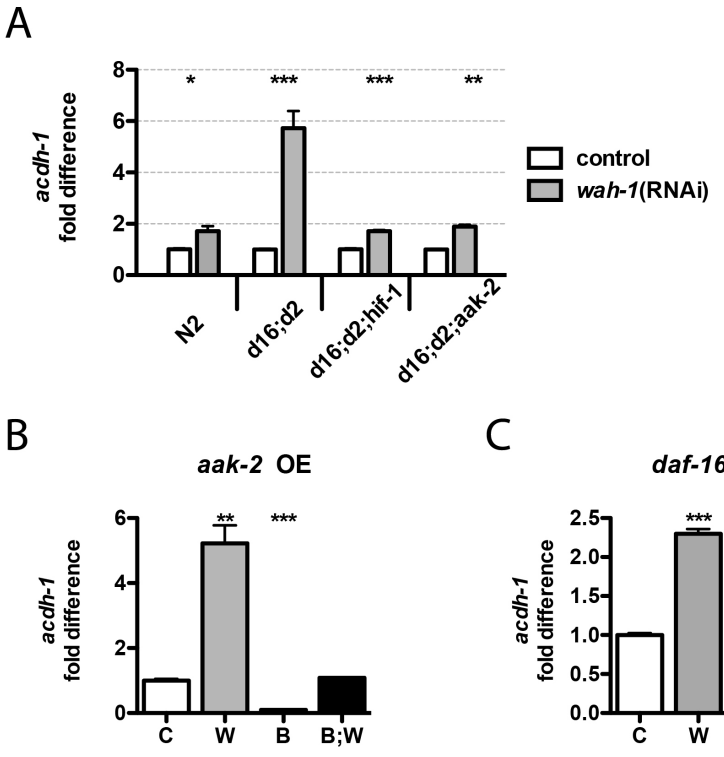


Figure 3-11: ***acdh-1* mRNA levels are dependent on *hif-1*, *aak-2* and autophagy. (A)** mRNA of *acdh-1* measured by qRT-PCR in wild type N2, *daf-16(mu86);daf-2(e1370), daf-16(mu86);daf-2(e1370);hif-1(ia4)* and *daf-16(mu86);daf-2(e1370)aak-2(ok524)* animals. *acdh-1* mRNA levels in **(B)** *daf-16(mu86);daf-2(e1370)* and **(C)** AAK-2 overexpressing nematodes treated with control, *wah-1*, *bec-1* and *wah-1;bec-1* (RNAi). (C: control, W: *wah-1* (RNAi), B: *bec-1* (RNAi), W;B: *wah-1;bec-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, **p< 0.01, *p< 0.05, two-tailed Student's t test)

AMPK itself has been demonstrated to be a key regulator of metabolism and showed to be crucial for *acdh-1* expression in *daf-16;daf-2*; *wah-1* (RNAi) animals. Therefore I asked weather overactivation of AMPK was sufficient to induce *acdh-1* expression. To this end, I measured *acdh-1* mRNA levels in a mutant nematode that overexpresses *aak-2* (*aak-2* OE). Strikingly, *aak-2* OE increased *acdh-1* levels when treated with *wah-1* (RNAi) similarly to *daf-16;daf-2* mutants (Figure 3-11 C). The additional downregulation of autophagy by *bec-1* (RNAi) suppressed the increase in *acdh-1* expression.

In summary, *acdh-1* expression levels suggest metabolic rewiring in mitochondrially impaired IIS mutants. This change in metabolism was dependent on the activity of HIF-1 and AMPK. Interestingly, AMPK overexpression was sufficient to induce *acdh-1* expression. Furthermore, autophagy seems to play a key role in this metabolic adaption and was required for *acdh-1* gene expression.

## 3.7 HIF-1 is activated by *wah-1* (RNAi) and important for the lifespan extension by *daf-16;daf-2*

In interaction with IIS/DAF-2 signalling, HIF-1 is an important mediator of lifespan (Chen *et al.*, 2009; Leiser & Kaeberlein, 2010; Zhang *et al.*, 2009) and shares a subset of target genes with the transcription factor DAF-16 (Hoogewijs *et al.*, 2007; McElwee *et al.*, 2004). Although, DAF-16 is dispensable for the promotion of lifespan in WAH-1 deficient animals (Chapter 3.4 ), active HIF-1 might mediate lifespan extension. Furthermore, *acdh-1* expression levels were dependent on the hypoxia-inducible factor HIF-1 in *daf-16;daf-2* mutants with impaired mitochondrial respiration. Therefore, HIF-1 might play an important role in metabolic rewiring reflected by *acdh-1* expression levels.

To address HIF-1 activity, HIF-1 protein and *nhr-57* mRNA levels were measured by western blot and qRT-PCR, respectively. In line with the effect of *wah-1* downregulation on respiration (Chapter 3.1 ), an increase in HIF-1 protein levels as well as an increase in the expression of the HIF-1 target nuclear hormone receptor *nhr-57* was observed. This increase occurred in both wild type (Figure 3-12 A-B) and insulin/IGF-1 mutant animals (Figure 3-12 C-D). To test the involvement of *hif-1* in the

animal's survival, a lifespan analysis was performed in *hif-1* loss-of-function mutants. *hif-1* did not affect the lifespan of *wah-1 (RNAi)* treated worms in the wild type background (Figure 3-12 E). However, *hif-1* (RNAi) abrogated the lifespan extension observed in *wah-1* (RNAi) treated *daf-16;daf-2* mutants (Figure 3-12 F). Therefore, HIF-1 seems to play an important role in IIS dependent lifespan extension of OXPHOS deficient *wah-1* (RNAi) animals.

The results suggest that the transcription factor HIF-1 plays an important role in the lifespan extension of ETC-impaired IIS mutants.

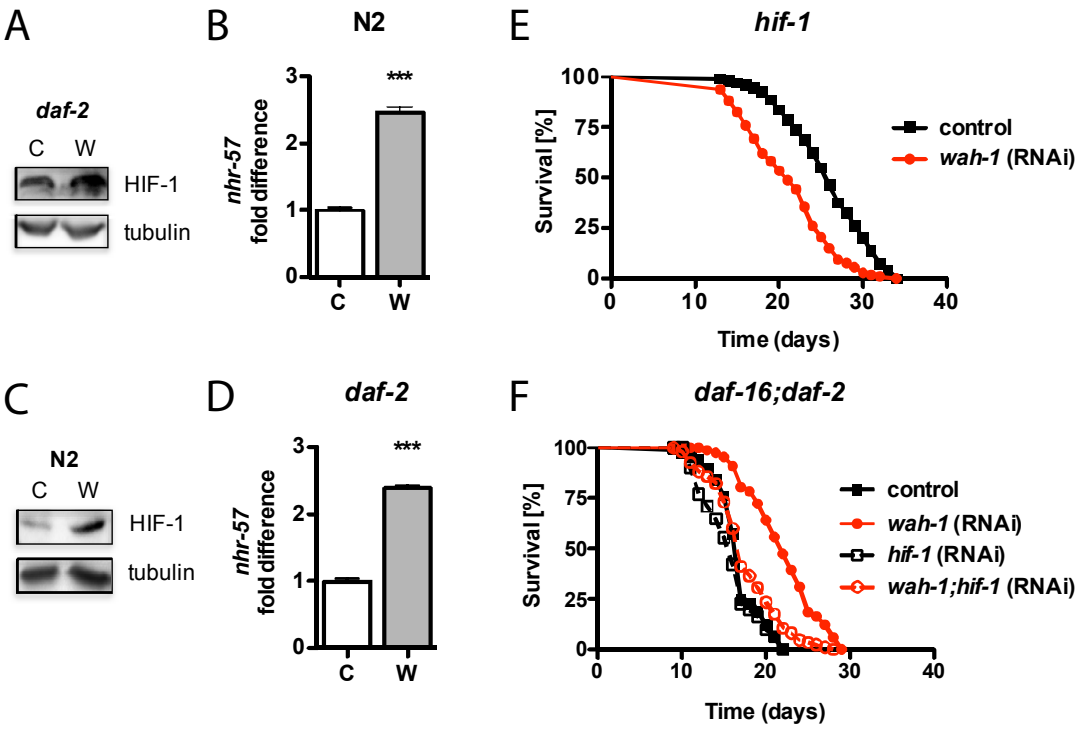


Figure 3-12: **Activation of Hypoxia-Inducible Factor is required for the lifespan extension of WAH-1 deficient *daf-16;daf-2* animals.** HIF-1 protein levels measured by western blot and *nhr-57* mRNA analysed by qRT-PCR in **(A-B)** wild type animals and **(C-D)** insulin/IGF-1 mutants. Lifespan of **(E)** *hif-1(ia4)* mutants treated with control and *wah-1* (RNAi) and **(F)** *daf-16(mu86);daf-2(e1370)* mutants treated with control, *wah-1*, *hif-1* and *wah-1;hif-1* (RNAi). (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, two-tailed Student's t test)

## 3.8 The lifespan extension of WAH-1 deficient animals is mediated by AMPK/AAK-2

Apart from the activation of protective pathways under hypoxic conditions by HIF-1, other cellular sensors such as AMPK/AAK-2 play an important role in the maintenance of cellular function upon mitochondrial impairment. AMPK/AAK-2 is a nutrient sensor that monitors the energy status of the cell and tunes metabolism accordingly. Active IIS blocks the activation of AMPK/AAK-2 and the nutrient sensor has been found to be required for the long lifespan of *daf-2* mutants (Curtis *et al.*, 2006) and animals subjected to dietary restriction (DR) (Greer *et al.*, 2007).

As impaired OXPHOS can activate AMPK/AAK-2, the energy sensor might also be activated by the consequences of *wah-1* (RNAi)-induced mitochondrial dysfunction. However, in wild type animals, active IIS might inhibit AMPK/AAK-2. In contrast, IIS is inactive in *daf-16;daf-2* mutants. Consequently, the inhibition of the energy sensor might be abrogated and lead to AMPK/AAK-2 activity. Thereby, active AMPK/AAK-2 could mediate lifespan extension in mutant animals. To test this, I first measured phospho-AMPK (the active form of AMPK/AAK-2) protein levels by western blot analysis. Indeed, phospho-AMPK levels were increased in *daf-16;daf-2* but not in wild type animals subjected to *wah-1 (RNAi)* (Figure 3-13 A). To test whether AMPK/AAK-2 activity also has an impact on the lifespan of *daf-16;daf-2* nematodes, the mutants were crossed with a *aak-2* loss-of-function mutation in the catalytic α subunit of AMPK/AAK-2. Lifespan analysis of these animals demonstrated that the increased survival of *daf-16;daf-2* animals was abolished (Figure 3-13 B). Since AMPK/AAK-2 activity was indispensable for the lifespan extension of *daf-16;daf-2* animals, it is possible that the sole overexpression of AAK-2 was sufficient for a lifespan extension in *wah-1 (RNAi)* treated nematodes. To test this hypothesis, *aak-2* OE animals were fed with control bacteria and *wah-1* (RNAi) and the effect on lifespan was observed. In contrast to the lifespan reduction by the *aak-2* loss-of-function mutation, the overexpression of AAK-2 was sufficient to increase the lifespan of *wah-1* (RNAi) animals (Figure 3-13 C).

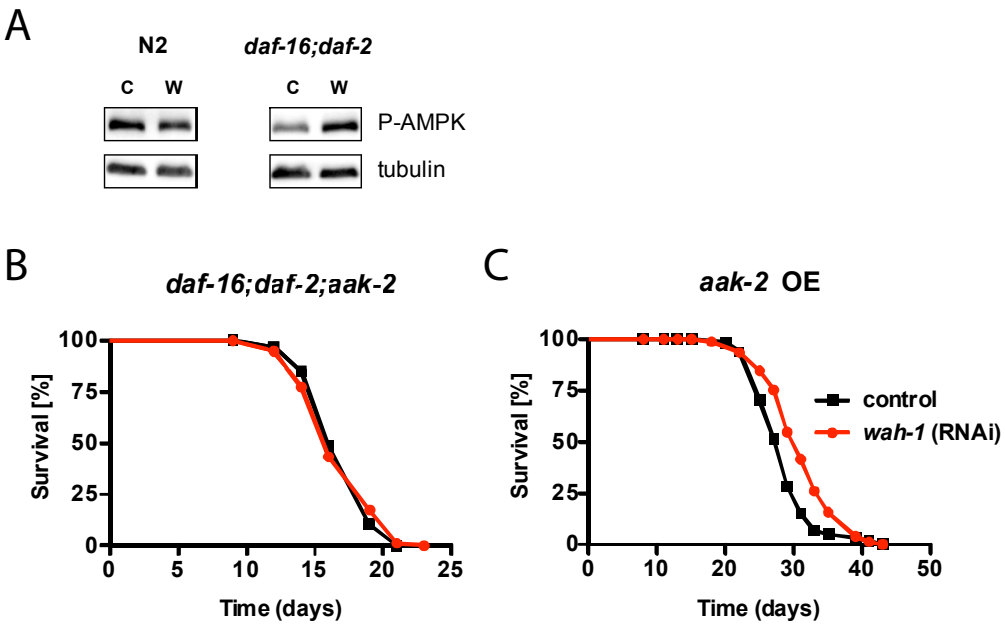


Figure 3-13: **Lifespan extension requires AMPK/AAK-2 activity. (A)** Western Blot analysis of Phospho-AMPK in wild type and *daf-16;daf-2* animals treated with *wah-1* (RNAi). Lifespan analysis of **(B)** daf-16;daf-2;aak-2 and **(C)** AAK-2 overexpressing animals. (C: control, W: *wah-1* (RNAi) treatment)

Taken together, these results highlight the important role of the energy sensor AMPK/AAK-2 in the regulation of lifespan. AMPK/AAK-2 activity was indispensable for the lifespan extension of *daf-16;daf-2* mutant worms with reduced WAH-1 levels. Moreover, its overexpression was even sufficient to extend the lifespan of *wah-1* (RNAi) treated wild type *C. elegans*. These results make AMPK/AAK-2 an interesting target for potential pharmacological treatment of higher organisms suffering from mitochondrial impairment (Wu *et al.*, 2014).

## 3.9 *daf-16;daf-2* and AAK-2 mediated lifespan extension requires autophagy

Under low energy conditions and upon mitochondrial damage, AMPK/AAK-2 activates autophagy (Hardie, 2011a; Salminen & Kaarniranta, 2012; Zhang, 2013). Notably, autophagy has been reported to play an important role for the lifespan extension under various conditions and is also linked to changes in metabolism (Hansen *et al.*, 2008; Hars *et al.*, 2007; Jia & Levine, 2007; Lim *et al.*, 2014; Melendez *et al.*, 2003; Toth *et al.*, 2008). To determine whether activated AMPK/AAK-2 also led to an increased autophagic flux after *wah-1* (RNAi) treatment, a transgenic *daf-2* line expressing a LGG-1/LC3::GFP reporter under the control of the *lgg-1* promoter was analysed for the number of GFP puncta. The number of puncta correlate with autophagic activity. Fluorescent puncta in the hypodermal seam cells were increased by *wah-1* (RNAi) treatment in IIS deficient mutants but not in wild type animals (Figure 3-14 A). To address the involvement of autophagy in the lifespan extension by *daf-16;daf-2* mutation, RNAi for *bec-1* was used. BEC-1 is a crucial protein for autophagic function and knockdown blocks autophagy. Decreased BEC-1 levels abolished the lifespan extension of *daf-16;daf-2* mutants subjected to *wah-1* (RNAi) (Figure 3-14 B). This means that autophagy was necessary for the lifespan extension of *wah-1* (RNAi) treated animals by the mutations in IIS. Furthermore, autophagy was also required for the lifespan extension by *aak-2* overexpression. The simultaneous decrease of *wah-1* and *bec-1* in animals overexpressing AMPK/AAK-2 diminished the lifespan extension compared to the exclusive downregulation of *wah-1* (Figure 3-14 C) emphasising the importance of autophagy in the lifespan extension by AMPK/AAK-2.

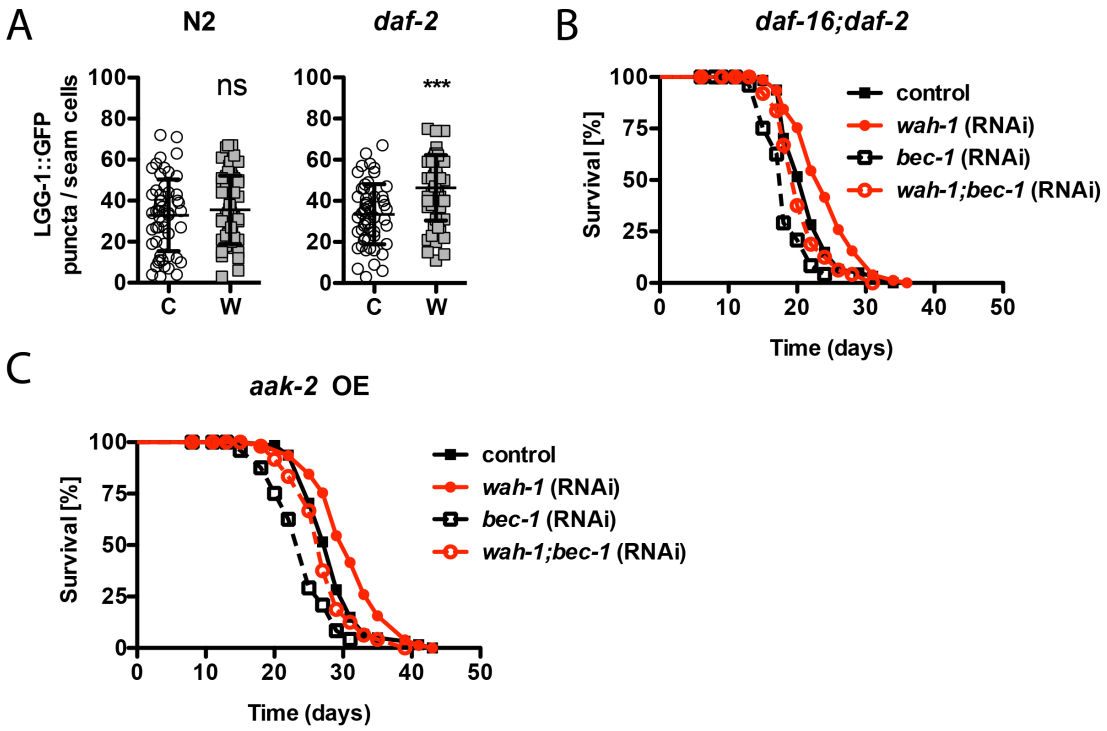


Figure 3-14: **Autophagy is required for lifespan extension. (A)** Microscopy analysis of LGG-1::GFP puncta in seam cells of wild type and *daf-2(e1370)* mutant *C. elegans* as a marker of autophagy. Lifespan of **(B)** *daf-16(mu86);daf-2(e1370)* and **(C)** AAK-2 overexpressing animals treated with *bec-1* (RNAi). (C: control, W: *wah-1* (RNAi) treatment. Mean ± S.E.M., ***p< 0.001, ns: not significant, two-tailed Student's t test)

In conclusion, while AMPK/AAK-2 activation in *wah-1* (RNAi) treated animals induced autophagy and increased lifespan, the block of the autophagic pathway abolished this lifespan extension. Therefore, autophagy plays a key role in the lifespan regulation of ETC deficient animals in IIS mutants and AMPK/AAK-2 overexpressing animals.

# 4 Discussion

Mitochondria are complex organelles that execute essential functions for the activity and survival of cells. These include energy and ROS production, biosynthesis and degradation of amino acids, fatty acid β-oxidation, calcium homeostasis and apoptosis. Due to the complex nature of mitochondrial architecture and mitochondrial function, it is not surprising that their dysfunction can result in the development of various pathological conditions. Inherited and spontaneous mutations in many different nuclear or mitochondrial DNA genes can cause severe mitochondrial dysfunction and result in mitochondriopathies and neurodegenerative diseases (Koopman *et al.*, 2013). Yet, cause and consequence of many syndromes are difficult to diagnose as genotype and phenotype are often poorly correlated, resulting in inadequate therapy of many patients with mitochondrial disorders (Chinnery, 2014). The heterogeneity of symptoms and the unique character of mitochondrial genomics make disease management further challenging. Although, the underlying genotype can be highly heterogeneous, many mitochondrial diseases share common cellular consequences and phenotypes. Thus, understanding common pathways downstream of ETC dysfunction could result in the development of treatments for many mitochondrial disease phenotypes.

In the present study, I induced mitochondrial dysfunction by reducing the expression of AIF/WAH-1 in *C. elegans*. Furthermore, I explored genetic ways to protect the organism from the detrimental consequences (Figure 4-1). Low levels of AIF protein resulted in ETC deficiency and OXPHOS dysfunction in *C. elegans*. Mitochondrial ETC complexes were destabilised and led to a decrease in oxygen consumption and ATP production. Mitochondrial morphology was affected and protective stress response mechanisms were induced, indicating profound impairment of mitochondrial function. As a result, the lifespan of nematodes was significantly reduced when fed with bacteria expressing *wah-1* (RNAi). However, IIS/DAF-2 signalling has been reported to extend the lifespan of various organisms (Bartke, 2008). In WAH-1 deficient nematodes, impaired IIS did revert the short lifespan. In fact, lifespan of the animals was further increased. This effect on lifespan extension was independent of the tran-

scription factor DAF-16 but dependent on HIF-1 and AMPK/AAK-2 as well as the crucial cellular process of autophagy. Strikingly, overexpression of AMPK/AAK-2 was sufficient to increase lifespan. Interestingly, levels of the metabolic marker ACDH-1 showed strong correlation with the lifespan of treated animals.

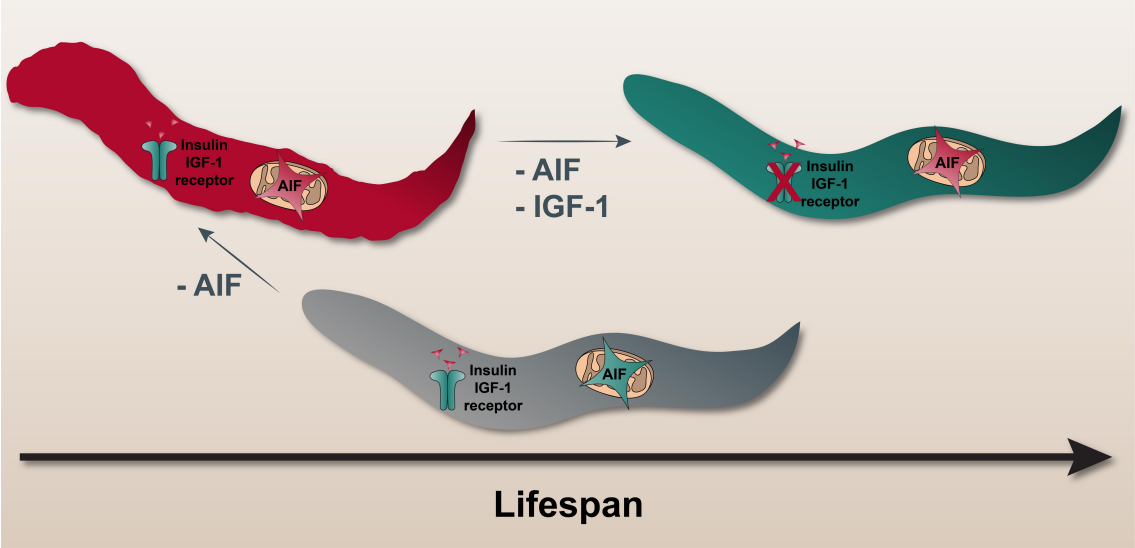


Figure 4-1: **Effect of AIF/WAH-1 decrease and impaired insulin/IGF-1 signalling on *C. elegans* lifespan.** Wild type *C. elegans* (grey) have a mean lifespan of ~20 days. Downregulation of AIF/WAH-1 protein levels (red) affects mitochondrial function and decreases their lifespan of ~20 %. However, additional decrease of insulin/IGF-1 signalling (green) extends the lifespan of the animals and they live even longer than wild type controls.

In summary, I present evidence, that the manipulation of ageing-related pathways can counteract the detrimental effects of mitochondrial dysfunction caused by an impaired ETC in *C. elegans*. Furthermore, the results promote the idea of a convergent therapy for various mitochondrial diseases that affect complex I and presumably other ETC dependent deficiencies. Specifically, AMPK and autophagy might serve as potent targets that could contribute to health in patients.

## 4.1 AIF/WAH-1 downregulation as a *C. elegans* model of mitochondrial deficiency

To mimic the situation in the harlequin mouse and patients that suffer from AIF dependent mitochondrial encephalomyopathy, RNAi against *wah-1*, the *C. elegans* orthologue of human *AIFM1*, was used. *wah-1* (RNAi) resulted in a profound down-regulation of mRNA levels to about 40 % compared to wild type levels. Although, AIF has not yet been shown to directly interact with any ETC subunits, there is growing evidence that the flavoprotein stabilises the ETC. Accordingly, reduced AIF levels destabilise the ETC and compromise OXPHOS (Klein *et al.*, 2002; Vahsen *et al.*, 2004). My findings revealed that AIF/WAH-1 deficiency disturbed ETC function in the nematode *C. elegans*. This resulted in reduced respiration rates and altered mitochondrial morphology. Furthermore, RNAi-induced mitochondrial defects decreased ATP levels, reduced oxygen consumption and altered mitochondrial morphology in *C. elegans*. Thus, the function of AIF/WAH-1 in the maintenance of the ETC and cellular respiration appears to be evolutionarily conserved. Moreover, decreased WAH-1 levels shortened the lifespan of wild type nematodes. As WAH-1 deficiency did not influence the lifespan of mutants that already carry a genetic lesion in C I (i.e. *gas-1(fc21)*), WAH-1 likely affects lifespan by altering mitochondrial respiration. This observation supports the hypothesis that distinctive mitochondrial mutations can affect common downstream pathways that ultimately affect the function of the cell.

## 4.2 ROS levels and ETC dysfunction

Commonly, mitochondrial deficiencies and defects in OXPHOS are associated with increased levels of ROS (Kirkinezos & Moraes, 2001). Impaired ETC function fails to efficiently process oxygen and produces free electrons that eventually lead to the generation of oxygen radicals. However, cells have their own ROS scavenging mechanisms that are required for cell survival under stress conditions in healthy individuals (Melov *et al.*, 1998). Notably, it has been demonstrated that under normal conditions incomplete scavenging results in approximately 1–3 % of remaining ROS (Boveris & Chance, 1973; Nohl & Hegner, 1978). Evidence arises that ROS are not just a toxic by-

product of mitochondrial respiration, but function as important physiological regulators of a myriad of cellular signalling pathways (Finkel, 2011; Reczek & Chandel, 2014).

Mutations in ETC subunits that decrease *C. elegans* lifespan commonly lead to an increase in ROS. Therefore, it is surprising that ETC dysfunction induced by *wah-1* (RNAi) revealed decreased ROS levels. Nonetheless, studies of impaired AIF function in mice also demonstrate contradictory results on the generation of ROS. Whereas Klein *et al.* (2002) and Apostolova *et al.* (2006) report an increase in ROS, Joza *et al.* (2005) and Pospisilik *et al.* (2007) report no changes in ROS levels. On the contrary, Urbano *et al.* (2005) find ROS levels to be decreased. Therefore, the concept that AIF serves as a potential antioxidant through its cofactors FAD and NAD is further challenged by the decreased ROS levels after downregulation of AIF/WAH-1 in *C. elegans* (Chen *et al.*, 2014; Klein *et al.*, 2002; Sevrioukova, 2011; van Empel *et al.*, 2005).

In accordance with studies of other mitochondrial deficiencies (Ishii *et al.*, 1998), AIF/WAH-1 loss activated transcription factors that mediate stress response such as the upregulation of genes encoding mitochondrial chaperones (HSP-6 and HSP-60), hypoxia-induced proteins (HIF-1 targets) and antioxidant proteins (SOD-3 and GST-4). This activation of detoxification pathways may ultimately result in ROS scavenging. Therefore, the relative decrease of ROS could arise from increased expression of antioxidant enzymes.

## 4.3 UPRmt in impaired mitochondria

The stress responses elicited by detrimental macromolecules have been characterised as mechanisms to prevent imbalance of cellular homeostasis. Furthermore, they play an important role in the lifespan extension of long-lived mutants, such as *daf-2* and *eat-2* (Durieux *et al.*, 2011). Moreover, RNAi knockdown of UPRmt components can suppress the lifespan extension from mutations in *isp-1* or *clk-1*. However, cellular stress response is insufficient to protect from abnormal insults and the activation of the UPRmt is neither sufficient for lifespan extension nor a predictor of longevity in *C. elegans* (Bennett *et al.*, 2014).

The expression of heat shock proteins in nematodes lacking AIF is a marker of induced mitochondrial stress. However, this expression was independent of the UPRmt signalling molecules DVE-1 and UBL-5 (Benedetti et al, 2006). Instead, the results of this study support the role of ATFS-1 as the main regulator of the UPRmt that can act independently of DVE-1/UBL-5 (Bennett *et al.*, 2014). Nevertheless, different kinds of mitochondrial insults might activate different UPRmt signalling molecules. Some insults might primarily activate ATFS-1, whereas others result in DVE-1/UBL-5 activation. For example, detriments that directly affect mitochondrial membrane potential or mitochondrial import mechanisms but do not have an acute effect on matrix homeostasis could induce UPRmt via ATFS-1 independently of DVE-1/UBL-5. In this scenario, import deficiency of ATFS-1 into the mitochondria would result in nuclear translocation and induce heat shock protein gene expression. Protein folding in the mitochondrial matrix would not (yet) be directly affected. Thereby, low CLPP activity resulted in little production of peptide products and export through HAF-1. In this way, the DVE-1/UBL-5 node of action would be bypassed. For instance, the downregulation of *ubl-5* by RNAi did not affect the lifespan in various long-lived mutants but suppressed the lifespan extension in *isp-1* (C III subunit) and *clk-1* (necessary for coenzyme Q synthesis), exclusively (Durieux *et al.*, 2011). Additionally, the partial effect of *ubl-5* (RNAi) on the lifespan extension from knockdown of *cco-1* (Cyt c oxidase subunit Vb) (Bennett *et al.*, 2014) indicates that induction of the UPRmt plays a causal role in lifespan extension from ETC inhibition. Nevertheless, a broader and more systemic adaption to organelle-induced stress that exceeds the UPRmt could increase the chance for cellular protection from adverse insults.

## 4.4 Insulin/IGF-1 signalling, AMPK and HIF-1 correlation

For more than 20 years, impaired IIS has been the single most effective mutation for lifespan extension in *C. elegans*. In *wah-1* deficient nematodes, *daf-2* loss-of-function also prolonged lifespan (Figure 4-2). Importantly, this effect was not specific to *wah-1* (RNAi) but *daf-2* mutation also extended the lifespan of the ETC mutant *gas-1*. Taken together, ETC deficiencies that decrease *C. elegans* lifespan showed a lifespan extension upon impaired IIS. A central protein for increased survival through IIS is the transcrip-

tion factor DAF-16. However, in OXPHOS impaired animals, the protective properties of IIS were independent of DAF-16. This suggests other regulatory factors being responsible for the lifespan extension in *daf-16;daf-2* nematodes. Nonetheless, the DAF-16-independent protection of *wah-1* (RNAi) animals does not principally understate the importance of the transcription factor DAF-16 for lifespan. The overall lifespan of *daf-16;daf-2; wah-1* (RNAi) animals was considerably shorter compared to the long life of *daf-2; wah-1* (RNAi) animals with active DAF-16 transcription. This finding enforces the importance of DAF-16 as the most potent mediator of lifespan extension and underlines the orchestrated and cooperative function of DAF-2 downstream transcription factors. Nevertheless, DAF-16 does not seem to play a role in the cellular protection from the detrimental consequences of ETC dysfunction.

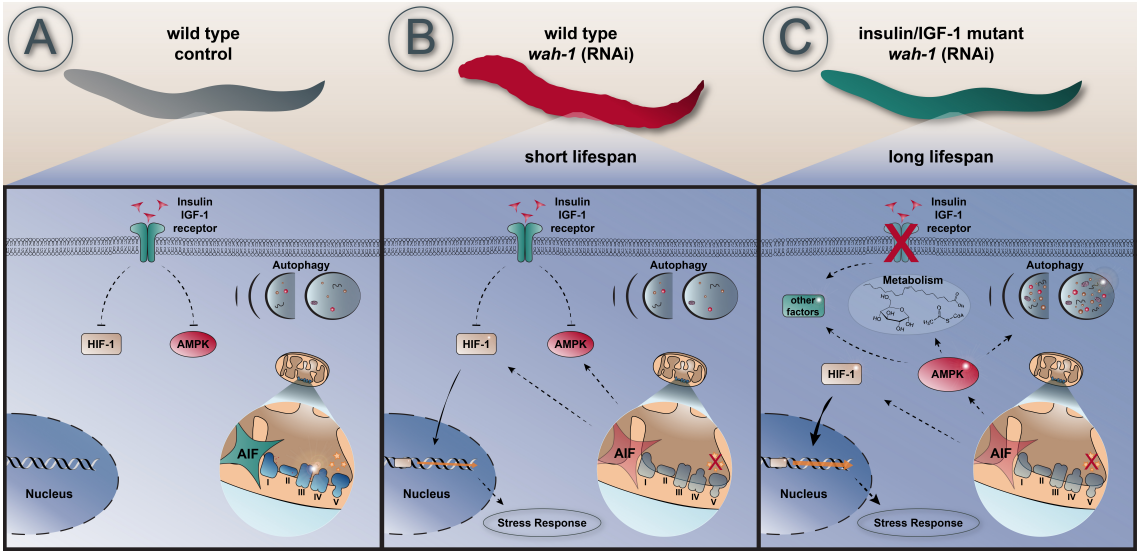


Figure 4-2: **Proposed model for the molecular mechanisms involved in the lifespan extension of nematodes carrying impaired OXPHOS. (A)** In wild type animals, mitochondrial OXPHOS produces sufficient energy for cellular function and maintenance. Active Insulin/IGF-1 signalling inhibits stress response mechanisms. **(B)** Loss of AIF/WAH-1 leads to impaired mitochondrial function and energy production. Despite the inhibitory effect of the active insulin/IGF-1 signalling cascade, OXPHOS impairment results in stress-mediated activation of HIF-1. The nuclear translocation of the transcription factor elicits a stress response, which is not sufficient to promote lifespan extension. **(C)** Under condition of low insulin/IGF-1 signalling, the metabolic sensor AMPK is activated along with HIF-1. The enhanced catabolism and the engagement of the autophagic machinery ultimately increase stress resistance and survival. All three components – nuclear-encoded gene expression, AMPK and autophagy – are required for the lifespan extension upon WAH-1 loss in *C. elegans*.

HIF-1, along with AMPK, is likely to be a key player in the regulation of metabolism and lifespan extension in *wah-1* (RNAi) treated worms. HIF-1 is activated under hypoxic conditions or reduced mitochondrial respiration and signals to the nucleus to express genes encoding for protective proteins (Lee *et al.*, 2010). Furthermore, HIF-1 acts as a metabolic switch (Kim *et al.*, 2006) and could mediate its protective effect by mitochondrial and mitochondria-to-nucleus signalling (Ristow & Zarse, 2010). For example, HIF-1 directly stimulates the glycolytic flux to pyruvate (Lee *et al.*, 2010) as detected in *wah-1* (RNAi) animals by increased *gpd-3* mRNA levels. However, HIF-1 was activated in wild type and IIS mutant animals and the effect on glycolysis was consistent in both backgrounds. Furthermore, it has been reported that HIF-1 can upregulate mitochondrial autophagy (Zhang *et al.*, 2008) and macroautophagy (Bellot *et al.*, 2009; Tracy *et al.*, 2007; Wilkinson & Ryan, 2009). In fact, autophagy plays a crucial role in the lifespan extension of mitochondrially-impaired nematodes (Chapter 4.5 ). Interestingly, it has been reported that AMPK is required for the activation of HIF-1 under hypoxic conditions in human prostate carcinoma cells (Lee *et al.*, 2003). The activation of both regulators might also be connected in WAH-1 deficient worms. Hwang *et al.* (2014) recently reported a common HIF-1/AMPK feedback loop activation by elevated levels of reactive oxygen species in *isp-1(qm150)* (complex III) mutants. Although, ROS levels were decreased in the AIF/WAH-1 model of mitochondrial deficiency in both wild type and *daf-16;daf-2* background, HIF-1 and AMPK were similarly activated as demonstrated in *isp-1(qm150)* mutants. Importantly, the mutation in *isp-1* results in an extension of wild type animal lifespan whereas *wah-1* (RNAi) reduced survival. Hence, HIF-1 and AMPK are hypothetically activated by different OXPHOS deficiencies through common pathways that are controlled by distinct regulators. Notably, I demonstrated that HIF-1 was activated in wild type as well as in IIS mutant nematodes. AMPK on the other hand was only activated in the mutant background.

For the development of disease treatments or the amelioration of phenotypes, a rather focused node of action is desirable, emphasising HIF-1 or more importantly AMPK/AAK-2 as potential targets. The activation of the nutrient sensor AMPK/AAK-2 was crucial for the lifespan extension of *wah-1* (RNAi) treated *daf-16;daf-2* mutants. A loss-of-function mutation in *aak-2* completely abolished the prolonged survival. Furthermore, overexpression of *aak-2* was enough to reverse the effects of WAH-1 loss in wild type animals and extended their lifespan. This makes AMPK a key player in the lifespan extension of AIF/WAH-1-impaired animals by inactive IIS. Therefore, it could potentially serve as a target for the treatment of mitochondrial deficiencies. Furthermore, AMPK is an interesting target as various chemical activators of the nutrient sensor are available. Some of them are already in clinical use for the treatment of other pathologies. AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide) is an analogue of AMP and a potent activator of endogenous AMPK/AAK-2. Strikingly, experiments in our laboratory recently demonstrated that pharmacologic activation by AICAR was sufficient to extend the lifespan of treated wild type animals after *wah-1* downregulation. Based on the finding that *daf-16;daf-2*-induced lifespan extension might not be restricted to mitochondrial dysfunction caused by AIF/WAH-1 deficiency but might also counteract ETC deficiency induced by other mutations, boosting AMPK activity might also be a putative treatment for other mitochondrial diseases. These results make AMPK/AAK-2 an interesting target for potential pharmacological treatment of higher organisms suffering from mitochondrial impairment.

## 4.5 Involvement of autophagy

Autophagy is a process that has the capability to rejuvenate cells by recycling cellular components and organelles. Autophagy plays a central role in the protective capacities of insulin/IGF-1 and AMPK/AAK-2 signalling, as it is necessary for the OXPHOS deficiency-induced lifespan extension by *wah-1* (RNAi). In support of a protective effect of autophagy, several studies demonstrate that genetic or extrinsic interventions that up-regulate autophagy can extend the lifespan of the organism (Bjedov *et al.*, 2010; Eisenberg *et al.*, 2009; Hansen *et al.*, 2008; Schiavi *et al.*, 2013). Accordingly, block of autophagy in various long-lived mutants reduces their lifespan (Toth *et al.*,

2008). Nevertheless, how could autophagy mediate lifespan extension under conditions of mitochondrial dysfunction? Autophagy is an evolutionarily conserved catabolic process that eliminates and recycles macromolecules and organelles, such as damaged mitochondria. Lack of autophagy is associated with an increased number of impaired organelles that can affect cellular function. For example, the deregulation of autophagy in cells with high energy demand, like neurons, results in the loss of synapses and culminates in apoptosis (Levine & Kroemer, 2008). In ETC deficient nematodes it is plausible that a compensatory increase of mitochondria, despite their intrinsic damage, might act as a reservoir for the autophagic pathway. They supply material essential for the progression of catabolic processes (Hailey *et al.*, 2010). Nevertheless, it is not clear how autophagy and other stress response mechanisms could interact to protect from the detrimental consequences of impaired OXPHOS.

A recent study by Papa and Germain (2014) in a breast cancer cell line elucidates an important crosstalk between various mechanisms involved in lifespan extension:

Accumulation of misfolded and unfolded proteins in mitochondria induces proteotoxic stress and increases ROS levels. Consequently, UPRmt is activated to counteract the negative effects of unfolded proteins. In parallel, autophagy is activated to clear the cell from molecular debris and defective organelles. When ROS is inhibited by the antioxidant NAC, the increase in LC3BII levels (formation of autophagosomes) is abolished. Also, the block of autophagy by ATG9 siRNA abolished the increase in LC3BII and led to a 40 % reduction in viability. Therefore, the activation of autophagy is required for the maintenance of cellular viability.

Although, in *C. elegans, wah-1* (RNAi) leads to a decrease in ROS instead of an increase, the UPRmt is still activated and chaperones are expressed. Nevertheless, the induction of the UPRmt alone is insufficient to protect the organism from a decrease in lifespan. Just upon activation of AMPK/AAK-2 and subsequent induction of autophagy, survival could be enhanced. This is another indication of the importance of active autophagy in cell survival.

## 4.6 Autophagy and metabolism

Recent studies on the important role of autophagy during stressful conditions illustrate the relationship between autophagy and systemic metabolism (Lim *et al.*, 2014). In mammals, Atg7 is an essential autophagy gene in skeletal muscle and liver. The cross of haploinsufficient mice for Atg7 with ob/ob mice that eat excessively and become obese does not alter the metabolic profile of the animals. Nevertheless, they fail to adapt to the metabolic load and develop diabetes and aggravated insulin resistance (Lim *et al.*, 2014). Subsequent induction of autophagy by the chemical enhancer Imatinib was sufficient to ameliorate the adverse affects of Atg7 haploinsufficiency. Despite the different model and disease in my study, these data support that autophagy is a crucial mechanism in the adaption to metabolic changes and protection from detrimental organelle dysfunctions. Furthermore, it provides the identification of another potential target for treatment of mitochondrial dysfunction with chemical compounds.

Regarding the potential for therapy, autophagy provides an important advantage over the targeting of transcription factors. At least in lower model organisms, tissue-specific regulation of DAF-2 signalling and *daf-16* expression levels have been demonstrated to signal to other tissues. Feedback regulation of insulin/IGF-1 and other unknown effects of *daf-16* gene expression ultimately affect all tissues in *C. elegans* (Kenyon, 2005; Murphy *et al.*, 2007). Also, tissue specific insulin/IGF-1 dysfunction in mice can affect the whole organism. This cell non-autonomous gene regulation is not specific for DAF-16 but applies to other transcription factors including HIF-1 (Bishop & Guarente, 2007). Autophagy, however, is regulated in a tissue-specific manner, providing the option of site-specific modulation of the autophagic flux, ameliorating potential side effects. Therefore, even organ-specific pathologies of mitochondrial dysfunction could be targeted by techniques that precisely regulate autophagy in the respective tissue.

## 4.7 Involvement of ACDH-1 and metabolism

HIF-1 and AMPK/AAK-2 sense metabolic perturbations caused by impaired OXPHOS and stimulate alternative pathways. The adaption of metabolism by the proteins involved in IIS eventually led to an induction of *acdh-1* expression. ACDH-1 is an important metabolic sensor that plays an active role in metabolic reprogramming through fatty acid β-oxidation and BCAA breakdown (Watson *et al.*, 2013). However, the activation of *acdh-1* by AIF/WAH-1 downregulation in IIS mutants did not have an immediate effect on BCAA levels. Furthermore, the block of *acdh-1* by RNAi was not sufficient to abolish the lifespan extension of *wah-1* (RNAi) treated *daf-16;daf-2* mutants. However, a minimum of 13 acyl-CoA dehydrogenase genes has been identified for *C. elegans* that could compensate for the loss of a single isoform. Additionally, such as IIS is a complex process, metabolism is an intricate interplay of hundreds of metabolites and other co-factors could be involved.

However, if metabolism plays a critical role in lifespan, why where no differences in important metabolites and key metabolic pathways between wild type and IIS mutant animals detected?

The majority of scientific studies that demonstrate the critical role of metabolic changes for cellular homeostasis have been conducted in cell lines. For example, absence of glucose, amino acids or other nutrients induces autophagy *in vitro* (Galluzzi *et al.*, 2014). However, such drastic alterations in the abundance of extracellular supplies do not occur *in vivo*. Food-restriction in mice for 24–48 hours leads to a reduction of 10 % – 20 % in body weight and induces autophagy in close-to-all nucleated cells of the body (Mizushima, 2009a; Mizushima, 2009b). However, a major depletion in amino acid levels or massive, life-threatening hypoglycemia was not observed. The authors speculate that this might be due to the autophagy-dependent mobilisation of cellular stores and the systemic response to starvation (He *et al.*, 2012; Kuma *et al.*, 2004). Although, *C. elegans* is a significantly less complex organism, the composition of the extracellular milieu in multicellular eukaryotes is preserved by multiple homeostatic circuits (Galluzzi *et al.*, 2014). Moreover, the cellular availability of nutrients is not mainly dictated by their abundance but, rather, by the regulation of their uptake via specific transporters

(Wieman *et al.*, 2007). Therefore, the metabolic analysis of *C. elegans* whole body extracts might not reflect the metabolic state of the cells inside the organism.

However, the involvement of the metabolic regulators HIF-1, AMPK and autophagy as well as the expression of the metabolic sensor *acdh-1* strongly suggest an involvement of metabolism in the regulation of lifespan under conditions of impaired ETC function. *acdh-1* expression levels strongly correlated with the lifespan of animals. The reduction of *aak-2* and *hif-1* in *daf-16;daf-2* animals abolished lifespan extension by *wah-1* (RNAi) and strongly suppressed *acdh-1* expression. The increased lifespan of *aak-2* OE animals when treated with *wah-1* (RNAi) also displayed increased *acdh-1* levels. Strikingly, *acdh-1* expression levels also resembled the changes in lifespan caused by impaired autophagy (*bec-1* (RNAi)) on both *daf-16;daf-2* mutants and *aak-2* OE animals. The reduction of *bec-1* in *wah-1* deficient animals resulted in a loss of the lifespan extension and decreased *acdh-1* expression levels approximately to control levels.

Taken together, *acdh-1* expression levels changed along with the effect of *hif-1* and *aak-2* on the lifespan of *C. elegans*. Furthermore, *acdh-1* levels also correlated with the influence of autophagy on lifespan in the various backgrounds and treatments. Thus, *acdh-1* might serve as a marker of lifespan in OXPHOS deficient animals.

## 4.8 Concluding remarks

In conclusion, this study demonstrates the evolutionarily conserved role of AIF/WAH-1 in the maintenance of the ETC and mitochondrial OXPHOS. Furthermore, I identified a new model of disease related to mitochondrial deficiency in the nematode *C. elegans*. Based on these findings, WAH-1 deficiency engages a series of molecular mechanisms that recapitulate detrimental effects of impaired mitochondrial function. Importantly, reduced insulin/IGF-1 signalling can protect from OXPHOS deficiency and activate programs that lead to lifespan extension in *C. elegans*. The response to insufficient AIF/WAH-1 by impaired IIS represents an important link between mitochondrial function, stress response and metabolism. In animals with OXPHOS impairment, the reduction of IIS leads to the activation of the transcription factor HIF-1 and the nutrient sensor AMPK/AAK-2. This in turn, upregulates autophagy that can prevent the accumulation of damaged molecules and organelles and controls metabolic reprogramming. Thus, AMPK kinase and autophagy may represent attractive targets for the development of new therapeutic strategies for the treatment of mitochondrial diseases. Furthermore, ACDH-1 was identified as a metabolic marker that reflects the effect of signalling pathways that promote *C. elegans* lifespan. Although, the effects of IIS, HIF-1, AMPK and autophagy in *wah-1* deficient nematodes on metabolism remain largely elusive, the results highlight the role of metabolic genes as regulators of cellular health, disease and longevity.

Finally, further studies are indispensable to identify the underlying processes that ultimately induce AMPK and autophagy. Furthermore, the protective functions of these factors need to be established for other ETC deficiencies that are independent of AIF/WAH-1. Various mitochondrial dysfunctions and pathologies that share common symptoms might also share common disease pathways. With focus on the treatment of patients these common pathways might provide the option of collective therapies. Importantly, FDA approved drugs that activate AMPK and autophagy are available and already in clinical use. For example, AICAR is used to stimulate AMPK activity to treat and protect against cardiac ischemic injury (Mentzer *et al.*, 1988). Conversely, autophagy can be induced by the inhibition of mTOR. Most prominently, rapamycin (sirolimus) has been shown to prolong the life of mice (Harrison *et al.*, 2009) and might be

useful in the treatment of mitochondrial deficiencies. Although mitochondrial diseases are rather poorly understood and require extensive research, the chance of collective treatment and the availability of potentially beneficial drugs emphasise the development of future therapies for patients.

# 5 Materials and Methods

## 5.1 Caenorhabditis elegans procedures

### 5.1.1 Strains

*C. elegans* strains were obtained from the Caenorhabditis Genetics Centre (CGC, University of Minnesota). Strains used in this study:

N2 (ancestral, wild type, Bristol isolate), AGD731 *uthEx299[aak-2(aa1-aa321)::GFP::unc-54 3'UTR, myo-2p::tdTomato]*, BAN-1 *daf-2(e1370)III*, BAN-41 *daf-2(e1370)III; Ex[lgg-1p::lgg-1::gfp]*, BAN-57 *daf-16(mu86)I; daf-2(e1370)III; gas-1(fc21)X*, BAN-61 *aak-2(ok524)X; daf-16(mu86)I; daf-2(e1370)III*, CF1553 *muls84*[*pAD76(sod-3::*GFP)], CL2166 *dvls19*[*pAF15(gst-4::*GFP::NLS)], CW152 *gas-1(fc21)X*, *jrIs1 [Prpl-17::HyPer]*, *jrIs2 [Prpl-17::Grx-1-roGFP2]*, RB867 *haf-1(ok705)*IV, SJ4100 *zcls13*[*hsp-6::*GFP], SJ4103 *zcls14*[*myo-3p::*GFP(mit)], SJ4151 *zcIs19[ubl-5p::ubl-5::GFP]*, SJ4197 *zcIs39[dve-1p::dve-1::GFP]*, TK22 *mev-1(kn-1)III*, VC1026 *rab-10(ok1494)*, ZG31 *hif-1(ia4)V*.

### 5.1.2 Crossing of mutant and transgenic worm strains

In order to obtain *C. elegans* strains with more than one mutation or to express transgenes in mutant nematodes, worms were crossed with other *C. elegans* strains. In general, *C. elegans* males are only observed at a frequency of ~0.02 % in the nematode population. To increase the frequency of males in the population, about 30 late L4 hermaphrodites were heat shocked at 31 °C for ~5 hours and subsequently transferred back to 20 °C. For the cross, four to six male L4s were mated with one hermaphrodites of the desired strain, keeping the ratio in favour of male worms. After 24 hours, the crossed hermaphrodites were transferred onto individual 55 mm NGM plates. The newly hatched progeny was singled out on individual NGM plates and after animals had laid eggs the parental worms were used for genotyping. This step was repeated until homozygous worms were obtained.

### 5.1.3 *C. elegans* maintenance

Nematode growth medium (NGM) plates seeded with *E. coli* OP50 bacteria ( 5.1.4.1 ) were used as standard maintenance plates for *C. elegans*. In case of contamination, worms were treated with hypochlorite bleaching solution and eggs were transferred to fresh plates ( 5.1.6 ). Before each experiment, animals were kept on sufficient food supply for at least two generations to prevent developmental effects and adverse gene expression caused by starvation response mechanisms.

### 5.1.4 Preparation of NGM plates

Nematodes were maintained at 20 °C on NGM as described (Brenner, 1974), unless stated otherwise. NGM agar plates were prepared by an automated peristaltic pouring system (Mediajet, Integra) using 90x20 mm and 55x20 mm petri dishes (nerbe plus). Plates were left at room temperature (RT) for at least two days before use. The *Escherichia coli* strain OP50 was used as a food source for standard maintenance ( 5.1.4.1 ); HT115 bacteria were used for RNA interference (RNAi) experiments ( 5.1.4.2 ). For experiments that included treatment of *C. elegans* with ethidium bromide, the chemical compound was added at a final concentration of 50 μg/ml to the NGM after autoclaving, before pouring the media.

#### 5.1.4.1 Maintenance plates – E. coli OP50

To generate *E. coli* OP 50 starter colonies a 90 mm LB pate was pre-warmed to 37 °C in an incubator and bacteria were grown by streaking of a glycerol stock on the LB plate and incubation over night (O/N) at 37 °C. The next day, LB was aseptically inoculated with an OP 50 starter colony picked from the streaked LB agar plate. A liquid culture of 100 ml was allowed to grow for 5-8 h at 37 °C on a shaker at 160 rpm. Subsequently, NGM plates were seeded with 1 ml (90 mm dish) or 100 μl (50 mm dish) of OP 50 solution per plate, evenly distributed over the agar surface. Residual OP 50 liquid culture was stored at 4 °C to be used for up to three days. The distributed bacterial lawn was allowed to dry and grow O/N at RT. Plates were then stored at 4 °C.

#### 5.1.4.2 RNAi plates – *E. coli* HT115

*E. coli* HT115 (DH3) strains were taken from the Ahringer library (Source BioScience). The bacteria express stable dsDNA for RNAi induction in *C. elegans*. RNAi strains for genes that were not available from the library were individually generated. Therefore, the desired RNAi fragment was cloned into the pL4440 vector construct and subsequently transformed into competent HT115 bacteria. HT115 transformed with the empty pL4440 vector were used as a control. Bacteria carrying control pL4440 vector or RNAi-encoding plasmids were streaked out on pre-warmed (37 °C) LB plates with 100 µg/ml ampicillin and 12.5 µg/ml tetracycline and incubated O/N at 37 °C. Single HT115 colonies were used to inoculate 3 ml of LB supplemented ampicillin and incubated O/N at 37 °C on a shaker at 160 rpm. The next day, an over day culture was prepared by inoculation of LB + ampicillin with the fresh O/N culture (ratio ~1:25). The culture was grown for 5-8 hours and used to seed NGM plates. For the induction of double stranded RNA expression in the HT115 bacteria, Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the bacteria suspension at a final concentration of 1 mM before seeding of NGM plates. Seeded bacteria were allowed to grow O/N at RT.

### 5.1.5 Generation of RNAi constructs

#### 5.1.5.1 Plasmid DNA isolation

4 ml of inoculated LB medium supplemented with ampicillin were centrifuged at 3,000 *g* for 10 min after incubation O/N at 37 °C. Supernatant was discarded and Plasmid DNA was isolated and purified using the Jetquick Plasmid Mini Spin Kit (Genomed). The protocol was followed according to the manufacturer's users manual provided. Plasmid DNA was resuspended in 40 µl of sterile water.

#### 5.1.5.2 DNA extraction from *C. elegans*

*C. elegans* genomic DNA for cloning purposes was extracted using the Illustra tissue & cells genomicPrep Spin Kit (GE Healthcare). Approximately 1,000 worms were grown to adulthood on standard NGM plates with OP50 bacteria. Worms where rinsed

off of the plates with M9 buffer and thoroughly washed free from remaining bacteria. Worms were left in fresh M9 buffer for 15 min to get rid of remaining bacteria in the animal's intestine. Subsequently, the supernatant was discarded and the animals cleaned another time. Afterwards, DNA was extracted following the manufacturer's protocol.

#### 5.1.5.3 RNAi construct cloning

The mixing of two bacterial strains carrying individual RNAi results in low gene regulation efficiency. To prevent the dilution effect, *bec-1* RNAi was cloned into the existing *wah-1*-pL4440 RNAi construct. For this purpose, the *wah-1* plasmid was extracted from a 4 ml O/N culture in LB medium. The *bec-1* RNAi sequence was amplified by PCR from genomic *C. elegans* DNA. The two primers contained restriction sites for XhoI 5' and KpnI 3', respectively. Then, DNA was purified and cloned by T4 ligation after restriction digest for 2 h. Vector was then transformed into HT115 bacteria. Bacteria colonies were checked for the *bec-1* insert by PCR. Positive clones were sent for sequence analysis (GATC biotech) and subsequently used for RNAi experiments.

#### 5.1.5.4 Transformation of *E. coli* HT115

RNAi clones were transformed into *E. coli* HT115. To this end, 25 µl of competent HT115 bacteria solution were slowly thawed on ice and 50-100 ng of plasmid DNA were added to the bacteria and incubated on ice for 30 min. Transformation was then promoted by heat shock in a heating block at 42 °C for 45 s and bacteria were immediately chilled on ice for 1 min. Subsequently, 500 µl of Super Optimal broth with Catabolite repression (SOC, Invitrogen) were added to the bacteria and incubated at 37 °C for 1 h at 200 rpm. In the meantime, LB plates supplemented with 100 µg/ml ampicillin where allowed to pre-warm to 37 °C in an incubator. Between 25-150 µl of bacteria solution were distributed on LB-plates and clones were grown at 37°C O/N. The next day, single colonies were isolated and transferred to 6 µl of fresh LB. Subsequently, 2 µl of the solution were used for single clone analysis by PCR and the residual 4 µl of the bacteria were transferred to 3 ml LB medium with ampicillin and grown O/N for subsequent plasmid extraction ( 5.1.5.1 ).

### *5.1.6* Synchronisation of *C. elegans*

Synchronous nematode populations obtained by bleaching using a sodium hypochlorite/NaOH solution. Incubation of nematodes with hypochlorite bleaching solution results in the lysis of *C. elegans* tissue, while eggs survive the treatment for an extended period of time. Gravid hermaphrodites carrying fertilized eggs were collected with M9 buffer and transferred to a 1.5 µl reaction tube. Animals were washed three times with M9 buffer and supernatant containing bacteria and young larvae was aspirated. Worms were resuspended in 200 µl of double-distilled water (dd$H_2O$). Subsequently, 600 µl of freshly prepared sodium hypochlorite solution were added. Following this, the suspension was thoroughly mixed for approximately 5 min in a vortex until animals were dissociated. To avoid extensive sodium hypochlorite treatment, 500 µl of M9 were added and eggs were immediately spun down at 9,000 *g* for 30 s. Eggs were then washed three times with 1 ml of M9, mixed and resuspended thoroughly and spun down at 9,000 *g* for 30 s between each washing step. After the last washing cycle all eggs were resuspended in M9 buffer and distributed to fresh NGM plates seeded with HT115 (DH3) or OP50 bacteria for RNAi experiments or maintenance, respectively.

### *5.1.7* Lifespan assays

Synchronous nematode populations were established by hypochlorite treatment of gravid adults to obtain synchronised embryos as described ( 5.1.6 ). The HT115 bacteria were transformed with either control pL4440 vector or RNAi-encoding plasmid ( 5.1.4.2 ). Bleached *C. elegans* progeny was grown at 20 °C unless noted otherwise, and transferred to fresh HT115-seeded plates every other day after they reached adulthood. Lifespans were performed at groups of 30-50 nematodes per plate for a total number of 120-150 individuals per experiment. The day of hatching was defined as $t$=0. Animals were examined every second day for touch-provoked movement and pharyngeal pumping. The mean lifespan is defined as the time point when 50 % of the animals are still alive. Worms which died abnormally due to internally hatched eggs, protruded vulva or dried on the edge of the plates were considered censored. The survival curves were generated using the method of Kaplan and Meier with the GraphPad Prism Soft-

ware package (GraphPad Software Inc., San Diego, USA). Lifespans were performed at least three times and the figures report representative experiments. *p* values for the evaluation of the differences were determined using the log-rank (Mantel-Cox) test.

## 5.2 Molecular Biology

### 5.2.1 Quantitative real-time PCR

#### 5.2.1.1 RNA isolation

At adulthood, 200 worms were collected from NGM plates and cleaned from the bacteria. Then, nematodes were frozen and stored at -80 °C. Total RNA isolation was performed using QIAshredder (Qiagen) for disruption of nematode tissue and RNeasy RNA isolation kit (Qiagen) according to the manual. RNA isolation was carried out in an automated QIAcube system (Qiagen) including an on column DNAse digest. Briefly, 600 µl RLT lysis buffer were added to the frozen nematodes and samples were vortexed for 5 min. Lysates were frozen and vortexed for a second cycle. In the meantime, the QIAcube was loaded and buffers for RNA extraction were prepared and provided in the designated compartments. Dependent on the number of samples, respective amounts of RNAse free DNAse (Qiagen) was diluted in RDD buffer in a 2 ml reaction tube and loaded to the machine. RNA was collected from the column in 35 µl RNAse free dH2O. After isolation, RNA concentration was determined using the NanoDrop 2000c/2000 UV-Vis Spectrophotometer (Thermo Scientific).

#### 5.2.1.2 Quantitative real-time PCR (qRT-PCR)

Gene expression analysis in *C. elegans* was assessed by quantitative real time PCR (qRT-PCR). Furthermore, qRT-PCR was used to validate the downregulation of target genes by RNAi for each experiment.

(A) Equimolar amounts of RNA were transcribed into cDNA by reverse transcription. Isolated RNA ( 5.2.1.1 ) was translated into cDNA in a thermal cycler (Biometra TProfessional Trio) by the qScript cDNA Synthesis Kit (Quanta Bioscciences)

according to the manufacturer's protocol. cDNA was analysed by real-time PCR analysis.

(B) The real-time PCR reaction was carried out in a Step One Plus Real time PCR System (Applied Biosystems) using the Fast SYBR Green Master Mix (Applied Biosystems). The following cycling conditions were used: Initial AmpliTaq Polymerase activation at 95 °C for 20 s followed by 40 cycles of denaturation (95 °C for 3 s) and Annealing/Extension (60 °C for 30 s). Data was analysed using the comparative ΔΔCt method (Livak & Schmittgen, 2001). An average of at least 3 technical repeats was used for each sample.

Table 5-1: **Primers used for qRT-PCR**

| | | | |
|---|---|---|---|
| *beta-actin* | 5′-tgtgatgccagatcttctccat-3′ | and | 5′-gagcacggtatcgtcaccaa-3′ |
| *wah-1* | 5′-gctgatgctgtcgaggaga-3′ | and | 5′-tggtggtgttctcttctgtaga-3′ |
| *hsp-6* | 5′-aaccggaaaggaacaacagat-3′ | and | 5′-tctcgatttggtccttggaa-3′ |
| *hsp-16.2* | 5′-tgcagaatctctccatctgagt-3′ | and | 5′-tggtttaaactgtgagacgttga-3′ |
| *dve-1* | 5′-gcattcagcacccactca-3′ | and | 5′-gagtgggtcgaacatcagg-3′ |
| *nhr-57* | 5′-tcggaatgaatccggaagt-3′ | and | 5′-atgcaggggaagatgaacag-3′ |
| *bec-1* | 5′-tgcagctctaggacaaattgtttt-3′ | and | 5′-gggcatcagttcgtgatgttg-3′ |
| *acdh-1* | 5′-gttcgtgtccacaaatcagc-3′ | and | 5′-caatggcaattctacctgcat-3′ |

### 5.2.2 Western Blot

#### 5.2.2.1 SDS-PAGE

*C. elegans* proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on nitrocellulose membranes for immunoreaction with primary antibodies. To this end, 200 worms were collected from NGM plates and thoroughly cleaned from bacteria with M9 buffer. Supernatant was discarded and the samples were frozen and stored at -80 °C. After thawing of the samples, 40 µl of 2x Laemmli buffer were added and the nematodes were disrupted by ultra-sonication at an amplitude of 11 µm (23 kHz) for 30 s (MSE Soniprep 150 plus). Proteins were denatured at 95 °C for 5 min and immediately transferred to ice. After the samples were cooled down, the lysates were centrifuged at 10,000 *g* for 10 min to spin down tissue remains. Next, 20 µl of the prepared samples were loaded onto 12 – 15 % polyacrylamide gels and allowed to penetrate the stacking gel at a voltage of 80 mV for 20 min. Subsequently, the voltage was increased to 130 mV to separate the proteins until bromophenol blue in the loading buffer reached the desired distance in the gel. The gels were subsequently used for western blotting.

#### 5.2.2.2 Western Blot transfer

Separated proteins were transferred to a nitrocellulose membrane by wet-blotting (Bio-Rad) for 85 min at 300 mA. Following, membranes were briefly washed with TBST buffer (15.3 mM Tris/HCl, 140 mM NaCl, pH 7.6, 0,1 % Tween). Blocking of the membrane with 5 % non-fat milk powder in TBST-buffer for 30 minutes prevented unspecific binding. Primary antibodies were diluted in 5 % milk in TBST and incubated O/N at 4 °C or for 2 h at RT. After incubation, the antibody was removed and the membrane was washed with TBST buffer 3 times for 10 min. The conjugated secondary antibody (horseradish peroxidase conjugated, HRP) was diluted 1:10,000 in 5 % milk in TBST and incubated for 1 h. Membranes were washed 3 times with TBST and finally developed with peroxidase substrate for enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, Thermo Scientific) according to the manufacturer´s instructions. The chemiluminescent detection was performed using the ChemiDoc XRS+ Gel

documentation system (BioRad). Protein bands were quantified by densiometric analysis. Figures show one representative experiment.

Table 5-2: **Primary antibodies used for immunoblotting**

| | | |
|---|---|---|
| Anti-ATP synthase subunit alpha | Mouse monoclonal | MitoSciences, MS507 |
| Anti-Ndufs3 (Complex I) | Mouse monoclonal | MitoSciences, MS112 |
| Anti-MT-CO1 (Complex IV) | Mouse monoclonal | MitoSciences, MS404 |
| Anti-COXIV (nuclear encoded) | Rabbit polyclonal | Cell Signaling |
| Anti-Pyruvate dehydrogenase (PDH) subunit E1 alpha | Rabbit monoclonal | MitoSciences, MSP07 |
| Phospho-AMPK | Rabbit monoclonal | Cell Signaling |

## 5.3 Imaging and stress-response analysis

### 5.3.1 STED microscopy

Super resolution microscopy of mitochondria in the muscle was performed with a Leica TCS SP8 gated stimulated emission depletion (STED) microscope. The inverted microscope (Leica DMI6000 CS) was equipped with a 100x-oil-objective, and a White Light Laser (WLL). Images were taken with a resolution of about 50 nm at 488 nm following laser depletion at a wavelength of 592 nm.

### 5.3.2 ROS quantification by $H_2$DCF-DA and 2-NDBG staining

6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) and 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) are fluorescent dyes that have been used to monitor reactive oxygen species (ROS) and glucose uptake in live cells, respectively. Staining with the fluorescent compounds was performed *in vivo* in *C. elegans* and protocols were adapted from Klapper *et al*. (2010) and Schulz *et al*. (2007). Young adult nematodes were washed off of RNAi plates and rinsed with M9

buffer three times. After cleaning, worms were resuspended in 950 µl M9. To prevent starvation during incubation, 50 µl of concentrated control and *wah-1* (RNAi) bacteria were added. Subsequently, 2 µl of a 0.1 mM DCF-DA (Life Technologies) or 4 µl of a 0.1 mM 2-NBDG (Life Technologies) stock solution in DMSO were added to the suspension to obtain a final concentration of 200 µM or 400 µM, respectively. Worms were then incubated for 5 h at 20 °C on a shaking incubator at 200 rpm. After staining, animals were briefly centrifuged and washed three times with 1 ml fresh M9 buffer for 5 min to prevent unspecific fluorescence from DCF-DA or 2-NBDG-stained HT115 bacteria in the nematodes intestine and remove all dye that was not incorporated. Nematodes were resuspended in M9 and analysed using the COPAS Biosort (Union Biometrica). Fluorescence was measured at an excitation wavelength of 488 nm and a GFP emission filter.

### 5.3.3 Hyper and roGFP ROS analysis

Quantification of $H_2O_2$ levels in *jrIs1 [Prpl-17::Hyper]* and GSSG/2GSH ratios in *jrIs2 [Prpl-17::Grx-1-roGFP2]* animals was performed by spinning disc microscopy. The setup consisted of a fully motorised inverted microscope (Nikon) equipped with an auto focus stage in association with a Yokogawa Spinning Disk connected to a back-illuminated EM-CCD camera (Andor iXON DU-897, 512 x 512 pixels, 16 bit, 35 frames/s). This setup was equipped with a 60x and 100x-oil-immersion lens (Nikon). Treated animals at 4 or 7 days of age were paralysed with 25 mM levamisole (AppliChem) in M9 buffer, mounted on 2 % agarose pads on glass slides and closed with cover slips. Nematodes were then imaged with 488 nm (oxidised Hyper, reduced roGFP2) and 405 nm (reduced Hyper, oxidised GroGFP2) excitation lasers and a 525 nm emission filter. Regions of interest (ROIs) were analysed by using Fiji software and oxidised/reduced ratios were calculated. GraphPad (Prism) software was used for statistical analysis.

### 5.3.4 Analysis of fusion-GFP strains

GFP expression in fusion-GFP worm strains (i.e. *hsp-6*::GFP, *hsp-60*::GFP, *ubl-5p*::GFP, *dve-1p*::*dve-1*::GFP, *gst-4*::GFP, *sod-3*::GFP, *acdh-1*::GFP) was analysed with the Complex Object Parametric Analyzer and Sorter (COPAS) Biosort (Union Biometrica). A minimum of 200 animals were analysed for each experiment and treatment. Fluorescence was measured at an excitation wavelength of 488 nm and a GFP emission filter. Laser intensity and gain settings were individually adjusted to GFP expression signals of each strain and kept consistent for all the experiments. The LGG-1/LC3::GFP reporter strain has been widely used for the estimation of autophagosomes in *C. elegans* (Melendez *et al.*, 2003). Autophagic activity was analysed by quantification of fluorescent puncta in the hypodermal seam cells under a fluorescence stereoscope.

## 5.4 Metabolite analyses

### 5.4.1 Oxygen consumption rates

Oxygen consumption rates were analysed with the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). The day before the assay was performed, a XF24 FluxPak sensor cartridge (XF24 extracellular flux assay kit, Seahorse Bioscience) was hydrated in 1 ml calibrant solution at 37 °C O/N in an incubator without $CO_2$. The next day, calibration of the cartridge was performed at 20 °C. Young adult worms were collected from NGM plates in M9 buffer and washed three times. Subsequently, 50 animals per well were transferred into a 24-well XF24 microplate in quadruplicates or quintuplicates per genotype and treatment. The volume was brought up to 500 µl/well with M9 buffer. For the background correction, empty wells were filled with 500 µl of M9. Measurement was performed at 20 °C with a repetition of ten cycles.

### 5.4.2 ATP content

ATP levels were measured with the Luminescent ATP Detection Assay Kit (abcam). The lysis buffer provided in the kit irreversibly inactivates ATP degrading enzymes. The ATP content in the cells/tissue is then quantified by ATP dependent luciferase activation and analysed by fluorescence detection. A synchronised nematode population was grown to adulthood on NGM plates seeded with HT115 *E. coli* bacteria containing control or RNAi plasmid. 200 worms were collected in a 1.5 ml sample tube and washed with M9 buffer three times. Animals were centrifuged and the supernatant was discarded. To inactivate ATPases, 50 µl of the detergent provided with the kit were added and worms were immediately homogenised with a motor-driven pestle. After homogenisation, 150 µl of $dH_2O$ were added to the sample. The homogenate was centrifuged at 4 °C for 10 min at 10 000 g. Samples were measured in triplicates at a volume of 50 µl in a black 96-well plate according to the assay kit's protocol. Luminescent signals were detected by an EnVision® Multilabel plate reader (Perkin Elmer).

### 5.4.3 Lactate Dehydrogenase activity

Lactate Dehydrogenase (LDH, EC 1.1.1.27) activity was measured by the reduction of NAD to NADH with the Lactate Dehydrogenase Assay Kit (abcam). For the analysis, 200 young adult worms of a synchronised nematode population grown on control and RNAi NGM plates were collected in a 1.5 ml tube and washed with M9 buffer three times. Animals were centrifuged and the supernatant was discarded. Nematode bodies were cracked by a freeze-thaw cycle, resuspended in 500 µl cold assay buffer and mechanically homogenised with a motor-driven pestle. The homogenate was centrifuged for 15 min at 10,000 *g* at 4 °C. For the assay, 50 µl of the supernatant was used in triplicates in a clear 96-well plate (Brand) according to the assay protocol. Optical density at 450 nm was measured kinetically every 30 min using an EnVision® Multilabel plate reader (Perkin Elmer). Data analysis was performed on values of time point T2-T1.

### 5.4.4 Fumarate levels

Fumarate levels were measured using the Fumarate Detection Kit (Abcam). For the analysis, 200 young adult worms were collected in a 1.5 ml tube and washed with M9 buffer three times. Animals were centrifuged and the supernatant was discarded. Nematode bodies were cracked by a freeze-thaw cycle, resuspended in 200 µl cold assay buffer and mechanically homogenised with a motor-driven pestle. Homogenate was centrifuged for 10 min at 13,000 *g*. For the assay 50 µl of the supernatant was used in triplicates in a clear 96-well plate (Brand) according to the assay protocol. Fumarate specific substrate reaction was measured at 450 nm using an EnVision® Multilabel plate reader (Perkin Elmer).

### 5.4.5 Branched Chain Amino Acid Levels

BCAAs were detected by oxidative deamination and measurement of the subsequently produced NADH with the BCAA Assay Kit (abcam). For the analysis, 200 young adult worms were collected in a 1.5 ml tube and washed with M9 buffer three times. Animals were centrifuged and the supernatant was discarded. Nematode bodies were cracked by a freeze-thaw cycle, resuspended in 100 µl assay buffer and mechanically homogenised with a motor-driven pestle. Homogenate was centrifuged for 10 min at 15,000 *g*. For the assay 25 µl of the supernatant was used in triplicates in a clear 96-well plate (Brand) and brought to 50 µl with Assay Buffer. BCAA specific enzyme reaction was measured at 450 nm using an EnVision® Multilabel plate reader (Perkin Elmer).

## 5.5 Materials

**LB (1 l)**

10 g tryptone (Sigma); 5 g Bacto-yeast (Sigma); 5 g NaCl (Sigma); made up to 1 l with $dH_2O$; adjusted to pH 7.0 using 1 M NaOH (Roth)

**NGM agar (1 l)**

3 g NaCl (Sigma); 17 g agar (Sigma); 2.5 g peptone (Sigma); 1 ml Cholesterol (Sigma); made up to 1 l with $dH_2O$

*autoclave*

add 1 ml of 1 M $CaCl_2$ (Sigma); 1 ml of 1 M $MgSO_4$ (Sigma); 25 ml of 1 M $KPO_4$ (pH 6) (Sigma)

**S-medium (1 l)**

1 l S-basal

5.5 g NaCl (Sigma); 1 g $K_2HPO_4$ (Sigma); 6 g $KH_2PO_4$ (Sigma); 5 mg cholesterol (Sigma); $dH_2O$ to 1 l

*autoclave*

10 ml 1 M potassium citrate (Sigma)

10 ml trace metals solution

1.86 g disodium EDTA (Sigma); 0.69 g $FeSO_4 \bullet 7\ H_2O$ (Sigma); 0.2 g $MnCl_2 \bullet 4\ H_2O$ (Sigma); 0.29 g $ZnSO_4 \bullet 7\ H_2O$ (Sigma); 0.025 g $CuSO_4 \bullet 5\ H_2O$ (Sigma); $H_2O$ to 1 l

*autoclave*

3 ml 1 M $CaCl_2$ (Sigma); 3 ml 1 M $MgSO_4$ (Sigma)

**Sodium hypochlorite solution (6 ml)**

1 ml 10 M NaOH (Roth); 2 ml 5 % sodium hypochlorite (GPR rectapur); 2.4 ml $ddH_2O$

**M9 (1 l)**

3 g $KH_2PO_4$ (Sigma); 6 g $Na_2HPO_4$ (Sigma); 5 g NaCl (Sigma); 1 ml 1M $MgSO_4$ (Sigma); made up to 1 l with $dH_2O$

**Worm DNA lysis buffer**

20 mM Tris pH 7.5 (Sigma); 50 mM EDTA (Roth); 200 mM NaCl (Sigma); 0.5% SDS (Sigma)

**Western Blot Buffers**

separation gel buffer

1.5 M Tris-Base; 0.4 % SDS; pH 8.8

stacking gel buffer

1 M Tris-Base; 0.4 % SDS; pH 6.8

running buffer

25 mM Tris-Base; 192 mM glycine; 0.1 % SDS

transfer buffer

25 mM Tris; 192 mM glycine; 20 % methanol

blocking buffer

5% w/v non-fat dried milk in TBS-Tween 0.05%

**Laemmli Buffer (4x)**

250 mM Tris/HCl pH 6.8 (Sigma); 8 % SDS (Sigma); 40 % glycerol (Sigma); 20 % β-mercaptoethanol (Sigma); 1 mg/ml bromophenol blue (Applichem)

**TBS (10x)**

153 mM Tris-HCl (Sigma); 1.4 M NaCl pH 7.6 (Sigma)

**TBST (1 l)**

15.3 mM Tris/HCl (Sigma); 140 mM NaCl (Sigma); pH 7.6; 0.1% Tween 20 (Calbiochem)

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# Author contributions

Experiments in Figure 3-10 E and Figure 3-12 E,F were conducted by Kostoula Troulinaki.

Experiments in Figure 3-1 D and Figure 3-12 A,C were conducted by Daniele Bano.

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