Prevention of neuronal degeneration in human mitochondria-associated diseases (HMAD): *C. elegans* as a model organism for high-content screenings

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für dich, meine wichtige Entdeckung in diesen aufregenden Jahren

Abstract

Mitochondria play a pivotal role in controlling cellular homeostasis. The mitochondrial respiratory chain is severely compromised in different human mitochondrial-associated diseases (HMAD), resulting in oxidative stress and reduced ATP production, ultimately leading to degeneration of affected tissues. Similarly, in the nematode *Caenorhabditis elegans*, severe mitochondrial disruption leads to deleterious effects such as developmental arrest or lethality. On the other hand partial mitochondrial disruption in different species, including *C. elegans*, prolongs lifespan.

Different and distinct reproducible phenotypes are therefore associated in the nematode *C. elegans* with different levels of mitochondrial stress and were used in the first part of my study as a readout for an RNA-interference screening aimed at developing different HMAD models, which mimic different phases of the disease progression. I then systematically characterized some of the new models with a special emphasis on neuronal deficits evaluation.

Investigating the molecular mechanisms underlying the transition in animal phenotypes (from mild to severe mitochondrial stress), and in particular the characterization of the adaptive beneficial pathways extending lifespan in response to mild mitochondrial stress, may lead to the identification of genes likely relevant for the prevention or delay of neurodegenerative or age-related diseases associated with progressive mitochondrial deterioration. With this aim in mind, in the second part of my thesis I assessed neuronal structure and functions during animal aging and investigated the involvement of specific neuronal genes (chemosensory-related genes and globins) in lifespan specification, upon mild mitochondrial stress. I demonstrated that a moderate mitochondrial alteration leads to increase animal's lifespan through neuronal hormesis.

Finally, taking advantage of an automated microscopy platform (the Cellomics ArrayScan VTI HCS Reader) coupled with the typical phenotypic readouts observed in *C. elegans* in response to different level of mitochondrial stress, I optimized and validated the conditions to carry out an *in vivo*, high-content screening (HCS) aimed at identifying interventions inducing beneficial mitochondrial stress responses. This

platform will offer the double opportunity to screen for potential HMADs therapeutics as well as for general anti-aging drugs (**Figure 1**).

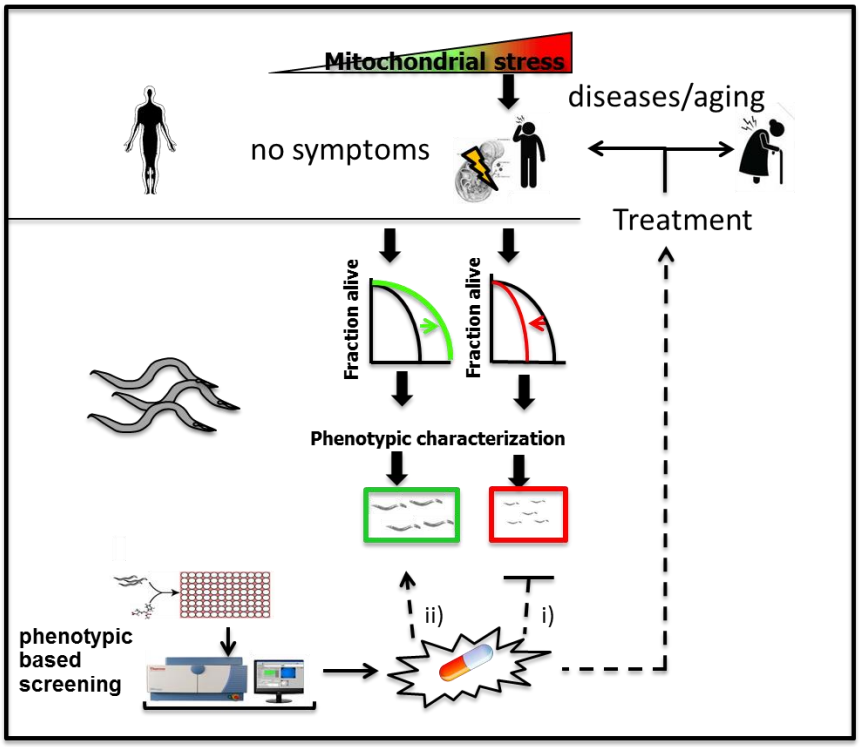


Figure 1. In humans mild mitochondrial dysfunction is asymptomatic while severe mitochondrial deterioration leads to diseases and is associated with the aging process. Similarly, in the nematode *C. elegans*, strong impairment of mitochondrial functionality leads to short lifespan or lethality, while mild mitochondrial dysfunction activate protective pro-longevity pathways. The bimodal responses triggered by mild or strong silencing of mitochondrial proteins also lead to very reproducible and distinct animal phenotypes, which allowed the optimization of a screening platform to identify interventions able to i) rescue the arrest of development due to strong mitochondrial dysfunction (potential HMADs treatments) and to ii) induce the typical phenotypes associated with increased animal robustness (potential anti-aging drugs).

Introduction

1 The Mitochondrion

1.1 Origin

Symbiosis is a general term that defines the beneficial coexistence of organisms belonging to different species; the particular case of endosymbiosis implies that an organism lives inside another (Margulis and Chapman, 1998). The mitochondria originated about two billion years ago by a relationship between a eubacterium and a primordial eukaryotic cell. Many features of the organelle reflect its endosymbiotic origin: a double membrane, its own circular genome, an autonomous system of transcription and translation (Schatz, 1996). Mitochondria are able to grow and reproduce inside the cell in a partially independent way. Almost all eukaryotic cells contain mitochondria; the number and the shape can differ according to the species but also according to the metabolic activity inside the cell (Alberts et al., 2007).

1.2 Structure

Mitochondria are small cytoplasmic organelles, whose size is usually 0.5-1 μm, and are characterized by the presence of two membranes mainly composed of a phospholipid bilayer. The two membranes, that have different properties, make possible to distinguish in this organelle, five distinct compartments that are: the outer mitochondrial membrane, the inner mitochondrial membrane, the intermembrane space (the space between the outer and inner membranes), the cristae space (formed by folding of the inner membrane), and the matrix (space within the inner membrane) (Karp, 2013). Mitochondria are highly dynamic organelles

that adopt different shapes depending on the cell type and the metabolic demands of the cell. Their two predominant morphologies are reflected in the Greek name of the organelle: ‘mitos’ (thread) and ‘chondros’ (grain). The smooth outer membrane contains many copies of a transport protein called porin, which forms aqueous channels allowing molecules with a maximal molecular weight of 5000 Daltons to freely penetrate the layer. The inner membrane forms numerous invaginations, tubular or lamellar structures called cristae, which are connected by narrow tubular structures, cristae junctions (**Figure 2**).

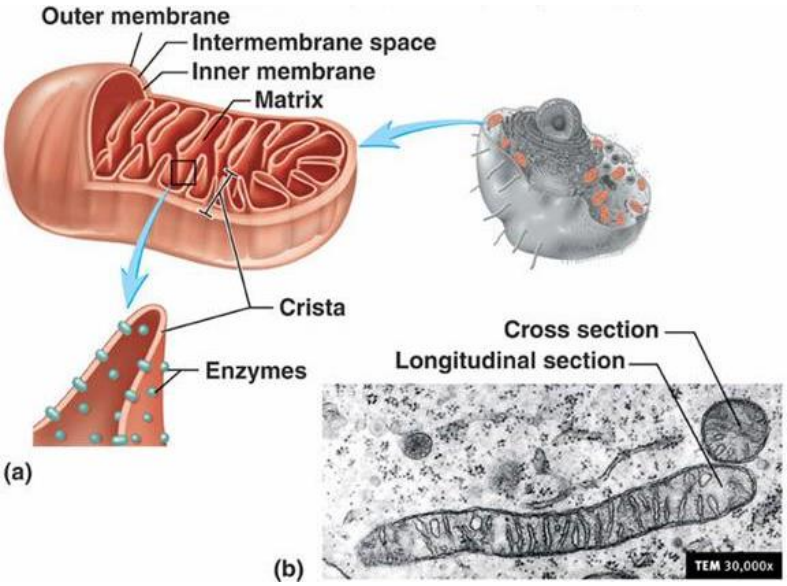


Figure 2. (a) Mitochondria structure representation and (b) electron microscopy microphotography (Copyright © The McGraw-Hill Companies, Inc).

The inner membrane is rich in an unusual phospholipid, cardiolipin, containing four fatty acids rather than the normal two, and may help to make the inner membrane impermeable (Alberts et al., 2007). There is also a membrane potential across the inner membrane, formed by the

action of the enzymes of the electron transport chain. The space within the inner membrane foldings is the matrix that hosts the mtDNA.

1.3 Genome

Although most of the mitochondrial genes are nuclear-encoded, mitochondria contain their own DNA, which is maternally inherited. In most species mtDNA is a small circle of double stranded DNA of 15-17Kb (*C. elegans* size is 13,8 Kb). In humans it codes for 13 respiratory chain key polypeptides of respiratory complexes I, III, IV, and V and 24 nucleic acids necessary for intra-mitochondrial protein synthesis of which 22 transfer RNAs (tRNA) and two ribosomal RNAs (rRNA) (Anderson et al., 1981). The 13 mRNAs are translated into proteins on mitochondrion specific ribosomes, using a mitochondrion-specific genetic code. A modified tRNA wobble base interaction with mRNA codons allows mitochondria to translate all codons with only the 22 tRNAs coded in mtDNA genome (Taanman, 2003).

However, mitochondria are not self-supporting units in the cell and the majority of mitochondrial proteins are nuclear-encoded, synthesized in the cytosol and successively imported into the organelle. Since mitochondria have a low activity DNA repair system and are continuously exposed to oxygen radicals coming out from the mitochondrial electron-transfer chain, somatic mutations in mtDNA are quite common (Taylor and Turnbull, 2005).

1.4 Dynamics

Dynamics is a prominent feature of mitochondria. These organelles are capable of fusion (the combination of two mitochondria into a single organelle) and fission (the separation of long, tubular mitochondria into two or more smaller parts), and they are organized in a dynamic architecture with transitions between a network-like structure and a more

disperse one. Mitochondria use microtubules to move and this can give the distinctive orientation and distribution of mitochondria in different types of cells (Alberts et al., 2007). The adequate balance between fusion and fission is crucial for the maintenance of mitochondrial function. For instance, mitochondrial fusion is required for the proper respiratory activity of the mitochondria and has been associated with cell survival (Chen et al., 2007b). On the other hand, mitochondrial fragmentation precedes, and it is required for, the proper elimination of mitochondria through the autophagic pathway (Rubinsztein et al., 2011), a protective degradation process activated for the elimination and turnover of old or dysfunctional mitochondria, which we recently found to be required for lifespan extension in response to mitochondrial stress (Schiavi et al., *under revision*).

1.5 Functions

Mitochondria are often referred to as the powerhouses of the cells. In fact these organelles provide the energy that our cells need to live. Mitochondria generate chemical energy, in the form of adenosine triphosphate (ATP). They are the sites of cellular respiration, also known as aerobic respiration (because dependent on the presence of oxygen), which ultimately generates fuel for the cell's activities. This is done by oxidizing the major products of glucose, pyruvate, and NADH, which are produced in the cytosol, through three major reaction pathways: Glycolysis, Krebs Cycle and Electron Transport. The machinery that the mitochondria use to produce ATP is called the electron transport chain. This chain is located in the inner membrane and is made up of 5 complexes (I-V), which are groups of proteins that work together to carry out their function. Except for complex II, complexes I to IV are oxidoreductases able to transport electrons with the passage of protons over the inner membrane. The created electrochemical proton gradient is then used by complex V (ATP synthase) to generate ATP (Dudkina et al., 2010).

In detail, fatty acids, pyruvate and amino acids are transferred from the cytosol into the mitochondrion where they are metabolized to acetyl coenzyme A (AcCoA). AcCoA is further metabolized through the citrate cycle, causing the reduction of oxidized nicotinamide adenine dinucleotide (NAD^+) and flavine adenine dinucleotide (FAD) and production of CO_2 (Alberts et al., 2007).

The respiratory chain implies the oxidation of NADH by O_2. It takes place in a chain-like, sequential manner catalysed by three protein complexes bound to the inner mitochondrial membrane: Complex I, Complex III (or cytochrome bc_1complex) and Complex IV (or cytochrome c oxidase). In addition, ubiquinone and cytochrome c participate as redox carriers that functionally connect complexes I and III, and III and IV, respectively (**Figure 3**). Reducing equivalents (electrons or hydrogen atoms) are transferred sequentially, until they reach O_2 at the active site of Complex IV, where O_2 is reduced to H_2O (Wikström, 2003). Apart from the oxidoreductase function, another important feature of respiratory chain enzymes is the ability to conserve free energy of the redox reactions for ATP synthesis, by pumping protons across the inner membrane from the matrix into the intermembrane space (Burte et al., 2014). The established electrochemical proton gradient drives the ATP generation via the H^+-ATP synthase, also called Complex V. The H^+-ATP synthase allows protons to flow back into the matrix, using the released energy to synthesize ATP (Burte et al., 2014).

Besides energy generation, mitochondria are also important for other cellular processes such as heme and iron-sulfur cluster biosynthesis, calcium homeostasis, cell death regulation and control of the cell cycle (Osellame et al., 2012).

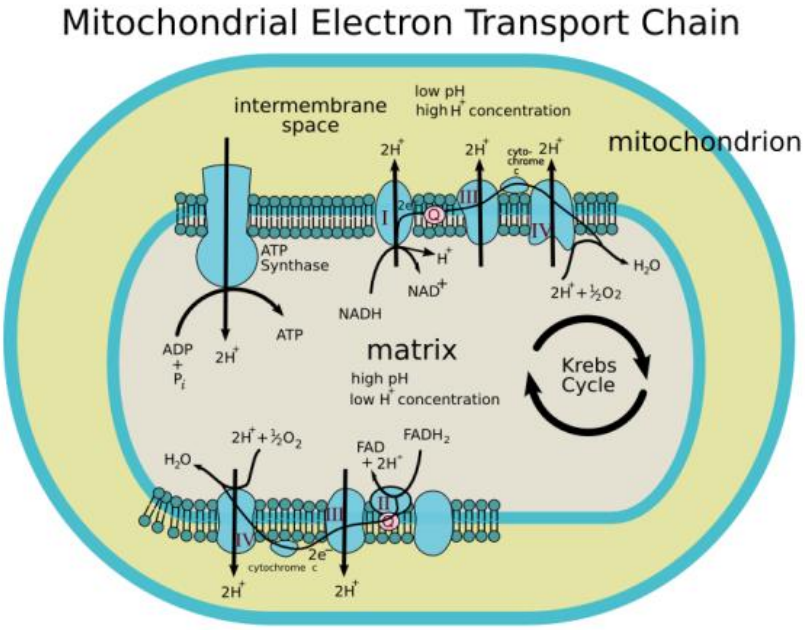


Figure 3.The mitochondrial respiratory chain (from www.wikidoc.org)

1.6 Human Mitochondria-associated diseases (HMAD)

Mitochondria are notable mobile and plastic organelles, able to change their shape, to fuse (fusion) each other and to separate (fission) again; the equilibrium between fusion and fission is likely a major determinant of mitochondrial number, length and degree of interconnection. When the equilibrium moves from fusion to fission, the mitochondria tend to become more elongated and interconnected while when it moves to fission the mitochondria tend to becomes more numerous and distinct (Karp, 2013). Mitochondrial fission and fusion are finely regulated, and disruption of this processes leads to abnormal mitochondria and

consequently in most of cases to diseases, most notably neurodegenerative disorders. (Itoh et al., 2013; Knott and Bossy-Wetzel, 2008; McInnes, 2013; Nikoletopoulou and Tavernarakis, 2014).

Besides diseases resulting from altered mitochondrial dynamics, the vast majority of mitochondrial diseases are in fact a clinically heterogeneous group of disorders that arise as a result of direct or indirect dysfunction of the mitochondrial respiratory chain. The mitochondrial respiratory chain is the essential final common pathway for aerobic metabolism, and tissues and organs that are highly dependent on aerobic metabolism are preferentially involved in mitochondrial disorders (Wallace, 1999) such as brain, heart, liver, skeletal muscles, kidney and the endocrine and respiratory systems. Depending on which cells are affected, symptoms may include loss of motor control, muscle weakness and pain, gastro-intestinal disorders and swallowing difficulties, poor growth, cardiac disease, liver disease, diabetes, respiratory complications, seizures, visual/hearing problems, lactic acidosis, developmental delays and susceptibility to infection.

Mitochondrial diseases are the result of either inherited or spontaneous mutations in mtDNA or nDNA which lead to altered expression and/or functions of the proteins or RNA molecules that normally reside in mitochondria. More than 70 different polypeptides interact on the inner mitochondrial membrane to form the respiratory chain. Most of the subunits are encoded by nuclear DNA and synthetized within the cytosol, but 13 essential subunits are encoded by the 16.5-kb mitochondrial DNA (mtDNA) (Schon et al., 2012). Every human cell contains thousands of copies of mtDNA. At birth these are usually all identical (homoplasmy). Different mtDNA mutations may instead result in a mixture of mutant and wild-type mtDNA within each cell (heteroplasmy), a phenomenon responsible for the clinical heterogeneity of these disorders. Indeed, the percentage level of mutant mtDNA may vary among individuals within the same family, and also among organs and tissues within the same individual. Mutant mtDNA must exceed a critical threshold level before a cell expresses a biochemical abnormality of the mitochondrial respiratory chain (the so called threshold effect or the percentage of mutation needed to manifest clinically, that depends on each particular tissue) (Koopman et al., 2012), but it seems that also age

and the environment can influence the pathogenesis of mitochondrial disorders (Vafai and Mootha, 2012).

Mitochondria-associated disorders may present at any age and can either be due to sporadic or spontaneous gene mutations in mtDNA or nuclear DNA, or alternatively to exogenous factors such as drugs, toxins and infections (Finsterer, 2004). They can affect a single organ (e.g. the eye in Leber hereditary optic neuropathy and the ear in nonsyndromic hearing loss with or without aminoglycoside sensitivity), but many involve multiple organ systems and often present with prominent neurologic and myopathic features (Vafai and Mootha, 2012). In children, approximately one-third of the inherited metabolic disorders are attributable to mitochondrial defects (Finsterer, 2004). In addition, increasing evidence shows that acquired mtDNA mutations and mitochondrial dysfunction are involved in aging and age-related diseases (Taylor and Turnbull, 2005).

1.7 Mitochondria in neurodegenerative disorders and aging

Neurons require a lot of energy. They are therefore particularly vulnerable to mitochondrial dysfunction, and many mitochondrial diseases result indeed in neurodegeneration. An increasing number of evidences relate common neurodegenerative diseases and the aging process with mitochondrial respiratory chain dysfunction and mutations in mtDNA (Reeve et al., 2008).

Nevertheless, if mitochondrial respiratory chain function is impaired in many neurodegenerative diseases, the original causes of this alteration and the role it plays in the pathogenesis of the disease may vary in different disorders. Several observations suggests that mutation in mitochondrial genes are involved in the pathogenesis of Parkinson disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer Disease (AD) (Swerdlow, 2009). It has been also suggested that increased excitotoxicity (Huntington's disease, ALS), altered mitochondrial biogenesis and increased oxidative stress and damage (ALS, PD and Friedreich's ataxia), may account for the changes in mitochondrial

respiratory chain function found in neurodegenerative diseases (Swerdlow, 2009). In fact, it is not clear whether in these pathologies mitochondrial impairment and oxidative stress are involved in the onset and progression of neurodegeneration or actually a consequence of the degenerative process (Federico et al., 2012; Miquel et al., 1980)

The focus on mitochondria as an important player during aging exponentially increased in the past decades. The first mitochondrial theory of aging was postulated by Denham Harman already in 1972 as a modification of his first theory of aging, dated 1956 (Harman, 1956). Then, in 1986, Miquel and Fleming, reformulating Harman's idea, presented the "oxygen radical-mitochondrial injury hypothesis of aging" implicating the mitochondria as the chief target of radical damage (Miquel et al., 1980). As previously said (paragraph 1.3) the reactions occurring on the mitochondrial respiratory chain have an intrinsic danger because of electron leakage leading to the production of reactive oxygen species (ROS). Because mtDNA is spatially close to the source of ROS, it is particularly vulnerable to ROS-mediated mutations. Moreover, mtDNA mutations that reduce the accuracy of electron transfer increase the probability of ROS production and further mtDNA lesions, leading to a "vicious cycle". From this scenario the hypothesis that mitochondrial dysfunctions play a critical role in the aging process takes form. In this perspective, aging is caused by the ROS-accelerated accumulation of mtDNA damage, leading to a progressive decline in respiratory function over time (Chan, 2006). To corroborate this hypothesis, many tissues from elderly individuals have found to have lower respiratory function compared to those from younger persons (Boffoli et al., 1994; Trounce et al., 1989). Aging has also been associated with clonal mtDNA deletions and respiratory incompetence in single neurons in the substantia nigra (Bender et al., 2006; Kraytsberg et al., 2006). Although these and other studies show the association between aging and the decline of respiratory functionality, accumulation of mtDNA deletions and point mutations, and accumulation of oxidative damage to mtDNA, there still remains a substantial concern about whether these changes are causal or only collateral in the aging process (Chan, 2006).

2 *C. elegans* as a model organism

The establishment of *C. elegans* as a model system for basic biological research started in 1963 with the molecular biologist, Sydney Brenner, at the Medical Research Council (MRC) Laboratory of Molecular Biology in Cambridge, United Kingdom. In a letter that the biologist wrote to the head of the laboratory, Max Perutz, the biologist declares his intents (Brenner, 1988) to extend the basic biological research to other fields, particularly development and the nervous system.

In his opinion the successes that were reached at that time in defining the molecular bases of biological processes in bacteria suggested that a similar approach would work also in more complex organisms. Brenner wanted to identify a metazoan animal to study development directly that could be handled similarly to bacteria and viruses.

Brenner then was asked to submit a formal proposal to the MRC and he in fact propose, in October 1963, to study the genetics of differentiation in the nematode *Caenorhabditis briggsae*. The possibility to use the nematodes in genetic research had already been shown by Dougherty and Calhoun (Dougherty and Calhoun, 1948). In his proposal Brenner listed all the numerous experimental advantages of this model to identify genes and mechanisms regulating development. The worm can be easily cultured in the laboratory, has a short life cycle (**Figure 4**) and produces numerous progeny. Hermaphrodite worms use self-fertilization and this allows homozygous worms to breed true and greatly facilitates the isolation and maintenance of mutant strains. It is also a useful resource if mutant animals are uncoordinated or paralyzed since they don't need to move and find a male for reproduction. Nevertheless, mating with males is essential to move mutations between strains. Ultimately, *Caenorhabditis*' precise and relatively low number of somatic cells for a highly differentiated animal (<1,000), offers a tractable system for studies of cellular function, development and differentiation. Brenner finally proposed to identify every cell in the worm and trace their lineages, to investigate the constancy of development and to study how that is controlled by looking at mutants. Brenner's proposal was judged

audacious but very promising, his aim was accomplished and the lineage of every nematode cell was studied. This work enables Sydney Brenner, in 2002, to win the Nobel Prize in Physiology or Medicine, shared with John Sulston and H. Robert Horvitz. They have been awarded for their discovery in *C. elegans* of genes that regulate organ development and programmed cell death. In the past two decades, however, the amazing experimental power of *C. elegans* has been exploited above and beyond the developmental biology field. This little animal has been, and is still employed to study cell cycle control, apoptosis, ageing, sensory physiology, neuronal biology, and many more.

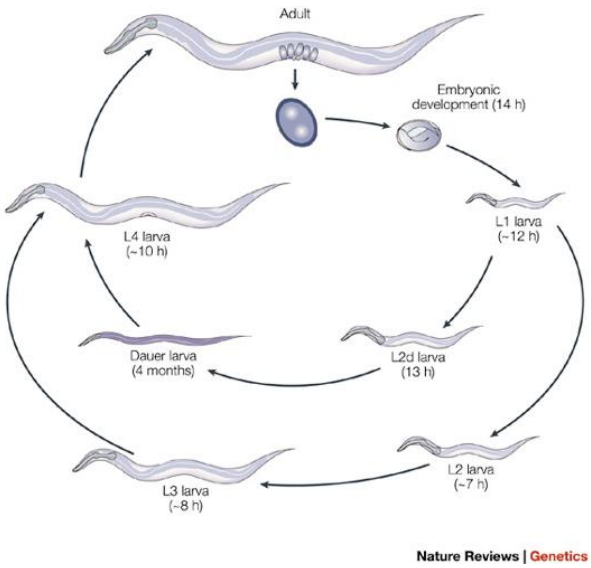


Figure 4. Lifecycle of *C. elegans* at 25°C. *C. elegans* development under favourable conditions goes through four larval stages in about 35 hours before molting to an adult. The end of each larval stage is marked with a molt, during which a new, stage-specific cuticle is synthesized and the old one is shed (during this period, pharyngeal pumping ceases and the animal enters a brief lethargus). When conditions turn adverse (low food or crowding) animals can switch to a facultative diapause stage called dauer larva that can survive 4 to 8 times the normal 3-week life span. Dauer larvae are capable of living for months in poor conditions and exit the dauer stage and matures to adulthood when

conditions become favourable. Figure from Jorgensen and Mango Nature Reviews Genetics 3:356-369 (Jorgensen and Mango, 2002)

2.1 *C. elegans* nervous system

The *C. elegans* nervous system (**Figure 5**) has been so far well characterized both from the anatomical and functional point of view and also several assays to assess the functionality of specific sets of neurons have been developed; for these reasons the round worm is an excellent model to study neurodegenerative diseases and to investigate the action of neurotrophic substances.

The nervous system is the most complex organ in *C. elegans*, whose body is composed for one third of neurons (to be precise 302 out of 959 cells in the adult hermaphrodite are neurons). 20 of these neurons are located inside the pharyngeal bulb, which has its own nervous system. The residual 282 are located in various ganglia in the head and tail and also along the ventral cord, the main longitudinal axon tract.

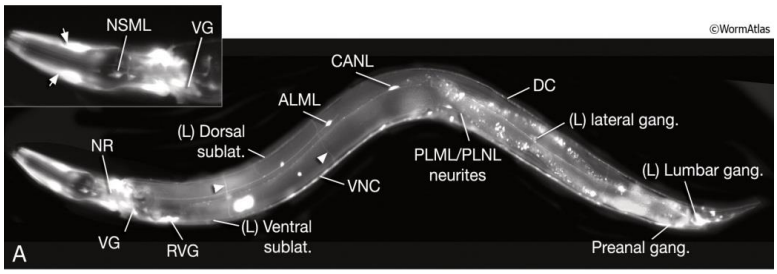


Figure 5. Epifluorescent image of a transgenic animal expressing a panneural reporter, left lateral view. The largest collection of neurons is around the nerve ring (NR) within several head ganglia, including the retrovesicular ganglion (RVG) and ventral ganglion (VG; also, inset). The second largest collection of cell bodies is in the tail ganglia (the left lumbar ganglion and preanal ganglion are shown). A few single neurons, including ALM, CAN, or small groups of neurons (lateral ganglia), are also found along the lateral body wall. Longitudinal nerve tracts travel along the body at ventral, subventral, lateral, subdorsal, and dorsal positions and connect cell bodies to major neuropils. Anterior to the ring, several sensillar nerves reach the tip of the head. VNC (ventral nerve cord) motor neurons are scattered along the VNC and send processes to the

DC via commissures (arrowheads). The pharyngeal nervous system is an autonomous network of 20 pharyngeal neurons (NSML, which is situated within the anterior bulb, inset). Note that this reporter is also expressed in head muscle cells (arrows, inset). Magnification, 400x. Strain marker: unc119::GFP. (from http://www.wormatlas.org/)

Most neurons develop already during embryogenesis, but 80 neurons (mainly motoneurons) develop postembryonically. The structure of the nervous system has been described in detail by electron microscopic reconstruction (White et al., 1986). With electron microscopic images White and colleagues mapped all the connections, identified at high resolution all the synapses (about 5000 chemical synapses, 2000 neuromuscular junctions and some 500 gap junctions), and determined the entire neuronal circuit.

2.2 *C. elegans* and neurodegenerative diseases

C. elegans is a small roundworm with a defined, precise anatomy, a rapid reproductive cycle, a short lifespan, and a transparent body (http://www.wormbook.org/). Each of these characteristics contributes to make of *C. elegans* a powerful tool for understanding the functional genomics of diseases. Gene expression can be indeed modulated by promoter-driven expression, knock down or gene deletion. Thanks to neuronal specific promoters, transgenes can be expressed to particular neuronal subtypes, such as chemosensory neurons or dopaminergic neurons. The structure of the neurons can be also examined in live animals during their aging using fluorescent markers, and these neurons can be followed through the entire animal lifespan from the larval stage until the worm naturally dies (**Figure 6**). In this way it is possible to study the involvement of specific genes in the detrimental structural changes during *C. elegans* aging. (Wolozin et al., 2011).
Even if it seems difficult to compare the complexity of the human brain with its billions of connections with the relative simplicity of *C. elegans* nervous system in the past decades several neurodegenerative diseases have been modeled in the worm. The nematode is considered by the Scientific Community as a "scout" system that has the potential to generate important findings regarding mechanisms of genetic or environmental induced neurotoxicity, with the warning that all findings

are conditional until extended to mammalian systems. Nevertheless *C. elegans* already provided a yield of information regarding the function of human disease-relevant proteins (Teschendorf and Link, 2009) and several neurodegenerative diseases have been modeled in the worm (**Table I**).

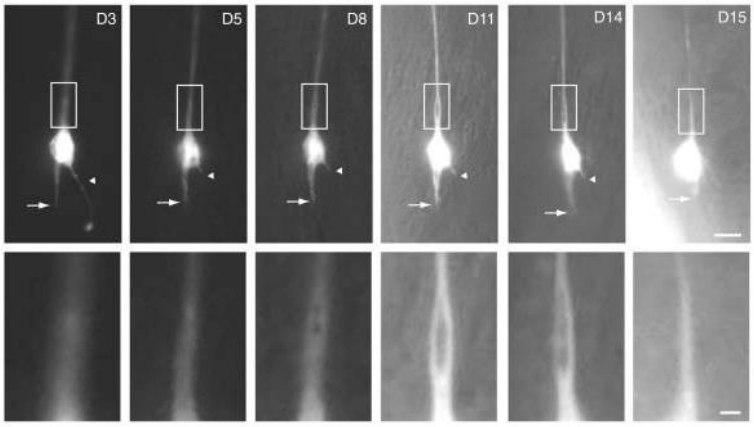


Figure 6. Example of neuronal structure examination during aging. Longitudinal fluorescence imaging of *C. elegans* mechanosensory neurons during the course of physiological aging, from day 3 to day 15. Figure adapted from (Chen et al., 2013)

Until now, different models have been developed for the major pathologies that affect the nervous system during aging (e.g. Parkinson and Alzheimer) but not many models exist for mitochondrial diseases, except for Friedreich's Ataxia and a few others (Dancy et al., 2014; Dingley et al., 2010; Grad and Lemire, 2004; Grad et al., 2005). But the possibilities to use this model organism to look for potential therapeutic drugs are shown in the last years in an increasing number. Even if the *C. elegans* high-content, high-throughput screenings belong to the recent history the results already obtained in this field are very encouraging. Recently the studies involving *C. elegans* and the nervous system are

taking advantage of new technologies and successfully showing that the nematode can provide a platform both for the discovery of new bioactive compounds and for target identification and therefore represents a tool to fight neurodegenerative diseases. First examples in this direction have been the work of Braungart in 2004, which showed the specific chance to identify anti-Parkinson treatment using the worms (Braungart et al., 2004) or of Ellerbrock in the research for potential drugs against Alzheimer (Ellerbrock et al., 2004). Different studies aimed with these screenings to identify neuroprotective interventions (Choe and Strange, 2008) and others used them to hit neurotoxins (Boyd et al., 2010).

Table I

Neurodegeneration-associated protein	Transgene expression	Promoter Sequence
Huntingtin::polyQ	chemosensory neurons	*osm-10*
	mechanosensory neurons	*mec-3*
	muscle	*unc-54*
DRPLAP::polyQ	muscle	*unc-54*
GFP::polyQ	muscle	*unc-54*
	pan-neuronal	*rgef-1*
α-synuclein	pan-neuronal	*aex-3*
	dopaminergic neurons	*dat-1*
	dopaminergic neurons	*dat-1*
	dopaminergic neurons	*dat-1*
	pan-neuronal	*unc-51*
α-synuclein::GFP	muscle	*unc-54*
α-synuclein::YFP	muscle	*unc-54*
β-amyloid peptide	muscle	*unc-54*
	inducibe muscle	*myo-3*
	inducible pan-neuronal	*snb-1*
tau	pan-neuronal	*aex-3*
	mechanosensory neurons	*mec-7*
	pan-neuronal	*rgef-1*
SOD1	muscle	*myo-3*
	heat shock inducible	*hsp-16.2*
	pan-neuronal	*snb-1*
SOD1::YFP	muscle	*unc-54*
LRRK2	pan-neuronal	*snb-1*
mouse prion protein	muscle	*unc-54*

Overview of neurodegenerative pathologies modeled in *C. elegans*, adapted from (Teschendorf and Link, 2009)

2.3 *C. elegans* and aging

During its lifespan the roundworm *C. elegans* shows a physiological decline of some of its features and basic structures. Biochemical changes occurring at a cellular and tissue level, lead finally to physiological alterations (Collins et al., 2008).

Specifically different features change during animal aging at a cellular/tissue and behavioral level:

- increase DNA breaks especially single-stranded, and the incorporation of 5-methylcytosine;
- alterations in genes expression;
- increase of level of the carboxylation of proteins;
- decrease in metabolic and enzymatic activity;
- increase susceptibility to (oxidative) stress;
- increase of green and red fluorescence signals, consistent with the accumulation of distinct fluorophores (Coburn et al., 2013);

While, at a phenotypic level it can be observed:

- decrease of pumping rate. The pharynx is a neuromuscular organ that contracts rhythmically about 300 times per minute in the adults; it begins to degenerate when the animal stops releasing eggs (around the sixth day of life) and gradually stops to function;
- decrease of locomotor ability: the characteristic sinusoidal and well-coordinated movement, progressively lose coordination and becomes slower in relation to muscle deterioration;
- decrease in the ability to sense food, chemicals and thermal stimuli: wild-type animals show a progressive decrease in the ability to sense, due to the deterioration of motor functions and the structural degeneration of the nervous system;
- decrease in fertility due to the exhaustion of self-sperm cells;

Also the research for age-related compounds, as it is happening for neurodegenerative diseases treatments, is taking advantage, in the last years, of automated screenings technologies. The first automated high-throughput screening for survival using the nematode *Caenorhabditis elegans* was done in 2003 by Lithgow and colleagues (Gill et al., 2003)

and this work showed the possibility to accelerate the process of identifying drugs that affect aging using the round worm. In the following years several screenings have been done and showed *C. elegans* as a powerful platform for *in vivo* high-throughput, high-content screenings for the identification of genetic or pharmacological interventions with anti-aging effects (Artal-Sanz et al., 2006; Burns et al., 2006; Kolesnick, 2002; Petrascheck et al., 2007; Stroustrup et al., 2013).

Materials and Methods

3 *C. elegans* methods

3.1 *C. elegans* strains and maintenance

We employed standard nematode culture conditions (Stiernagle, 2006). All strains were maintained at 20 °C on Nematode Growth Media agar supplemented with *Escherichia coli* (OP50 or transformed HT115). Strains employed in this work were as follows: N2 (wild-type), *che-3(e1124), nmur-1(ok1387), osm-3(p082), odr-7(ky4), tax-4(p678), isp-1(qm150); ctb-1(qm189), gcy-7p::gfp;lin-15*.

3.2 RNAi feeding

The following dsRNA transformed bacteria for feeding were derived from the Ahringer *C. elegans* RNAi library (Kamath and Ahringer, 2003): *nuo-1* (C09H10.3), T20H4.5, F53F4.10, *cco-1* (F26E4.9), *nuo-5* (Y45G12B.1), *nuo-2* (T10E9.7), *spg-7* (Y47G6A.10), *lpd-5* (ZK973.10). For additional RNAi used in the phenotypic screening described in Chapter 4 refer to Table II. Feeding RNAi constructs against *frh-1* and *isp-1* have been generated as previously described (Rea et al., 2007; Ventura et al., 2005). All dsRNA bacterial clones were grown to a concentration of 0.9 OD and diluted 1/10 (except for *frh-1*) with empty vector expressing bacteria as previously described (Rea et al., 2007). For studies involving *frh-1* animals were cultured on dsRNA transformed bacteria for three consecutive generations as described previously (Ventura et al., 2005).

3.3 Drug Assays

Chemicals used in this work were all acquired from Sigma: Rotenone (R8875), Paraquat (36541), Doxycycline (1822), Oligomycine (75351) and Rotenone (R8875).

Treatment of *C. elegans* was carried out on NGM agar plates containing the mitochondrial targeting drugs. All agar plates were prepared from the same batch of NGM agar, whereas treatment plates were supplemented with the respective compound and control plates with the respective solvent. Drugs treatment was performed using UV killed bacteria (HT115) to avoid interference by the xenobiotic-metabolizing activity of *Escherichia coli*. Drugs have been spotted on the dried bacteria lay. Distilled water or DMSO used as solvent. Spotted plates were dried overnight at room temperature and used the day after.

3.4 Multiwell plates preparation for screening

On the day of the screen, assay NGM plates containing the animals under treatment (2x6cm plates per condition) were washed with S-medium. Animals washed off were transferred into 15 ml conical tubes and allowed to settle by gravity for 5 minutes. After discarding the supernatant, animals were washed again with 5 ml of S-medium to remove excess bacteria and other debris that could interfere with worm imaging. Aliquots of 150 µl of suspension were then transferred in each well of a 96 well plate.

Prior to imaging, worms were anesthetized by adding 2 µl of 1 M NaN_3 in S-medium to each well and the plate closed with a clear sealing film. Images were acquired with the ArrayScan V^{TI} HCS Reader fitted with a 2.5× objective and a 0.63× coupler using a 2-channel (TRITC and GFP) assay. Real-time analysis was performed using the SpotDetector BioApplication optimized to quantify fluorescent protein expression in *C. elegans*. Image acquisition and analysis of a 96-well plate was completed in <25 minutes.

3.5 Chemotaxis assays

Animal's neuronal functionality was assessed by quantifying attraction or repulsion to different concentration of chemicals as follow: sodium azide (NaN3), used to anesthetize worms, was placed on buffered agar 180 degrees opposite on a 10 cm dish; the attractant (or repellent) was then placed on one NaN3 spot, and ethanol (neutral odor for the worms in which chemical was diluted) on the other spot; a population of 80-100 age-synchronized animals was placed in the center of the testing plate and the number of worms at attractant and control was counted every 15 minutes for two hours to calculate the Chemotaxis Index (CI). CI=(A-B)/(A+B+C), where A is the number of worms at attractant, B is the number of worms at control and C is the number of animals which didn't reach any of the two spots at the end of the two hours. For a population of 3 days old, wild-type animals, a good CI is around 0,8 for attractants and –0.8 for repellents after two hours (CI=0 means no attraction, while CI=1 or –1 represent maximum attraction or repulsion, but there are always some animals that, also for a wild-type strain, remain randomly dispersed in the assay plate or reach the control spot instead of the attractant).
che-3(e1124), *osm-3(p082)*, and *tax-4(p678)* sensory defective mutants were used as positive controls. Compounds used in chemotaxis assays were all acquired from Sigma-Aldrich: ammonium acetate (A1542), was diluted in distillated water and 2-Methyl-3-(methylthio)pyrazine (545791), 1-Butanol (9B7906), Benzaldehyde (418099), 2-nonanone (W278505), all diluted in Ethanol 99.8%.

3.6 Locomotion assays

Ability to move: the number of animals moving spontaneously and upon touch was counted every day during the lifespan assay and then reported as the fraction of animals moving on total animal alive at each scored day.

Speed: was assessed by counting the number of body bends (changes in the body bend at the mid-body point per minute), per minute for each

worm on solid agar plates with no bacteria. One bend was counted every time the mid-body reaches a maximum bend in the opposite direction from the bend last counted. Body bends were checked in at least 20 single worms in 2 or 3 independent biological trials.

3.7 Lifespan assay

Survival curves and statistical analyses were carried out as previously described (Ventura et al., 2009).

Survival analysis starts from hatching and is carried out at 20°C using synchronous populations of 80 animals per condition. Animals are scored as dead or alive and transferred every day on fresh plates during the fertile period, and then every other day or every 3 days until death. Worms are considered dead when they stop pharyngeal pumping and responding to touch. Worms that die because of internal bagging, desiccation due to crawling on the edge of the plates, or gonad extrusion are scored as censored. These animals are included in lifespan analyses up to the point of censorship and are weighted by half in the statistical analysis. We calculate mean lifespan, standard deviation of the mean, and P value (Mantel-Cox regression analysis) from Kaplan-Meyer survival curves of pooled population of animals coming from at least two independent replicas. For statistical analysis we use the Online Application for Survival analysis OASIS (Yang et al., 2011).

As a measure of health-span, the ability to move spontaneously or upon prodding is also scored during the entire lifespan. Data from survival assays are summarized in Table VI and Appendix B, Table II and II.

3.8 Neuronal structure

The structure of the ASE neurons was assessed using a strain reporter for ASEL neurons, *gcy-7::gfp*. Amphid neurons' (AWB, ASI, ADL, ASH, ASJ, ASK) structure was instead analyzed through Dye-Filling (DiO)

staining. In all the experiments for neuronal analysis 3-days-old animals where observed using a Zeiss Imager 2 microscope equipped for fluorescence microscopy at 400× magnification. 20 animals per condition in two independent replicas were acquired. Staining with DiO was performed as previously described (Starich et al., 1995). Briefly, a stock solution of 2 mg/ml DiO (Molecular Probes) in dimethyl formamide was stored at −20° in a foil wrapped tube. Dilute solution from the stock was prepared fresh 1:200 in M9. 150 µl of diluted solution were dispensed in a well of a microtiter plate, and L4 worms were transferred with a platinum worm pick into the dye. Plate was incubated 2 hours at room temperature. Worms were then washed 3 times in M9 to remove non-specifically bound DiO and mounted on slides to be observed the microscope.

3.9 Single worm genotyping PCR

The genomic DNA was extracted from single wild-type and mutant worms, as described (Barstead et al., 1991). Briefly, single animals were picked up with a platinum wire and placed in a 5µl drop of lysis buffer (20µg/ml proteinase K in 10 mM Tris (pH 8.2), 50 mM KCL, 2.5 mM MgCl2, 0.45% Tween 20 and 0.01% gelatin) in the cap of a separate 0.2 ml PCR tube. The drops were moved to the bottom of the tubes by a brief microfuge spin and heated to allow lysis and DNA denaturation (60°C, 1 hr followed by 95°C, 15 min). After cooling to 4°C, 25 µl of a master mix were added to each tube. The reaction mixture is Master mix contain PCR Buffer, 0,5 µM primers, 0,2mM/each dNTPs, 0,25 µM iProof™ High-Fidelity DNA Polymerase. After a brief microfuge spin the reactions were rapidly heated to 98°C and cycled 35 times: 98°C for 10s, 57°C for 1min, and 72° for 1min. Gel lanes were loaded with 5µl of each reaction mixture. The gel image was acquired with an Intas UV transilluminator.

3.10 Animal size quantification

Pictures for animal size quantification were acquired with a Leica MZ10 F modular stereo microscope, 40× magnification, connected to a color digital camera. Length measurements were performed with the free Java image processing program ImageJ (http://imagej.nih.gov/). Total length of the worms is the length of the line drawn from the nose to the tail tip using a freehand selection, and is calibrated within the software using an image of a scale bar as reference. 5 to 8 animals per condition were measured.

3.11 Generation of transgenic animals

Translational reporter animals were generated using the MosSCI technique. MosSCI is a method to insert a single copy of a transgene into a well-defined location in the *C. elegans* genome (http://www.wormbuilder.org/). The method works by breaking a chromosome at a particular location by excising a Mos1 transposon. The excision creates a double-strand DNA break that is repaired by the cell. In the presence of a DNA template with homology to the breakpoint then the repair process will incorporate DNA from the repair template into the genome. We used a positive selection marker to select for this event (*unc-119*) and fluorescent markers to identify extra-chromosomal arrays. The transgene cloned in the appropriated targeting vector is injected in a uncoordinated (Unc) strain containing a Mos1 transposon at the site corresponding to the targeting vector. We use the EG6699 strain (Genotype: ttTi5605 II; unc-119(ed3) III; oxEx1578). The positively injected worms are easily recognizable because they rescue the uncoordinated phenotype. The vector in injected with other plasmid containing other three fluorescent markers, the transposase and the toxin peel-1, these last two activated by a two hour heat-shock at 34°C allowing to recognize the single copy transformed worms that will be the only moving animal without fluorescent marker but GFP.

Aims

Until recently Friedreich's Ataxia was one of the few Human Mitochondrial Associated Diseases (HMAD) successfully modeled in *C. elegans.* We decided to create new models for other HMADs, which could mimic different phases of the disease progression, with the ultimate goal to find potential targets for preventive therapies. Moreover, we wanted to investigate specific neuronal phenotypes and signaling pathways associated with mild mitochondrial stress extension of lifespan.

To this extent my PhD project followed three main aims:

1. To develop new models for human mitochondrial-associated diseases (HMAD) and characterize their neuronal dysfunction.
2. To identify neuronal signaling pathways involved in mitochondrial stress extension of lifespan.
3. To set up an *in vivo* phenotypic-based screening platform to identify interventions, which induce beneficial mitochondrial stress response pathways: (i) to rescue HMAD pathological phenotypes; (ii) to extend lifespan.

The findings obtained during my PhD studies resulted in the publication of one peer-reviewed paper (chapter 5) entitled "Mitochondrial stress extends lifespan in *C. elegans* through neuronal hormesis" and in two manuscripts currently in preparation (chapter 4 and 6 of my thesis). Some of my findings are also included in a manuscript currently under revision entitled "Reduced frataxin expression induces an iron starvation response that extends *C. elegans* lifespan via Parkin/Bnip3-regulated mitophagy". Moreover, during my PhD work I have also participated to additional collaborative papers and reviews (see Pag.95-96 for complete list of my publication record).

Results

4 Development and characterization of new *C. elegans* models for human mitochondrial associated diseases

4.1 Abstract

The functionality of the mitochondrial respiratory chain is severely compromised in different human mitochondrial-associated diseases (HMAD), resulting in oxidative stress and reduced ATP production, ultimately leading to neuromuscular cell degeneration. On the other hand partial mitochondrial disruption in different species including *C. elegans* has pro-longevity effects. This apparent paradox suggests that cells can induce adaptive, beneficial responses to cope with mild mitochondrial dysfunction, which may represent attractive candidates for preventive therapies.

Until recently Friedreich's ataxia was one of the few HMAD successfully modeled in *C. elegans*. We decided to create models for additional HMADs, which mimic different phases of the disease progression, with the ultimate goal to find potential targeted compounds for a preventive therapy.

With this aim we silenced the expression of a panel of 40 *C. elegans* genes, orthologous of known nuclear-encoded genes that when mutated in humans cause mitochondria associated diseases. We titrated RNAi against the genes under study and screened for specific phenotypic effects on lethality, development, size, color, and fertility. We identified for each gene: (i) a "mild" concentration of RNAi that caused slight decrease of size and fertility and slow development, phenotypes previously observed in long-lived mitochondrial mutants and therefore associated with a

compensatory, beneficial mitochondrial stress response (Rea et al., 2007). And (ii) a "strong" concentration of RNAi that induced animals sterility or growth arrest, reflecting the deleterious effect of severe mitochondrial dysfunction. We also characterized specific behaviors revealing animal neuronal functionality (e.g. locomotion and chemosensation) and observed a slight alteration in neuronal functioning upon mild mitochondrial stress, associated nonetheless with lifespan extension, (Maglioni et al., 2014) and as expected, a very strong neuronal deficiency upon severe mitochondrial stress.

4.2 Introduction

Over the last few decades, mitochondrial dysfunction, attributable to mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA), has been established as a cause for numerous human diseases (Kwong et al., 2006; Wallace, 2005). In these pathologies the mostly affected cells are those highly dependent on oxidative energy metabolism such as cardiac and skeletal muscle, renal and pancreatic beta cells and neuronal cells. Accordingly, many Human Mitochondria-Associated Diseases (HMAD) present with signs and symptoms of myopathy, cardiac defects (encephalocardiomyopaties) and neurodegeneration.

A number of human disease genes have homologs in the multicellular organism *C. elegans*, and the nematode system has already yielded important insights into the function of some of these (Artal-Sanz et al., 2006; Link, 2006; Silverman et al., 2009; Ventura et al., 2006). Since 1/3 of the *C. elegans* cells are represented by neurons, and its nervous system is very well characterized both at functional and structural level, this nematode is successfully used to study neurodegeneration during aging and in different diseases of genetic origin such as Alzheimer's disease and Parkinson's diseases (Alexander et al., 2014; Lublin and Link, 2013) and to screen for neurotrophic drugs (Helmcke et al., 2010; Teschendorf and Link, 2009). However, there are not many *C. elegans* models available for concerning HMAD, except the one for the Fredrich´s

Ataxia. Moreover, although *C. elegans* is an excellent tool for studying the action of compounds at the whole organism level, automated systems to screen big populations of animal and different physiological parameters at the same time are not yet completely optimized and established. Remarkable are the efforts in this direction with the development of the first large-scale drug screen reported in 2006 (Kwok et al., 2006). Or the one proposed at the same time by Lehner and colleagues (Lehner et al., 2006), an all-liquid workflow to allow HTS with the nematode in a 96-well format. And a few other liquid-based screens developed with the aim to identify compounds promoting the extension of lifespan in nematodes (Breger et al., 2007; Petrascheck et al., 2007, 2009). Also microfluidic devices recently emerged in the scene of HTS with *C. elegans* (Ben-Yakar et al., 2009).

Finally, in 2010, Gosai and collaborators combined automated high-content drug screen with automated worm transfer, image acquisition and data analysis (Gosai et al., 2010) taking advantage of the platform Arrayscan VTI (Thermo Scientific Cellomics) and the dedicated SpotDetector BioApplication. These devices are useful in isolating worms, delivering chemicals and observing worms in a high throughput manner but still improving the efficiency of these systems is desirable, especially advanced instrumentations to improve the image acquisition and data analysis to assess complex phenotypes are needed (O'Reilly et al., 2014).

In our laboratory, encouraged by the potentialities shown by the above-mentioned works, we are also using the Cellomics platform and its data analysis software to carry out drug-screenings.

The partial suppression of numerous genes encoding mitochondrial proteins has been shown to increase lifespan in *C. elegans* (Dillin et al., 2002; Lee et al., 2003b; Ventura et al., 2006). The nematode strains in which the expression of such genes is suppressed are generally indicated with the name of Mit (Mitochondrial) mutants (Rea, 2005), and they are characterized by an increased lifespan and by other typical phenotypes, such as slow development, reduced size and an extension of the fertile period (Rea et al., 2007). The majority of mitochondrial down-regulated proteins in these mutants are involved directly or indirectly in the functionality of the respiratory chain, so they have an important role in the organelle function (Hamilton et al., 2005; Hansen et al., 2007).

On the other hand a strong suppression of the same mitochondrial proteins, lead in the nematode (as it happens in human) to dramatic consequences, like arrest development and eventually lethality. Those two opposite phenotypes that appear in *C. elegans* upon mild or strong mitochondrial protein suppression represent a very useful tool allowing to mimic the severity of the disease, on one hand, and, on the other hand, to investigate the organismal response in the window of partial mitochondrial suppression, that in human is asymptomatic, while in our model is associated with a very specific and recognizable phenotype.

For the above mentioned reasons, Mit mutants can be exploited to study HMADs; in fact, understanding what are the protective pathways that are activated in *C. elegans* to compensate the initial, moderate, mitochondrial damage (that finally lead to an increased lifespan), may direct towards the identification of targets for possible therapeutic strategies to prevent or slow down the disease in humans.

Starting from the results previously obtained we wanted to generate and characterize additional models for other mitochondrial disorders, besides Friedrich´s Ataxia, and in particular to focus on the "mild" phase of the disease, that represents a window of time in which the mitochondrial damage is still not too severe for the organism to show the deleterious symptoms. Moreover, to exploit and make the best use of the *C. elegans* HMAD models we decided to set up an automated microscopy screening able to screen for drugs potentially useful in contrasting the diseases. In this prospective we worked on a high-content screening to look for interventions that rescue the pathological phenotype observed in response to severe mitochondrial dysfunction and potentially applicable to different mitochondrial-associated diseases. To this aim we optimized the screening platform Cellomics ArrayScan VTI HCS Reader, normally used for cell-based screening.

4.3 Results

4.3.1. Development of new HMAD models

With the aim of developing new models for HMAD we interfered a panel of 40 orthologous *C. elegans* genes (**Table II**) representing known nuclear-encoded genes that, when mutated in humans, lead to severe mitochondrial dysfunction and cause degenerative pathologies. We screened for the characteristic phenotypic effects associated with different doses of mitochondrial stress: lethality, development, size, color, and fertility. The aim of this screening was to investigate the response of the nematode to different levels of mitochondrial stress. We observed a substantial variance in the phenotype of the animals upon treatment with different doses of RNAi against the genes of interest. In three independent experiments, we observed the animals treated with the same RNAi for two consecutive generations, P0 and F1. The effect of the mitochondrial stress is in fact stronger in the second generation. (In the P0 the worms are fed with the recombinant bacteria only as adult while at the time of the F1 they lived on the interference their all life).

Table II

COSMID ID	GENE	SHORT GENE DESCRIPTION/DISEASE
ZK973.10	*lpd-5*	NDUFS4,NADH DEHYDROGENASE, Fe-S protein complex I subunit/complex I deficiency
Y47G6A.10	*spg-7*	paraplegin like/hereditary spastic paraplegia (SCA)
C01F1.2	*sco-1*	SCO-1 / Leigh Syndrome
C15F1.7	*sod-1*	SOD-1 (Cu-Zn SOD-1)/ Amyotrophic lateral sclerosis
D2013.5	*eat-3*	OPA-1 or mgm-1, dynamin family GTPase/dominant optic atrophy

C09H10.3	*nuo-1*	NDUFV-1, nuo-1/ Leigh Syndrome
Y57A10A.15	*polG*	POLG, MITOCHONDRIAL DNA POLYMERASE GAMMA l/progressive external ophtalmoplegia
Y46G5A.2	*cox-10*	COX10, complex IV farnesyl transferase/mitochondrial complex IV deficiency
T24C4.1	*ucr-2.3*	mutator phenotype
R10E4.5	*nth-1*	nth-1, Fe-S DNA repair enzyme/disease
F01F1.12	*aldo-2*	ADHUB, fructose bifosphate aldolase/fructosemia
T20H4.5	T20H4.5	NDUFS8/Leigh Syndrome
H14A12.2	*fum-1*	fum-1A-fum1B, fumarase1,FH /Fumarase deficiency, Leigh Syndrome
F54H12.1	*aco-2*	ACO-2 , aconitase / infantile cerebellar-retinal degeneration
M03C11.5	*ymel-1*	yme-1 (mitos escape) , AAA protease of the MMI/hereditary spastic paraplegia (SCA)
F54C9.6	*bcs-1*	BCSiL/Leigh syndrome and Gracile Syndrome
Y43C5A.5	*thk-1*	thk-1(thimidine Kinase)/associated with mitochondrial myopathy
Y38F2AR.7	*ppgn-1*	paraplegin/hereditary spastic paraplegia (SCA)
Y45G12B.1	*nuo-5*	NDUFS-1 or nuo-5 complexI/complexI deficiency
F46E10.8	*ubh-1*	ubiquitine C-terminal hydrolase, ortholog oh human UCHL1/parkinson
F53F4.10	F53F4.10	NDUFV2, Fe-S complex I/susceptibility to Parkinson disease
F23B12.5	F23B12.5	DLD, dihydrolipamide deidrogenase/lactic acidosis
T10E9.7	*nuo-2*	NADH Ubiquinone Oxidoreductase
C03G5.1	*sdha-1*	SUCCINATE DEHYDROGENASE COMPLEX, SUBUNIT A/complex II Leigh syndrome
F21G4.6	F21G4.6	Huntington/Huntington disease

List of orthologous *C. elegans* genes screened in this work. The genes are all known nuclear-encoded genes that, when mutated in humans, lead to severe mitochondrial dysfunction and cause degenerative pathologies.

We specifically identified for each gene: (i) a “mild” concentration of RNAi that caused slight decrease of size and fertility and slow development, phenotypes previously observed in long-lived mitochondrial mutants and therefore associated with a compensatory, beneficial mitochondrial stress response (Rea et al., 2007). And (ii) a “strong” concentration of RNAi that induced animals sterility or growth arrest, reflecting the deleterious effect of severe mitochondrial dysfunction (**Figure 8**).

Most of the clones screened showed these phenotype "mild" in the P0 and a "strong" in the F1 (see Table III and Table I in Appendix B)(**Figures 7, 8**). If in the parental generation a phenotype "strong" was already observed due to a strong effect of the RNAi, leading to the arrest of development or to infertility, it was decided to fed the P0 animals with a RNAi diluted with a factor 1:10 (or, in a few cases, 1:50) with bacteria transformed with the empty vector, thus obtaining the phenotype "mild" (**Table III**, clones labeled with *, and supplementary Table I in Appendix B) (**Figures 7, 8**). The clones in the three rounds of screening that gave consistent results at the phenotypic level were further investigated.

In the first category of clones then we observed a phenotype "mild" in the parental generation and a "strong" in F1; in the second category, conversely, both phenotypes were observed only in P0, but with different dilutions of the RNAi (1:10 = mild; undiluted = strong).

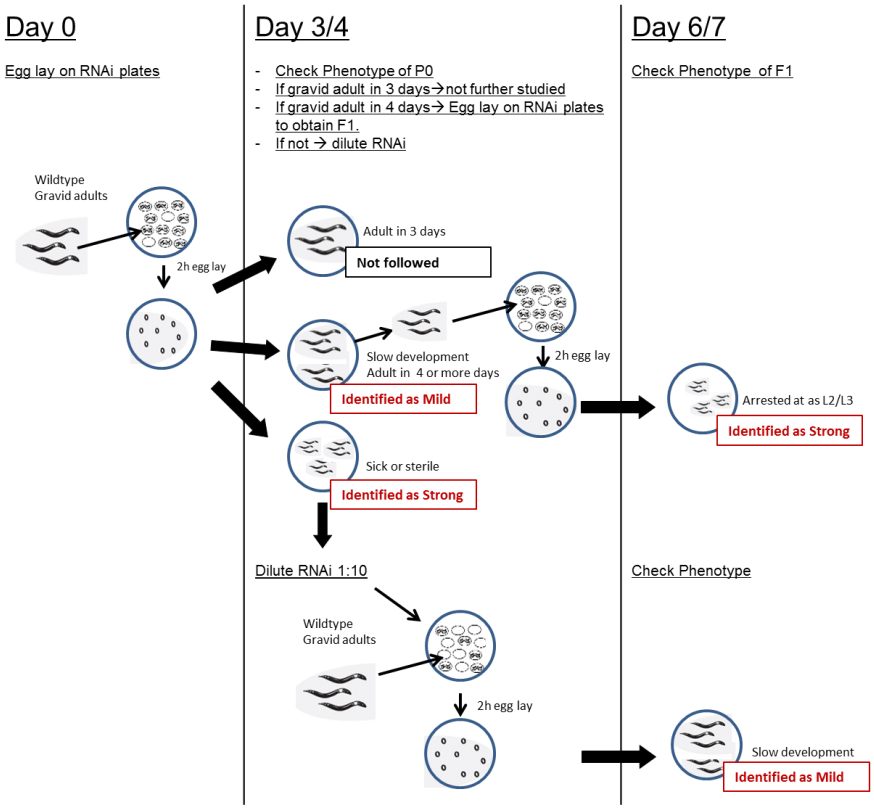


Figure 7. Phenotypic screening flow chart

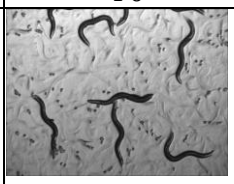
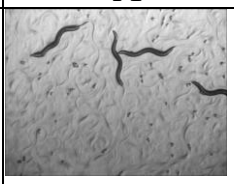
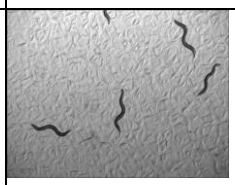
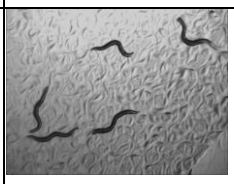
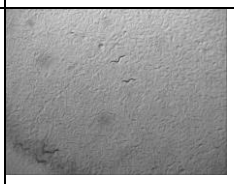
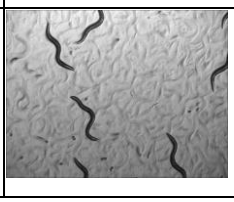
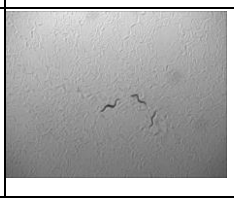
Clone	P0	F1
PL4440		
spg-7		
T20H4.5		
F53F4.10		

Figure 8. Representative pictures of different phenotypes induced by mild or strong RNAi (P0 vs F1 generation) at 2nd day of adulthood. Pictures were acquired with a Leica MZ10 F modular stereo microscope, 32× magnification, connected to a color digital camera 0.5x.

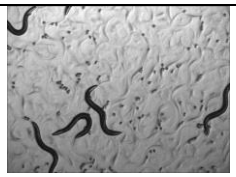
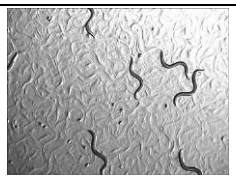
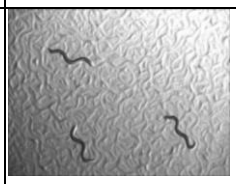
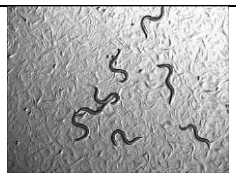
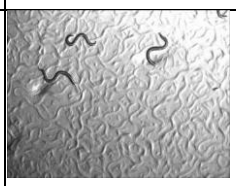
Clone	1:10	undiluted
PL4440		
tag-61		
W02F12.5		
F23B12.5		

Figure 9. Representative pictures of different phenotypes induced by mild or strong RNAi (diluted 1:10 vs undiluted) at 2nd day of adulthood. Pictures were acquired with a Leica MZ10 F modular stereo microscope, 32× magnification, connected to a color digital camera 0.5x.

Table III

cosmid ID	gene	Power of RNAi					
		01:10		P0		F1	
		Lifespan	Phenotype	Lifespan	Phenotype	Lifespan	Phenotype
ZK973.10	*lpd-5*			+	√	+	√
Y47G6A.10	*spg-7*	+	√	+	+	+	√
C01F1.2	*sco-1*			=		++	√
C15F1.7	*sod-1*			-		--	√
D2013.5	*eat-3**			=	√	++	√
C09H10.3	*nuo-1**			+	√	++	
Y46G5A.2	*cox-10*			=		-	
F01F1.12	*aldo-2*			nc			
T20H4.5	NDUFS8 *	-		+	√	+	√
H14A12.2	*fum-1*			--	√	-	√
F54H12.1	*aco-2*			-	√		√
M03C11.5	*ymel-1*			-		-	
K08E3.7	*pdr-1**	1:50 +	√	=	√	-	
T27E9.1	*tag-61**	+	√	+	√		
T12E12.4	*drp-1*			=		=	
T22B11.5	*ogdh-1*			=		+	
F45E4.9	*hmg-5*			nc			
T01B11.4	*tag-316*			+		+	
Y45G12B.1	*nuo-5*			+	√	++	√
F25B4.6	*hmgs-1**	1:50 -	√	-	√	--	
W02F12.5	*dlst-1**			nc			
F53F4.10	NDUFV2			+	√	+	√
F23B12.5	*dlat-1**		√	nc			
F13B9.8	*fis-2*			-		-	
C03G5.1	*sdha-1*			-		-	

Table Legend
*strong phenotype in P0 - lifespans completed on undiluted & 1/10 (some clones also did F1 undiluted).
All the others: mild phenotype on P0, strong on F1 - lifespans completed on P0 & F1 on undiluted dsRNAi expressing bacteria.
Lifespan: compared to PL4440 (Empty vector transformed bacteria) →
+ increase; - decrease; = no changes; √ alteration of the phenotype (see Table I in Appendix B for phenotypes description); nc not consistent result in the different replicates.

4.3.2. HMAD models characterization

4.3.2.1. Differential suppression of several HMAD proteins extends *C. elegans* lifespan

The lifespan of the animals upon RNAi against the genes under study have been investigated. P0, parental generation, was compared to empty vector treated animals. F1, first generation, was compared to P0 generation. When mild treatment was obtained diluting the RNAi 1:10 with empty vector, phenotypes have been compared to empty vector treated animals, animals fed with RNAi undiluted represent the strong treatment, phenotypes were compared to the 1:10 dilution.

Most of the clones analyzed were able to increase the lifespan of wild-type animals already at the mild dose of RNAi. Specifically, we observed a significant extension of lifespan in worms mild interfered with 15 out of 25 genes under exam, among them for example *spg-7*, *tag-316* and *hmg-5* (**Figure 10** and Table II and III in Appendix B). Almost all the genes which when mildly suppressed lead to an increased lifespan (13 out of 15) were also able to produce this effect at a strong dose, and often even to further increase it, as in the case of *nuo-1*, C01F1.2 and F53F4.10 and others (**Figure 11** and Table II and III in Appendix B). Only in one case an extended lifespan was detectable upon the strong treatment but not upon the mild (for W02F12.5).

Lifespan assays have been carried out in triplicate and the pool of the three experiments is summarized in **Table II** and **III** (in Appendix B).

A window of mitochondrial dysfunction where lifespan is increased likely indicates the induction of compensatory pathways that could be exploited to potentially offset disease appearance in patients.

It is important to keep in mind that the animals treated with strong mitochondrial interference arrest, in the most of the cases, at a larval stage. For this reason the increased lifespan of a strong interfered animal has to be considered cautiously, remembering that we cannot really compare a nematode which go through all its natural life stages with one that has an arrested growth (as it happen upon strong mitochondrial suppression). In fact the arrested animals, even if they live more days compared to the mild treated worms, are not healthier, they don't produce any progeny, and therefore we could say that their extended lifespan is from an evolutionary point of view, useless.

Nevertheless, keeping in mind these considerations we wanted to take track also of this aspect of the phenotypic changes as a pure descriptive parameter that we will not use for further investigations. For other parameters that we wanted to characterize in the strong mutants we compared them with the same stage control animal, but, obviously this was not possible for the lifespan analysis.

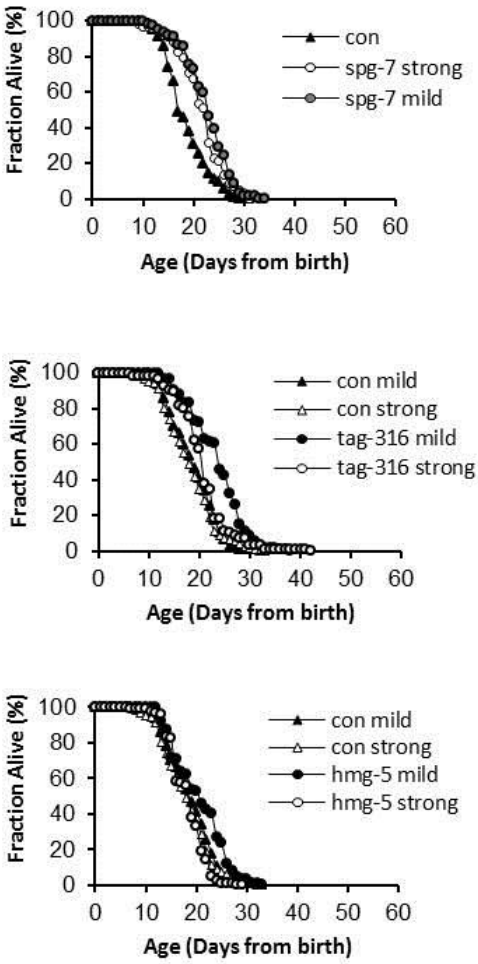


Figure 10. Representative Kaplan-Meier survival analysis of mutants in which a mild suppression of the specified genes lead to lifespan extension. Wild-type N2 worms fed bacteria expressing either empty-vector (con) or *spg-7* dsRNA (*spg-7*) or *tag-316* dsRNA (*tag-316*) or *hmg-5* dsRNA (*hmg-5*). See Table II and III in Appendix B for summary statistics from Kaplan-Meier survival analysis.

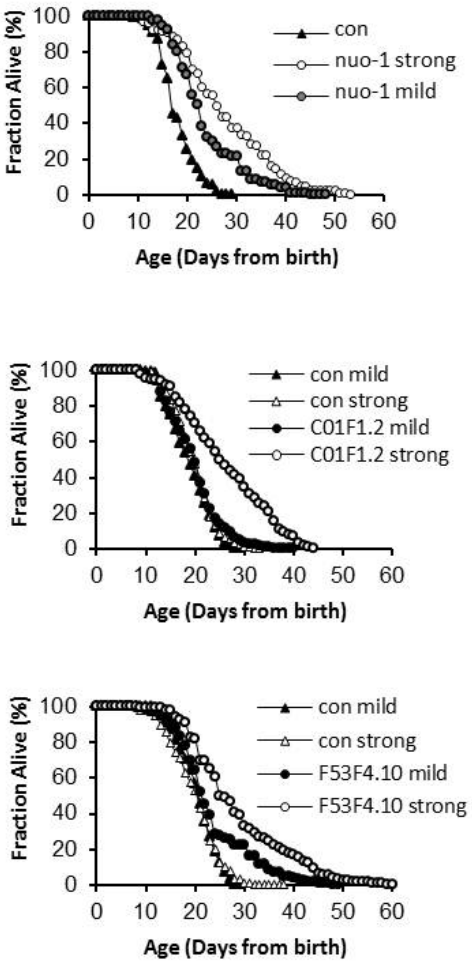


Figure 11. Representative Kaplan-Meier survival analysis of mutants in which a strong suppression of the specified genes lead to a further lifespan extension compared to the mild treatment. Wild-type N2 worms fed bacteria expressing either empty-vector (con) or *nuo-1* dsRNA (*nuo-1*) or C01F1.2 dsRNA (C01F1.2) or F53F4.10 dsRNA (F53F4.10). See Table II and III in Appendix B for summary statistics from Kaplan-Meier survival analysis.

4.3.2.2 Differential suppression of several HMAD proteins results in alteration of sensory functionality in *C. elegans*

The mutants analyzed, that gave in the phenotypic screening the specific mild and strong phenotypes have been further investigated. Specifically, we looked at their neurosensory functions using a chemotaxis assay. We used different attractants to verify the nature of a neuronal damage. In fact, it has been shown that different substances that are sensed in *C. elegans* by distinct class of sub-neurons.

From data previously collected in our laboratory (see chapter 5) we observed in animals with mild mitochondrial stress a slight impairment in their chemosensory abilities. In Table IV are summarized the changes in sensory abilities of adult worms upon mild mitochondrial suppression, compared with worms treated with the control empty vector transformed bacteria. These mutants all show a slight impairment in their ability to reach the attractant (**Figure 16A-D, 17A-B, Table IV and Appendix A, Figure S6A**).

Here we wanted to check the sensory neuron functionality upon strong mitochondrial suppression. We treated the animals with strong dose of RNA interference against the mitochondrial proteins under study and we found a very severe impairment in their sensory perception (**Table V**, red rows).

Since these mutants arrest their development at L2/L3 larval stage we repeated the same test in the mild interfered animals but instead of testing the adult worms we looked at them as larvae, to be able to make significant comparisons between the two treatments. At this stage, surprisingly, we actually observed a better performance of the mutants in the test, compared to the animals fed with the empty vector transformed bacteria. In detail, we tested mutants with mild suppression of *spg-7*, *nuo-2*, *nuo-5*, *F53F4.10* and *lpd-5* and we record a better general (not specific for any chemicals used) neurosensory functionality in these animals compared with control worms (**Table V**, blue rows) (Chemotaxis Index curves **Figure 12**). These findings are quite unexpected but may fit with our hypothesis of a hormetic response due to the mitochondrial deficit that sees as a central player the nervous system. It seems indeed that in the very young mild mitochondrial mutants there is the activation of a

positive response at the neuronal level that make them more efficient in the chemotaxis assay. This neuronal signal is then possibly activating pathways in other tissues and it is not promoting the sensory functionality itself at the middle stage of the worms lifespan, more likely a slightly decrease.

Table IV

		Stage : gravid adult				
RNAi clone	**RNAi power**	**NH_4Ac 2,5M (ASE)**	**2-nona none (AWB)**	**Pyrazine (AWA)**	**Benzal-dehyd (AWC)**	**Butanol (AWC)**
spg-7	**mild**	↓		↓		
nuo-2	**mild**	↓	↓	↓	↓	
atp-3	**mild**	↓	↓	↓	↓	
isp-1	**mild**	↓	↓	≈	↓	
nuo-5	**mild**	↓	↓	↓	↓	↓
F53F4.10	**mild**	↓		≈	↓	↓
cco-1	**mild**	↓	↑	≈	↓	
nuo-1	**mild**	↓		≈	↓	
T20H4.5	**mild**	↓		≈	↓	
lpd-5	**mild**	↓	↓	↓	↓	≈
fum-1	**mild**					≈
sdha-1	**mild**					↓

Table Legend
↑ Increased Chemotaxis index ; ↓ decreased Chemotaxis index or ≈ no changes in the Chemotaxis index, compared to N2 worms fed bacteria expressing the empty-vector (PL4440)

Table V

		Stage: L3 Larvae			
RNAi clone	**RNAi power**	**NH_4Ac 2,5M (ASE)**	**Pyrazine (AWA)**	**Benzaldehyd (AWC)**	**Butanol (AWC)**
spg-7	**mild**	↑	↑	↑	
	strong	↓	↓	↓	↓
nuo-2	**mild**		↑	↑	
	strong	↓	↓	↓	↓
nuo-5	**mild**		↑		
	strong	↓	↓	↓	↓
F53F4.10	**mild**		↑		
	strong	↓	≈	↓	↓
nuo-1	**mild**				
	strong	↓			
lpd-5	**mild**		↑	↑	
	strong	↓	↓	≈	↓
fum-1	**mild**				
	strong				↓
sdha-1	**mild**				
	strong				↑

Table Legend
↑ Increased Chemotaxis index ; ↓ decreased Chemotaxis index or ≈ no changes in the Chemotaxis index, compared to N2 worms fed bacteria expressing the empty-vector (PL4440)

But the activation of this hormetic response lead the animals to age slower, reason why when we compared old worms treated with mild mitochondrial interference and control ones we observed a better performance of the chemosensory abilities (**Figure 18**).

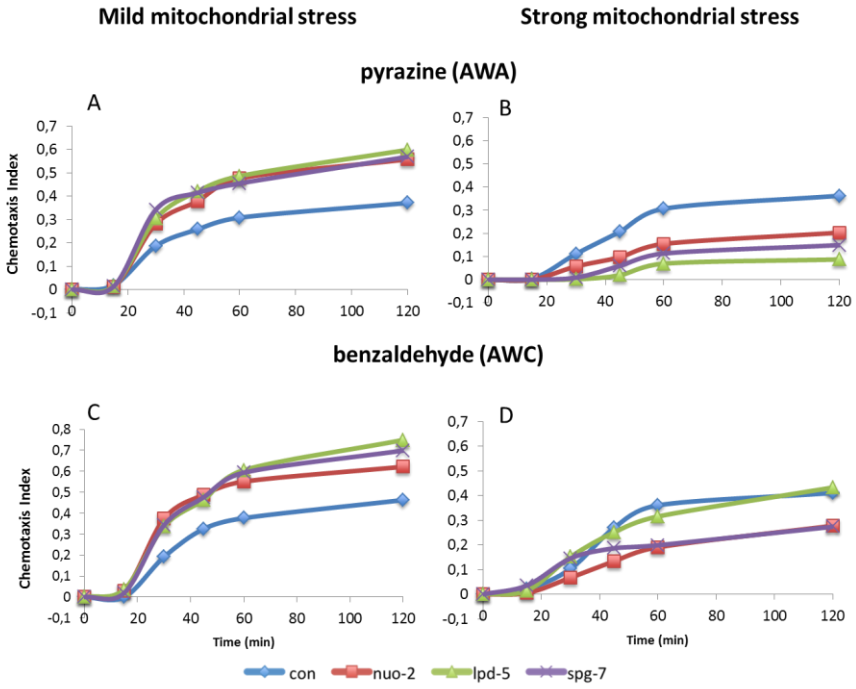


Figure 12. Mild and severe mitochondrial stress have opposite effect on chemosensory neurons functionality. Chemotaxis index curves with raw, not normalized values from wild-type (N2) animals fed bacteria transformed with the empty vector (con) or the indicated RNAi interference. Chemotaxis Index towards Pyrazine **A,B)** or Benzaldehyde **C,D**) is measured in L3 larvae. For mild treatment the parental generation (P0) is tested, for strong treatment test are carried out on the filial generation (F1).

4.3.2.3 Differential suppression of several HMAD proteins does not strongly impair locomotors ability in *C. elegans*

To further characterize our models we then tested whether reduced ability of animals to reach the attractants was specifically ascribed to sensory rather than motor-neurons activity defects, we monitored animals' ability to move by counting the number of body bends per minute. Worms were observed as L3 larvae; given the fact that some of the RNAi tested arrest the development we decided to carry out all the tests at this stage to be able to compare mild and strong doses of mitochondrial stress. Using a mild RNAi we didn't observe significant difference in locomotion ability in the treated animals, compared to the control ones (**Figure 13A**).

Strong RNAi treatment against some of the tested mitochondrial proteins reduced animal speed compared to wild-type animals (e.g spg-7) but in general the locomotor function was not affected as much as the chemosensory ability (**Figure 13B**).

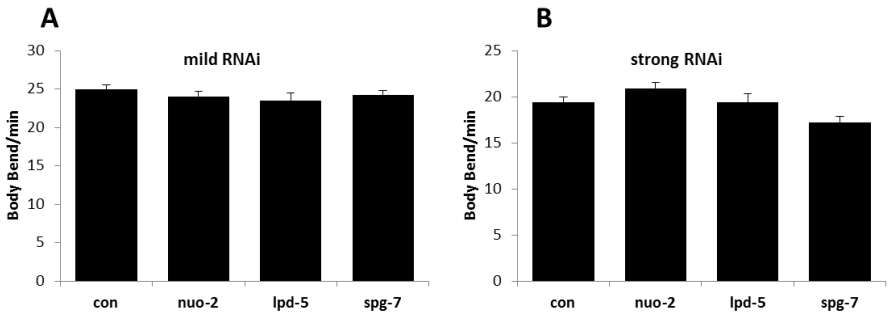


Figure 13. Representative graphs of locomotion assays carried out for some of the genes of interest. Locomotion activity (body bends per minute) in L3 larval animals fed bacteria expressing empty-vector (con) or the indicated dsRNA. A) mild RNAi and B) strong RNAi. Bars and errors indicate mean and SEM, unless otherwise indicated *p < 0.05 vs control, t-test. Body bends from 20 animals from one representative experiment out of three.

4.4 Discussion

In this study we developed and characterized new models of HMADs in *C. elegans.* Besides Friedrich's Ataxia, in fact, this class of deleterious disease did not found yet many models of study in the nematode. Even if a few models of Parkinson disease (PD) have been recently developed to study the relationship between the neurodegeneration of dopaminergic system and the dysfunction of mitochondria in the worms (Zhou et al., 2013), and more and more *C. elegans* has been used to model other neurodegenerative pathologies (Wolozin et al., 2011), models of pure HMADs are almost absent. Therefore, in this work we systematically described phenotypical, and behavioral changes in our model organism upon mild and severe suppression of several mitochondrial genes.

We identify for all the genes under study a dose-dependent response to the gene silencing generating two well distinct answers, one triggered by a moderate functional deficit in mitochondrial proteins and another, opposite one, generated by strong mitochondrial proteins deficiency. This is consistent with the hormetic idea that in Mit mutants there is the induction of compensatory protective pathways produced to cope with a mild mitochondrial stress.

Although further work is clearly required to elucidate the molecular mechanisms of this protective signal, the aim of this work was limited to the development of the new HMAD models and to the characterization of the altered features. This will make allow us to use the new models in a drug screening, with the final purpose to identify potential new therapeutic interventions.

Nevertheless in our characterization we found that i) differential suppression of several HMAD proteins differently affect animals development, fertility and extends *C. elegans* lifespan, ii) does not strongly impair locomotors ability but iii) results in differential alteration of sensory functionality. Interestingly, we confirmed the important role that the nervous system plays in the response to mild mitochondrial stress as we already previously pointed out (Maglioni et al, 2014). Indeed, here we were actually able show that in the early stages of life the nervous system is already responding to the mitochondrial dysfunction, since we

detected a better chemosensation in L3 larvae treated with the RNAi against mitochondrial genes compared to control animals at the same larval stage. It seems indeed that in the early developmental stages of mild mitochondrial mutants the nervous system is actively inducing some kind of protective response that makes the animal more efficient in the chemotaxis assay. This neuronal signal is possibly activating pathways in other tissues and it is not promoting the sensory functionality itself at the middle stage of the worms' lifespan but acting still to prolong the lifetime. We in fact already showed that these mutants live longer and their chemosensory abilities age slower. Recently, a couple of works nicely demonstrated that the nervous system plays a peculiar role in extending Mit mutants lifespan (Durieux et al., 2011; Walter et al., 2011) and our research aim to better understand which are those signals that likely act in a selected subset of cells to integrate cues from dysfunctional mitochondria and transfer to the whole animal to modulate longevity in a non-cell autonomous manner.

With this work we systematically characterized two response to the same stressor, precisely mitochondrial stress. On the one hand we found that all mitochondrial genes under study trigger a common response in *C. elegans*, such as arrest of development or reduction in body size. On the other hand we also observed different responses to the silencing of different mitochondrial proteins: for example the extent of the sensory deficit towards the different chemicals. Therefore if it is important to have common phenotypic readouts to use our models for suppressor drug screenings, it is also essential to differentiate between genes relevant for specific human mitochondrial diseases. Indeed, we have to keep in mind that we are talking about a heterogeneous group of disorders that can affect multiple organs with different severity. Our findings will therefore improve our understanding of the pathogenesis and treatment of mitochondria-associated neurodegenerative disorders **(Figure 14)**.

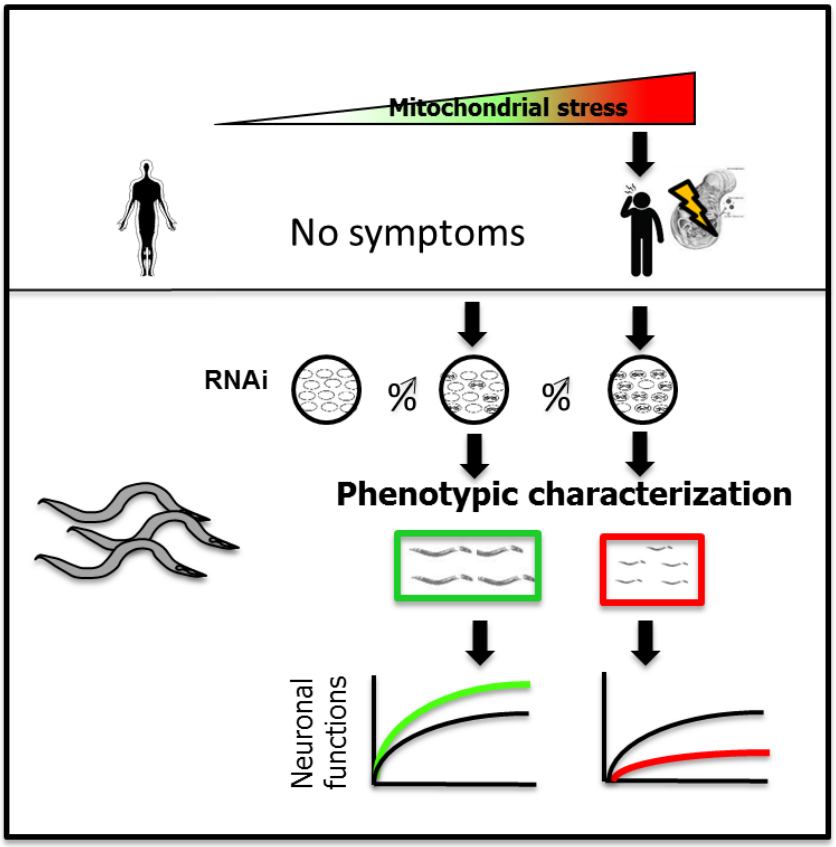


Figure 14. In humans mild mitochondrial dysfunction is asymptomatic while deleterious symptoms appear when mitochondria are severely compromised. Also in the model organism *C. elegans* a strong impairment of the mitochondrial functionality leads to detrimental effects, but on the contrary, a mild mitochondrial dysfunction leads instead to a very specific phenotype, associated with the activation of protective pathways. The characterization of the bimodal response triggered by mild or strong suppression of mitochondrial genes was the first purpose of this PhD thesis. The identification of two well distinct phenotypes permit to identify interventions able to ii) rescue the arrest of development due to strong mitochondrial dysfunction (often associated with shortening of lifespan); and iii) trigger the beneficial responses (associated with lifespan extension). The drugs identified using this screening represent therefore i) potential HMADs treatments, and ii) anti-aging drugs.

5 Mitochondrial stress extends lifespan in *C. elegans* through neuronal hormesis

5.1 Abstract

Progressive neuronal deterioration accompanied by sensory function decline is typically observed during aging. On the other hand, structural or functional alterations of specific sensory neurons extend lifespan in the nematode *Caenorhabditis elegans*. Hormesis is a phenomenon by which the body benefits from moderate stress of various kinds which at high doses are harmful. Several studies indicate that different stressors can hormetically extend lifespan in *C. elegans* and suggest that hormetic effects could be exploited as a strategy to slowdown aging and the development of age-associated (neuronal) diseases in humans. Mitochondria play a central role in the aging process and hormetic-like bimodal dose–response effects on *C. elegans* lifespan have been observed following different levels of mitochondrial stress. Here we tested the hypothesis that mitochondrial stress may hormetically extend *C. elegans* lifespan through subtle neuronal alterations. In support of our hypothesis we find that life-lengthening dose of mitochondrial stress reduces the functionality of a subset of ciliated sensory neurons in young animals. Notably, the same pro-longevity mitochondrial treatments rescue the sensory deficits in old animals. We also show that mitochondrial stress extends *C. elegans* lifespan acting in part through genes required for the functionality of those neurons. To our knowledge this is the first study describing a direct causal connection between sensory neuron dysfunction and extended longevity following mitochondrial stress. Our work supports the potential anti-aging effect of neuronal hormesis and open interesting possibility for the development of therapeutic strategy for age associated neurodegenerative disorders. (Maglioni et al., 2014)

5.2 Introduction

Neuronal deterioration is a well-established age-associated trait as exemplified in humans by the progressive decline in cognitive functions and the increased risk of developing neurodegenerative disorders (Yankner et al., 2008). Moreover, although often undervalued, it is common knowledge that a gradual loss in the functionality of all five senses typically accompanies the aging process. Sensory decline indeed appears early in life, can lead to social isolation, depression, disability and can represent symptoms or be predictive of age-associated diseases, thus representing an important social and economic burden for the rapidly expanding elderly population of our society (Lang et al., 2009; Nusbaum, 1999; Schumm et al., 2009).

The nematode *Caenorhabditis elegans* is an elective model organism for aging studies (Johnson, 2002; Torgovnick et al., 2013). Most genetic factors and external interventions which affect the aging process have been indeed at first identified in *C. elegans.* Moreover, many age-associated features observed in mammals are recapitulated in this model organism: cellular components progressively deteriorate, different physiological functions decline (i.e. pharyngeal pumping, locomotion, chemotaxis, learning behaviors, resistance to stress and infections) and mortality rate increase over time (Cai and Sesti, 2009; Collins et al., 2008; Glenn et al., 2004; Herndon et al., 2002; Kauffman et al., 2010; Murakami et al., 2005; Youngman et al., 2011). Although initial work surprisingly did not reveal any structural sign of neuronal aging in *C. elegans* (Herndon et al., 2002), recent studies described the accumulation of different neuronal aberrations, such as neurite branching, synapses deterioration, and formation of axonal beads and bubble-like processes mainly in touch sensory and motor neurons (Pan et al., 2011; Tank et al., 2011; Toth et al., 2012). Intracellular modification such as oxidation of voltage-gated K+ channels, altered synaptic vesicle formation and abnormal mitochondrial localization where also associated with (Liu et al., 2013; Toth et al., 2012), or causally involved to (Cai and Sesti, 2009), neuronal aging; and a couple of studies clearly demonstrated that motor neuron dysfunction precedes muscle deterioration (Glenn et al., 2004; Liu et al., 2013). On the other hand, besides the observed decline in attraction

to bacteria (Hosono, 1978), biotin (Cai and Sesti, 2009) and diacetyl (Glenn et al., 2004), age-associated changes in sensory functions have been so far largely overlooked.

In human, mitochondria play a central role in the regulation of the aging process and in the development of neurodegenerative disorders (Reddy and Reddy, 2011; Troulinaki and Bano, 2012; Yin et al., 2014). Similarly, in the nematode *C. elegans* severe reduction in the expression of mitochondria respiratory chain (MRC) regulatory genes leads to pathological phenotypes such as arrested development or curtailed lifespan (Rea et al., 2007; Ventura and Rea, 2007). On the other hand, mild mitochondrial disturbance can have pro-longevity effects from yeast to mice (Copeland et al., 2009; Dell'agnello et al., 2007; Dillin et al., 2002; Lee et al., 2003b). Also in *C. elegans* RNAi- or genetic-mediated depletion of different MRC regulatory genes has pro-longevity effects. This class of mutants, the so called Mit mutants (Rea, 2005; Ventura and Rea, 2007) live longer and have increased resistance to different types of stressors (Butler et al., 2010; Lee et al., 2003a; Ventura et al., 2005). These findings are consistent with the concept of hormesis, a phenomenon by which an organism benefits from moderate stress of various kinds which at high doses are harmful, and which can in turn increase resistance to the same or other types of stressors (Cornelius et al., 2013; Ristow and Zarse, 2010; Tapia, 2006; Ventura et al., 2006).

Several studies indicate that different stressors hormetically extend lifespan in *C. elegans* (Cypser and Johnson, 2002; Pietsch et al., 2011; Schmeisser et al., 2013b), and suggest that hormetic effects could be exploited to prevent the onset of various diseases (Calabrese, 2008, 2013; Calabrese et al., 2013), including neurodegenerative disorders, and to slow down the aging process (Marini et al., 2008; Matus et al., 2012). The sensory nervous system integrates environmental cues and endogenous signaling in turn affecting the aging process (Alcedo et al., 2013; Linford et al., 2011) and interestingly, non-lethal ablation, genetic or pharmacological manipulation of specific sensory neurons extend *C. elegans* lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Evason et al., 2005; Lans and Jansen, 2007; Maier et al., 2010; Petrascheck et al., 2007). Tissue-specific knock down and rescuing experiments clearly indicate that the nervous system is of particular relevance to extend Mit mutants lifespan (Durieux et al., 2011; Walter et

al., 2011). However, whether mitochondrial stress extends lifespan through alteration of specific neuronal subtypes or pathways is unknown and it was for the first time addressed in this work. We found that silencing of different nuclear encoded mitochondrial protein, some of which linked to neurodegenerative diseases in human, is indeed associated with alteration of ciliated neurons early in life. On the other hand, the chemosensory deficit of these long-lived animals is significantly rescued in old animals and they age healthier compared to wild-type animals. Notably, we showed that similar to ciliated chemosensory mutants, neuronal specific genes are causally involved in longevity extension following mitochondrial stress.

5.3 Results

5.3.1. Sensory functions decline faster than locomotion during *C. elegans* aging

With the aim of characterizing different aspects of *C. elegans* aging related to neuronal dysfunction, we compared the decline in animal locomotion activity and chemosensory functions at different age. The locomotion activity of wild type animals fed HT115 (DE3) bacteria containing empty vector (pL4440) decline very slowly: 7-day-old worm move marginally slower (20% reduction) and 10-days-old slightly slower (40% reduction) compared to 3 days old worms (**Figure 15A**). A severe locomotion impairment is only evident very late in life, starting from the 14th day (75% reduction in speed, **Figure S1C**) likely after motor neurons are severely dysfunctional (Liu et al., 2013). Of note the fraction of animals that move spontaneously or upon touching declines even slower than the speed (29% vs 75% reduction respectively in 14-days-old animals, **Figure S1A-C**).

We then tested the animals' chemosensory functions (olfaction and taste) by measuring their attraction to volatile and water-soluble chemicals which are sensed by different subtypes of chemosensory neurons (Ward, 1973). The volatile compounds pyrazine is specifically detected by AWA neurons while the water-soluble chemical ammonium

acetate (NH_4Ac) is sensed by ASE neurons. These are two pairs of amphid ciliated sensory neurons whose cell bodies are located in the nerve ring in the head of the animals. Like all other amphid neurons these cells are in strict contact with the external environment. Specifically, AWA have branched, flattened cilia located near the amphid pore, but enclosed by a support cell called the amphid sheath cell ("wing" cells). The two long thin cilia of the ASE neurons are instead directly exposed to the environment through the amphid pore ("exposed" cells). 7-days-old wild type worms exposed to either one of the two chemicals already showed a strong impairment of their sensory functions (more than 40% of reduction) and their attraction for the two substances was almost absent after 10 days (more than 85% reduction) (**Figure 15B-C**). Similar results were observed for other chemicals, such as the two volatile compounds 2-nonanone and benzaldehyde, which are sensed respectively by AWB and AWC neurons (**Figure S2A-B**). These neurons, the wing neurons, together with the AWA pair of neurons and their cilia terminate within a sheath cell, thus they are not exposed to the external environment. Strains carrying mutations in genes which regulate ciliated sensory neuron activity were used as control for our chemotaxis assays (**Figure S3A-E** *and data not shown*). Specifically *che-3* encodes a dynein heavy chain required for the structural integrity of sensory cilia and *che-3(e1124)* mutants have a severe structural defect to the cilia of all sensory neurons which dramatically impair animal sensitivity to many different substances (Dusenbery et al., 1975); *osm-3* instead encodes a kinesin motor proteins required for the formation of amphid neurons' cilia and *osm-3(p082*) mutants are consequently insensitive to soluble substances (Perkins et al., 1986); *tax-4*, an ortholog of the human gene coding for a channel-gate of the cGMP family, is expressed in different sensory neurons such as thermosensory, gustatory, and olfactory neurons and the mutant strain *tax-4*(*p678*) is defective in sensing all the substances perceived through the cationic TAX-2/TAX-4 channels, with the exception of 2-methyl pyrazine, which follows a different signaling pathway (Komatsu et al., 1996).

Our data clearly indicate that similar to mammals, deficits in sensory functions appear early in life and sensory neuron functionality declines faster than locomotion during animal aging.

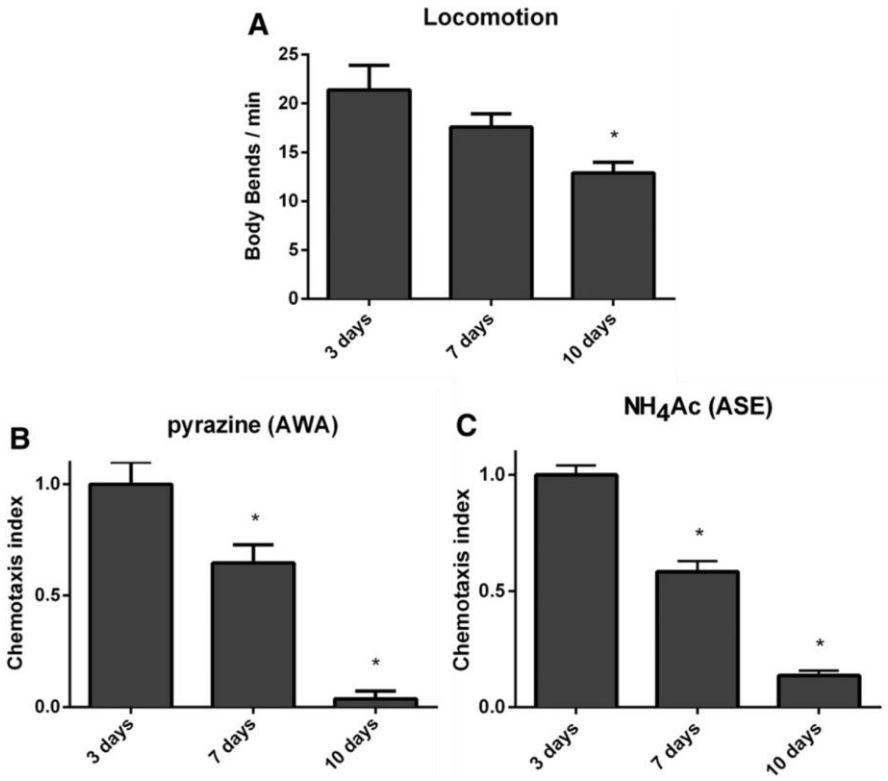


Figure 15. **Locomotion and chemosensory functions decline during aging.** A) Locomotion activity (body bends per minute) in wild-type (3, 7 and 10-days-old adults) animals fed with OP50 bacteria. Bars and errors indicate mean and SEM of body bends from 20 animals from one representative experiment out of three. B-C) Chemotaxis Index or animal ability to be attracted by pyrazine or ammonium acetate was quantified after 2 hours from the beginning of the assay in animals treated as in (A). Chemotaxis Index is normalized to the value reached at the end of the assay (2h) in 3 days-old animals. Bars and errors indicate mean and SEM from three independent replicates carried out with 80-100 animals each. See Supplementary Figure 3 (in Appendix A) for representative Chemotaxis Index curves with raw, not normalized values. All test were carried out on plates without bacteria.

These observations have different implications: first they imply that initial, subtle functional or structural modification must occur in sensory

neurons in turn leading to the observed behavioral defects in environmental perception and possibly in locomotion. Second, they suggest that sensory function alterations could represent accurate and earlier indicators of life expectancy. And finally, they support the notion that *C. elegans* could be an ideal genetic model organism to investigate molecular mechanisms underlying these evolutionarily conserved age-associated changes.

5.3.2. Mitochondrial stress reduces ciliated sensory neurons functionality in young animals

Tissue-specific knock down and rescuing experiments indicate that reducing mitochondrial function only in the nervous system is sufficient to extend Mit mutants lifespan (Durieux et al., 2011; Walter et al., 2011). However, how Mit mutant' neuronal function is affected during aging and whether mitochondrial stress extends lifespan through alteration of specific neuronal subtypes or pathways has not been yet characterized. We therefore carried out behavioral assays to estimate the functionality of different neurons upon feeding bacteria transformed with dsRNA against each of many MRC subunits which we know to extend lifespan in *C. elegans* (Rea et al., 2007; Ventura and Rea, 2007). As revealed by the reduced chemotaxis to different chemicals, RNAi-mediated depletion of subunits of Complex I, III and IV affected chemosensory neuron functionality already in 3-days-old young animals: a variably reduced attraction to the four compounds was observed in different Mit mutants (**Figure 16A-D**). The chemosensory defect was more evident for NH_4Ac and benzaldehyde, sensed by ASE and AWC neurons respectively, than for pyrazine or 2-nonanone, sensed by AWA and AWB respectively. Interestingly, ablation, genetic or pharmacological manipulations of some of the affected neurons (AWA and AWC) extend *C. elegans* lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Evason et al., 2005).

Gene silencing through feeding is not very effective in the *C. elegans* nervous system (Asikainen et al., 2005; Kamath et al., 2001). The plasticity in neuronal response in the different RNAi-mediated Mit mutants may therefore reflect either a variable ability of the different dsRNA to reduce gene expression in different neurons or a neuronal selective sensitivity to mitochondrial disturbance, or a combination of both. A reduced attraction to diacetyl, which is sensed by AWA and AWC neurons, has been observed for three genetic derived Mit mutants, *clk-1(qm30), clk-1(e2519), isp-1(qm150)* (Matsuura et al., 2009). Compared to stage-matched wild-type animals (first day fertile adults), the attraction of the genetic-derived Mit mutant *isp-1(qm150);ctb-1(qm189)* (Feng et al., 2001) to different chemicals was significantly and similarly reduced (**Figure 16E; Figure S4**), indicating that the difference in neuronal deficits observed in Figure 2A-D may be due to a variable RNAi efficacy in the different neurons. However, part of these differences are also likely ascribed to the variable sensitivity of the chemosensory assays to the different chemicals and concentrations (Bargmann et al., 1993; Bargmann and Horvitz, 1991; Ortiz et al., 2009) as clearly revealed by comparing the attraction of genetic and RNAi-mediated Mit mutants to butanol and benzaldheide, which are both sensed by the AWC neurons (**Figure 16F**): although in most cases the different Mit mutants display similar reduced attraction to the two substances, this is not always the case (*lpd-5* and *T20H4.5*), indicating that these assays do not permit comparative quantitative analysis between the levels of damage of each tested neuron but rather just indicate the presence of a deficit (unless the differences observed in *lpd-5* and *T20H4.5* underlie a specific gene-substance interaction).

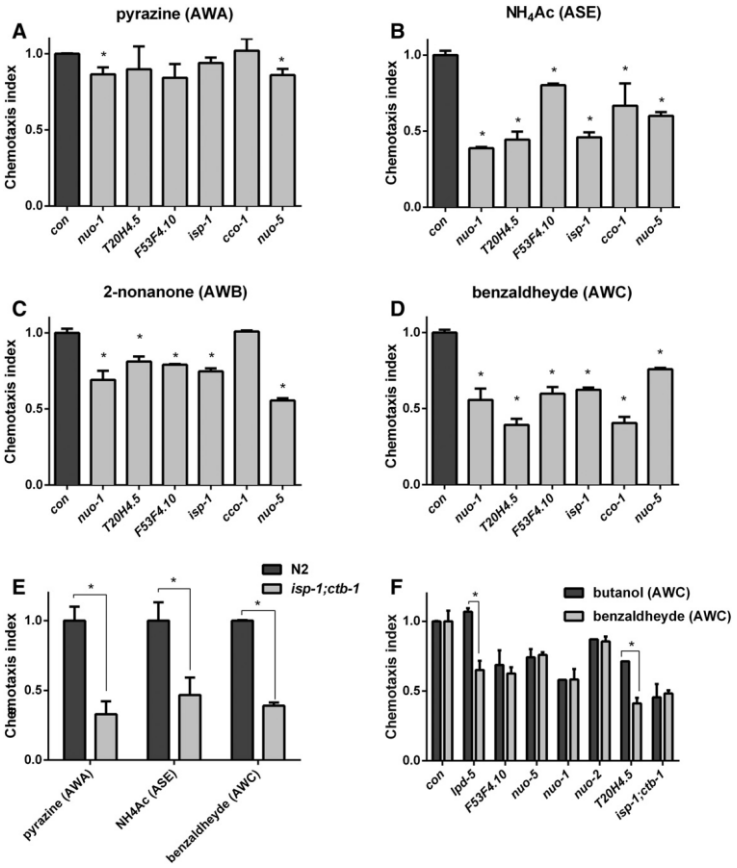


Figure 16. Mitochondrial stress reduces chemosensory neurons functionality in young animals. The functionality of specific chemosensory neurons was assessed in wild-type 3-days-old adults fed bacteria expressing empty-vector (con) or the indicated dsRNA, by calculating the Chemotaxis Index for pyrazine (**A)** ammonium acetate (**B)** 2-nonanone (**C)** or benzaldheyde (**D)** after 2 hours from the beginning of the assay. **E)** Chemotaxis Index for for pyrazine, ammonium acetate and benzaldheyde in stage-matched one-day gravid adults *isp-1;ctb-1* mutants compare to wild-type animals. Supplementary Figure S4A-D for Chemotaxis Index curves with raw, not normalized values. **F)** Comparison of the Chemotaxis Index for butanol and benzaldheyde in the indicated RNAi- or genetic-derived Mit mutants. Chemotaxis Index is normalized to 3 days-old wild-type control treated animals. Bars and errors indicate mean and SEM from two (E-F) or three (A-D) independent replicates. *$p < 0.05$ vs control, t-test.

Our results suggest that reducing mitochondrial function could extend lifespan in a hormetic-like fashion via alteration of specific subset of neurons (in a cell-autonomous or non –autonomous manner). Different studies indeed propose that hormetic effects could be exploited to prevent the onset of various diseases (Calabrese, 2008, 2013; Calabrese et al., 2013), including neurodegenerative disorders, and to slow down the aging process (Marini et al., 2008; Matus et al., 2012), and hypoxia preconditioning, a specific form of hormesis, protects mammalian and *C. elegans* neurons from severe hypoxia (Dasgupta et al., 2007; Sharp et al., 2004). To begin addressing this possibility we assessed the neuronal functionality upon silencing of three nuclear-encoded mitochondrial proteins whose mild suppression extends *C. elegans* lifespan while when severely defective lead to neurodegenerative disorders in humans and to pathological phenotypes in *C. elegans* (Chen et al., 2007a; Ventura and Rea, 2007; Ventura et al., 2006): *frh-1, nuo-2 and spg-7,* the *C. elegans* orthologs of human frataxin, NDUFS3 and paraplegin, respectively.

Severe frataxin deficiency in human leads to Friedreichs' ataxia, the most common inherited recessive ataxia; NDUFS3 disruption leads to a fatal degenerative disorder, the Leigh Syndrome, and mutations in paraplegin cause a form of spastic paraplegia (Wallace, 2005). As observed in the other tested RNAi-mediated Mit mutants (and in part showed for *frh-1* (Schiavi et al., 2013)), these three mutants also show a mild and variable decrease in their attraction for the same compounds compared to wild-type animals. Specifically RNAi against *spg-7* and *nuo-2* lead to a reduced functionality of AWA neurons (reduced attraction to pyrazine), with *frh-1* RNAi showing a slightly minor, although still significant, deficit compared to the other two mutants (**Figure 17A**). Instead ASE neurons (chemotaxis to NH4Ac) were only affected upon *frh-1* and *spg-7* RNAi (**Figure 17B**). The structure of some of the affected neurons (AWB and ASE) was instead not affected in *frh-1*(RNAi) or *nuo-2*(RNAi) 3-days-old animals (**Figure S5**). Further studies are required to assess the structure of additional neurons and the cell-autonomous or non-autonomous nature of the neuronal dysfunctions.

To then test whether reduced ability of animals to reach the attractants was specifically ascribed to sensory rather than motor neurons activity defects, we monitored animals' ability to move by counting the number of body bends per minute. RNAi-treatment against some of the tested

mitochondrial proteins reduced animal speed compared to wild-type animals but in general this was not significantly affected (**Figure 17C**). RNAi against *nuo-1* and *nuo-5* more dramatically reduced animals' speed and concurrently reduced chemotaxis to all tested chemicals (**Figure 16**). This indicates that *nuo-1* and *nuo-5* dsRNA have increased silencing activity and/or that movement defects contribute to the animal inability to reach move towards an attractant. However, the altered attraction to the different chemicals did not result from altered ability to move. Indeed, the two features do not always correlate, as clearly exemplified by the fact that *frh-1*, *nuo-2* and *spg-7* RNAi do not affect locomotion in 3-days-old animals while reducing animals' chemotaxis (**Figure 17A-C**). Consistent with this idea, the slope of the chemotaxis curves between control and RNAi-treated animals was almost identical (**Figure S6A**): all animals indeed reached their maximum chemotaxis index within one hour - although with slightly, non-significant difference in animal speed as reflected by the different time animals took to reach their plateau from the beginning of the test. Yet, after this point, the chemotaxis index (even when monitored up to 8 hours - *not shown*), did not further increase, indicating that the altered attraction to the different chemicals was not a result of their reduced ability to move but rather a specific sensory neuron defect. This is further supported by the fact that while the fraction of animals spontaneously moving after 3 days is not affected in long-lived *nuo-2* (RNAi) animals, the population of animals with an intact chemotaxis index is already reduced compare to control animals (**Figure S7A-C – 3 days)**. Our data suggest that the pro-longevity effects elicited in response to mild mitochondrial stress could be achieved in a hormetic-like manner by functional alterations of specific subset of neurons.

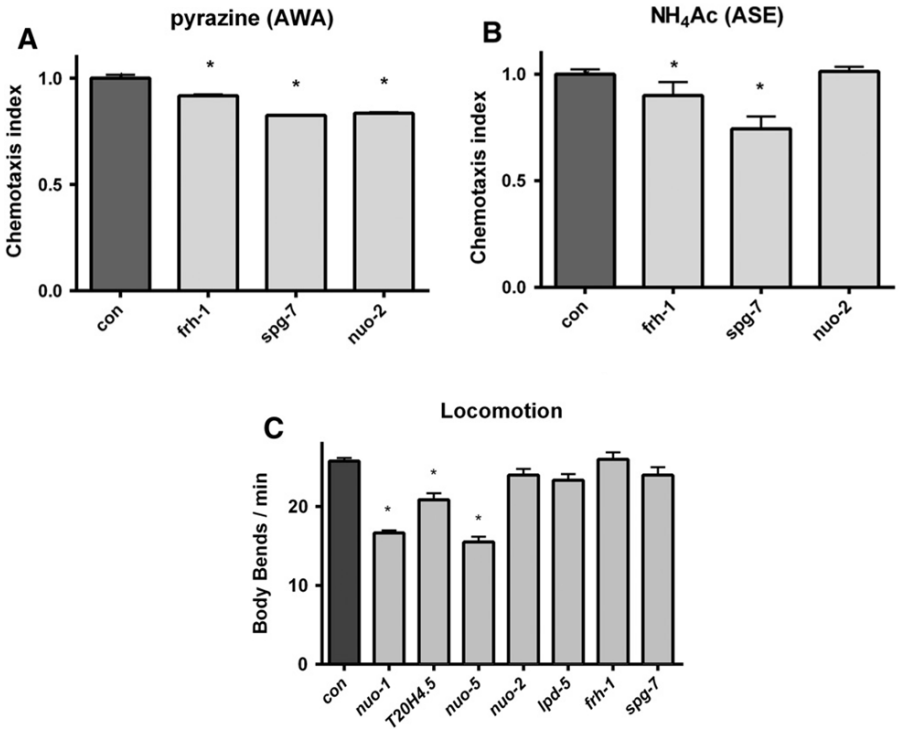


Figure 17. Mitochondrial stress affects chemosensory neurons in young animals more than locomotion. Chemotaxis Index for pyrazine (**A)** and ammonium acetate **(B)** was assessed in wild-type 3-days-old adults fed bacteria expressing empty-vector (con) or the indicated dsRNA after 2 hours from the beginning of the assay. Chemotaxis Index is normalized to 3 days-old animals. Bars and errors indicate mean and SEM from three independent replicates. **C)** Locomotion activity (body bends per minute) in wild-type 3-days-old adults fed bacteria expressing empty-vector (con) or the indicated dsRNA. Bars and errors indicate mean and SEM, unless otherwise indicated *$p < 0.05$ vs control, t-test. Body bends from 20 animals from one representative experiment out of three.

5.3.3. Mitochondrial stress rescue ciliated sensory neurons dysfunction in old animals

To assess whether reducing mitochondrial function indeed induced a hormetic, protective neuronal effect later in life, we quantified animals' neuronal functionality during aging in the three long-lived Mit mutants related to human diseases. Compared to control animals, the chemotaxis for pyrazine and ammonium acetate was further reduced in 7-days-old animals treated with RNAi against *frh-1, nuo-2* or *spg-7* (**Figure 18A-B; Figure S6B**), with the same trend of impairment observed in 3-days-old animals. However, the sensory ability of 14-days-old animals treated with the three RNAi was significantly improved compared to control animals of the same age (**Figure 18A-B; Figure S6C**). The chemosensory function linearly declined in control and in *frh-1*(RNAi) animals, although much slowly in the latter, thus ultimately resulting in improved ability to reach the attractant in *frh-1*(RNAi) 14-days-old animals compared to control. Even more strikingly, silencing of *nuo-2* and *spg-7* not only prevented chemosensory decline in 14-days-old animals compared to age-matched controls, but also improved neuronal functionality compared to younger, 7-days-old animals.

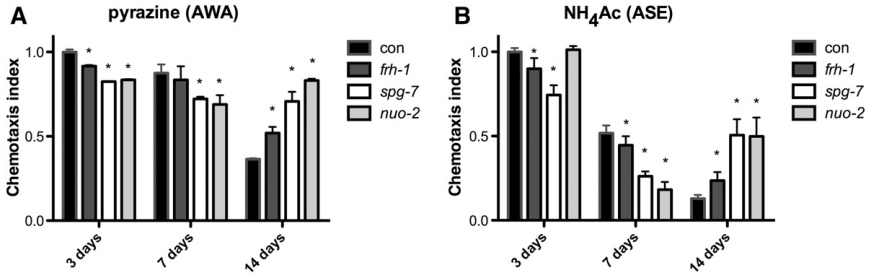


Figure 18. Mitochondrial stress rescues sensory neurons dysfunction in old animals. Chemotaxis index in 3- 7- and l4-days old animals fed bacteria expressing either empty-vector (con) or dsRNA against *frh-1, nuo-2*, or *spg-7*. Bars and errors indicate mean and SEM from three independent replicates carried out with 80-100 animals each. Unless otherwise indicated *p < 0.05 vs control, t-test. See Supplementary Figure S6A-C for Chemotaxis Index curves with raw, not normalized values.

Noteworthy, animal locomotion ability linearly declined in all conditions: the fraction of animals spontaneously moving at any given age was increased in response to mild mitochondrial stress compared to wild-type animals, but animal speed was similarly reduced in control and RNAi-treated animals at any age (**Figure S8A-B**). These observations indicate that reducing mitochondrial function specifically ameliorate sensory functions rather than motility during aging. A corollary of this observation is that pro-longevity interventions could differentially promote features associated with healthy aging, with some parameters being affected linearly while others in a hormetic - dose or time - dependent manner.

5.3.4. Mitochondrial stress and mutations that impair ciliated neurons functionality extends lifespan through partially overlapping mechanisms

The more selective effect on sensory neuron functionality led us to hypothesize that the extended longevity upon reduced mitochondrial function could be achieved by alterations of specific sensory neurons whose deficit has been already associated with lifespan extension. Indeed, ablation, genetic or pharmacological manipulations of some of the affected neurons (AWA, AWC) extend *C. elegans* lifespan (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Evason et al., 2005; Lans and Jansen, 2007; Maier et al., 2010; Petrascheck et al., 2007). We therefore compared the longevity effect elicited by *nuo-2* RNAi on wild-type, with the effect induced on long-lived chemosensory mutant strains. If our assumption is correct the longevity of these mutants should not be significantly extended by mild mitochondrial stress or at least the extension should be less substantial than that observed in the wild-type strain. As expected (Rea et al., 2007; Ventura and Rea, 2007), compared to animals fed empty vector transformed bacteria, *nuo-2* RNAi induced a significant extension of lifespan in the wild-type strain (**Figure 19A;**

Table VI). Notably, in support of our hypothesis, RNAi against *nuo-2* did not extend lifespan to the same extent in the different sensory mutants (**Figure 19C-F**). Specifically, the lifespan of two sensory mutants, *osm-3(p082*) and *che-3(e1124)* with structural defects respectively in the cilia of all sensory neurons or in the amphid neurons only (Dusenbery et al., 1975; Perkins et al., 1986), was still significantly extended by *nuo-2* RNAi (**Figure 19A-C; Table VI**). This indicates that although mitochondrial and neuronal damage possibly act through partially overlapping pathways, a structural alteration of the cilia is not the main mechanism through which mitochondrial stress is extending lifespan. This is supported by the lack of structural defects in AWB and ASE neurons upon *nuo-2* and *frh-1* RNAi treated animals (**Figure S5**).

We then focused on genes involved in the functionality of the ciliated neurons rather than in their structure: The TAX-2/TAX-4 cyclic nucleotide-gated channel transduces sensory signals at the ciliated endings of a subset of amphid neurons (Komatsu et al., 1996); *odr-7* belongs to the family of nuclear hormone receptors and its knockout specifically abolish the chemotaxis to all odorants sensed by the AWA ciliated neurons (Sengupta et al., 1994); *nmur-1*, a seven membrane neuropeptide receptor homolog to mammalian neuromedin-U-receptors, is expressed in sensory neurons, interneurons and the somatic gonad and the *nmur-1(ok1387)* mutants live longer in a food source-dependent manner (Maier et al., 2010). Consistent with partially overlapping mechanisms of action, the longevity of *tax-4(p678)*, *odr-7(ky4)* and *nmur-1(ok1387)* mutants was slightly extended by *nuo-2* RNAi (**Figure 19B-F; Table VI**). Mean lifespan normalized to the respective controls (**Figure 19G**) clearly revealed that *nuo-2* RNAi did not significantly extend lifespan of *tax-4(p678)* and of *odr-7(ky4)* mutants.

Besides a partial overlapping mechanism of action, the lack of additive effects could be ascribed to the sensory mutants affecting *nuo-2* RNAi efficacy or to a toxic effect induced by the combination of the two treatments which would surpass the threshold for maximum lifespan extension of the RNAi-mediated Mit mutants (Rea et al., 2007; Ventura et al., 2009).

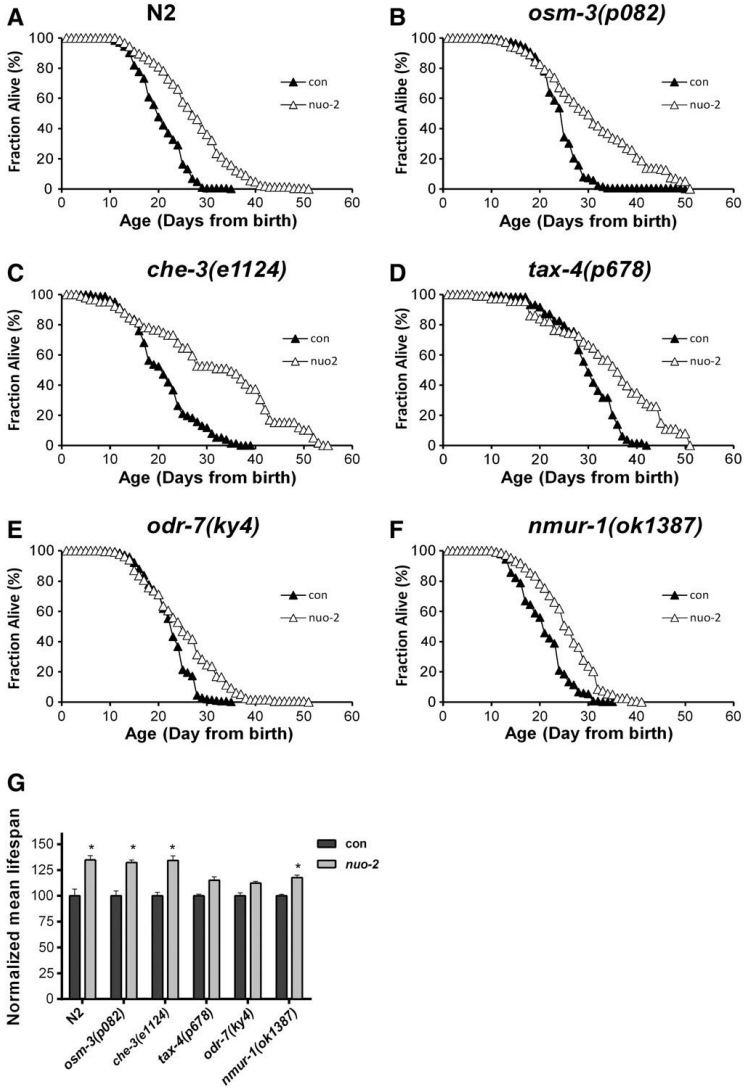


Figure 19. Mitochondrial stress differentially extends lifespan in wild-type and sensory mutant animals. Kaplan-Meier survival analysis of wild-type **(A)**, *osm-3(p082*) **(B),** *che-3(e1124)* **(C)**, *tax-4(p678)* **(D)**, *odr-7(ky4*) **(E),** *nmur-1(ok1387)* **(F)** strains fed bacteria expressing either empty-vector (con) or *nuo-2* dsRNA (*nuo-2*). See Table VI for summary statistics from Kaplan-Meier survival analysis. **G)** Normalized mean lifespan to respective control condition in each strain. Bars and errors indicate mean values + SEM from three independent replicates. *$p < 0.05$ vs control, t-test.

To rule out the first possibility we verified that our strains do not carry the *mut-16(mg461)* mutation (**Figure S9A**), which causes an RNAi-defective phenotype (Rde) (Zhang et al., 2011) and was recently found in the background of several strains. Moreover we quantified the reduction in animal body size, a parameter which directly correlates in the Mit mutants with the level of gene suppression (Rea et al., 2007), and showed that *nuo-2* RNAi reduced animal size to a similar extent in all strains (**Figure S9B-E; Table VI**). Importantly, this also argues against a toxic effect induced by the combination of the two treatments, which very likely would have instead further reduced animals' size or lead to developmental arrest (Rea et al., 2007; Ventura and Rea, 2007).

5.3.5. *glb-10* is causally involved in mitochondrial stress extension of lifespan

Globins are a family of small globular proteins with variant properties and functions. They usually consist of eight segments with α-helical structure (named A-H), displaying a characteristic 3-over-3 α-helical sandwich configuration that encloses an iron-containing heme group. Vertebrate globin genes mostly contain three exons separated by two introns inserted at highly conserved positions. Vertebrates normally express haemoglobin in red blood cells, neuroglobin in neurons, myoglobin in muscle and cytoglobin in a variety of non-neuronal cells. Globins in invertebrates comprise a more heterogeneous group of proteins in terms of structure and function. Single- and multi-domain globins have been characterized and they can be fused with non-globin subunits forming chimeric proteins (Hoogewijs et al., 2008). They have, as in vertebrates, O_2 storage and transport functions, but also a variety of other functions. In fact, they have been also shown to participate in: nitric oxide (NO) levels control in microorganisms; O_2 level control using NO in nematodes; binding and transportation of sulfide in endosymbiont-harboring species; protecting against sulfide and scavenging of O_2 in symbiotic leguminous plants; O_2 sensing in bacteria and archaebacteria (Weber and Vinogradov, 2001).

C. elegans globin-like proteins are mainly present in neurons. It is therefore possible that *C. elegans* globin-like proteins play different roles in neurons: they may help in the defense against the toxic effects of hypoxia/anoxia by functioning in detoxification of ROS and RNS; as proposed for mammalian Ngb (Brunori et al., 2005; Fago et al., 2004) they may work as O_2 sensors or carriers; finally they may have a role in redox reactions.

Due to their role in neurons and in oxygen sensing, we decided to investigate this class of genes and its possible involvement in lifespan extension upon mild mitochondrial stress. We quantified using RealTime PCR the transcript levels of all 33 *C. elegans* globins in response to *frh-1* RNAi and found that the expression of some of them was increased in the *rrf-3* mutant strain, which exhibits enhanced RNAi efficacy in several tissues (Pullarkat et al., 2014) and it is more sensitive to *frh-1* RNAi (Rea et al., 2007). In this mutant background, *frh-1* RNAi significantly increased the expression of *glb-10, glb-13, glb-19* and *glb-28*, and reduced the expression of *glb-26* (**Figure 20A** and **S10** in Appendix A). Automated fluorescence microscopy analysis of transcriptional globin::gfp reporters allowed unbiased quantification of *glb-10, glb-28, glb-19* and *glb-26* expression also in the wild-type strain (**Figure 20B**).

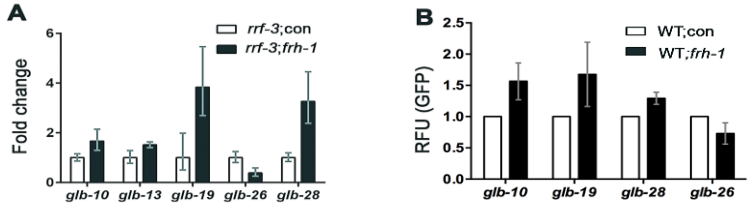


Figure 20. A-B) Transcript levels of 5 globins were measured by qRT-PCR in **A)** the RNAi hypersensitive mutants *rrf-3(pk1426) (rrf-3)* or in **B)** wild-type animals (N2). Animal in **A)** and **B)** fed bacteria transformed with empty-vector (con) or with vector expressing dsRNA against frh-1. All transcript levels were normalized to that of gamma-tubulin (tbg-1) and presented as average ± SEM from at least three independent experiments. *P<0.05, **P<0.01; Student's t-test.

We then looked at lifespan involvement of globin gene using the only available ko mutant strain (*glb-10)*. We treated these worms with RNAi

against *frh-1*, which extends the lifespan of wild type animals. Interestingly, *glb-10* mutant alleles significantly (but not completely) prevented the lifespan extension induced by *frh-1* RNAi (**Figure 21**).

Moreover, to better understand the involvement of these proteins in the context of mitochondrial dysfunction we decided to create translational reporter strain to be utilized in our experiment in addition to the transcriptional reporter already created in Braeckman Laboratory. To this purpose I spent a period of three month in the above-mentioned Laboratory to learn how to realize mutant animals using the Mos1 mediated Single Copy gene Insertion (MosSCI) technique (see paragraph 3.11).

The GFP reporter strains so generated will be then utilized to assess protein expression and their tissue and subcellular distribution.

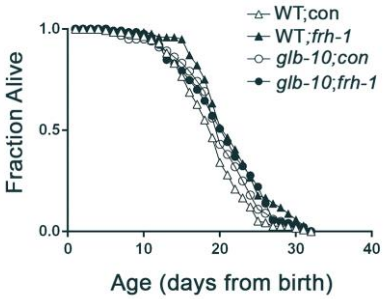


Figure 21. Kaplan-Meier survival analysis of wild-type and *glb-10* mutant animals fed bacteria expressing either empty-vector (con) or *frh-1* dsRNA (*frh-1*).

5.4 Discussion

Taken together our data indicate that mitochondrial stress generates a moderate functional deficit in chemosensory neurons, which, similar to the sensory neurons defective mutants, extends lifespan. This is consistent with the idea that neuronal hormesis may have anti-aging effects thanks to the induction of compensatory protective pathways triggered to cope with a mild mitochondrial-induced neuronal stress early in life. Autophagy is a fundamental cellular housekeeping process activated to protect neurons against hypoxia (Carloni et al., 2008; Samokhvalov et al., 2008) and in response to mitochondrial stress to extend *C. elegans* lifespan (Schiavi et al., 2013; Toth et al., 2008). Interestingly enough, two recent studies revealed a bidirectional functional interplay between autophagy and ciliogenesis, two processes primarily involved in responding to energy challenging environmental cues (Pampliega et al., 2013; Tang et al., 2013): in mammalian cells autophagy promotes ciliar growth in response to nutrient deprivation while altered ciliogenesis in normal nutrient conditions affects autophagy. Since mitochondria play a central role in neuronal functionality (Yin et al., 2014; Zsurka and Kunz, 2013), and ciliated neurons and autophagy are involved in mitochondrial stress control of longevity (*this work* and (Schiavi et al., 2013)), it is tempting to speculate that mitochondria may modulate physiological or environment-driven aging by integrating signaling between neuronal cilia and the autophagic process. Although further work is clearly required to elucidate the molecular mechanisms of this pro-longevity signal, subtle functional defects, such as signaling mediated by specific neuronal nuclear or membrane receptors, rather than overt structural alterations of the ciliated neurons seems to be at least in part causally involved in Mit mutant longevity. Different sensory mutant alleles (Apfeld and Kenyon, 1999), as well as temperature and food source (Maier et al., 2010) or modulation of the RNAi efficacy, will certainly reveal additional levels of complexity in lifespan regulation upon reduced mitochondrial function.

In this study we characterized behaviors associated with neuronal functionality decline during *C. elegans* physiological aging and in response to the pro-longevity effect of reduced mitochondrial function. We found that, similar to mammalian aging, chemosensory deficits appear earlier than motility defects and chemosensory neurons functionality decline faster than animal locomotion. We specifically

tested the hypothesis that neuronal hormesis might extend *C. elegans* lifespan in response to mitochondrial stress. In support of this idea we found that the pro-longevity effect elicited by silencing different nuclear encoded mitochondrial proteins, some of which linked to neurodegenerative diseases in human, is associated with mild alteration in specific subset of sensory neurons early in life. Yet, remarkably, the sensory deficit of these long-lived animals is rescued in old animals and they age healthier compared to wild-type animals. Finally, we demonstrated a causal connection between ciliated neuron dysfunction and extended longevity following mitochondrial stress. The molecular mechanisms underlying the anti-aging effect of mitochondrial stress-induced neuronal hormesis require further investigation but represent a very exciting and promising area of study for preventive medicine and healthy aging (Cornelius et al., 2013). Indeed, considering the wide variety of environmental (Meyer et al., 2013) and pharmacological (Reddy and Reddy, 2011) interventions which can target mitochondria, and the evolutionarily conservation of sensory neuron functionality (Linford et al., 2011; Prasad and Reed, 1999), our findings may be of relevance for the pathogenesis and treatment of age-related and/or mitochondria-associated neurodegenerative disorders.

We observed that depletions of *glb-10* shortens lifespan in response to *frh-1* RNAi and additional studies to accurately assess tissue specific expression and distribution of the globins are required to elucidate globins role in longevity specification in response to mitochondrial stress. Noteworthy, affected globins are mainly expressed in neurons *glb-1*, *glb-10*, *glb-13*, *glb-19* and *glb-28* (Hoogewijs et al., 2008), the tissues mostly involved in lifespan determination in response to mitochondrial disturbance (Durieux et al., 2011; Walter et al., 2011) (**Figure 22**).

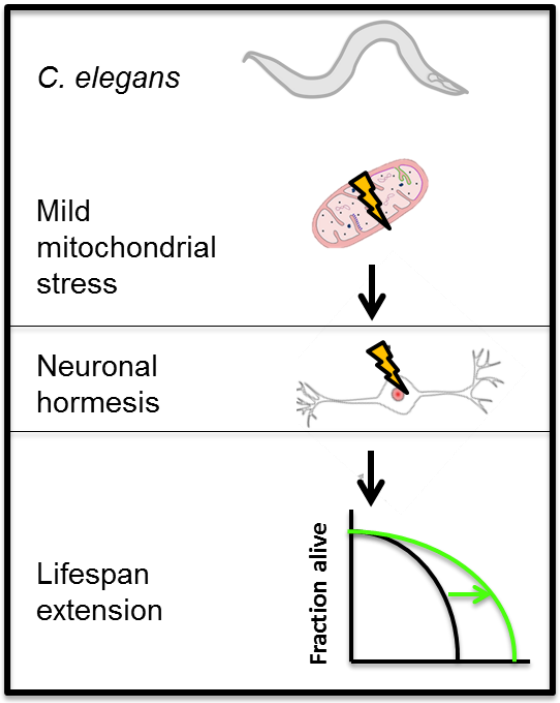


Figure 22. In the nematode *C. elegans* partial alteration of the mitochondrial functionality leads to extension of the lifespan. We demonstrated that mitochondrial stress generates a moderate functional deficit in chemosensory neurons, which, similar to the sensory neurons defective mutants, extends lifespan. This is consistent with the idea that neuronal hormesis may have anti-aging effects thanks to the induction of compensatory protective pathways triggered to cope with a mild mitochondrial-induced neuronal stress early in life.

Table VI. Lifespan summary statistics

Geno-type	RNAi	Mean Lifespan ± SE (Days)	Life-span change (%)[a]	P value[b] vs control	P value[c] vs wild-type	Sample size (censored)[d] /n trials	Mean animal size[e]/ worms (p value)
Wild-type (N2)	con	19,6 ± 1,3				300(47)/4	1417 /8
	nuo-2	26,1 ± 1,1	+34,7	< 0,0001		300(41)/4	1206/8 (< 0,0001)
osm- 3 (p082)	con	23,3 ± 1,1			< 0,0001	220(27)/3	
	nuo-2	30, 8 ± 0,8	+32,2	< 0,0001	< 0,0001	220(62)/3	
che-3 (e1124)	con	20,6 ± 0,9			< 0,0001	240(43)/3	
	nuo-2	27,7 ± 1,2	+34,4	< 0,0001	< 0,0001	240(39)/3	
odr-7 (ky4)	con	21,4 ± 0,6			0,0004	240(28)/3	1421/8
	nuo-2	24,1 ± 0,8	+12,3	< 0,0001	0.0031	240(44)/3	1155/8 (< 0,0001)
nmur-1 (ok1387)	con	20,9 ± 0,3			0.0036	240(66)/3	1345/8
	nuo-2	24,6 ± 0,6	+17,7	< 0,0001	0.0002	240(36)/3	1223/8 (0,0019)
tax-4 (p678)	con	28,9 ± 0,5			< 0,0001	140(11)/2	
	nuo-2	33,3 ± 1,1	+15,1	< 0,0001	< 0,0001	140(46)/2	

Table Legend

[a] % increase normalized mean lifespan compared to control ; [b] Kaplan-Meier survival analysis, Log-rank test against control; [c] Kaplan-Meier survival analysis, Log-rank test against wild-type; [d] Pooled worms from n replicate experiments (censored individuals were included in the analysis until the day they were censored); [e] In micron, 4 days old animals.

6 High-content phenotypic screen to identify compounds inducing bimodal mitochondrial adaptive responses

6.1 Abstract

C. elegans is an excellent candidate for high-content screening strategies analogous to those developed for cell-based systems. It has some specific characteristics that make it suitable for this purpose: manageability, low cost, fast reproductive cycle and lifespan, simple anatomy and a known genetics. Therefore, it offers the great possibility to carry out phenotypic-based screening also in automated systems. The history of *C. elegans*-based screening is quite recent but the progress reached in the last decade have shown clearly its potential.

We propose in this study a new high-content screening to take advantage of the phenotypic readout described in *C. elegans* upon mitochondrial alteration.

Mit mutants are an excellent model for studying human mitochondrial associated diseases (HMADs). In the context of the 'Mitochondrial Threshold Effect Theory', the processes induced to cope with mild mitochondrial alterations in the Mit mutants (very likely the same ones activated in early stages of HMADs) leads to extend their lifespan. On the other hand, upon strong mitochondrial suppression, *C. elegans* shows, as humans, deleterious phenotypical consequences.

Taking advantage of the distinct phenotypic readouts observed in response to different doses of genetic or pharmacological inhibitors of the mitochondrial respiratory chain, we optimized a screening platform, the Cellomics ArrayScan VTI HCS Reader, which will be exploited to identify potential preventive interventions. The here described screening offers the double opportunity to carry out direct and indirect studies. In the first case, targeting the specific phenotypes associated with the human pathologies, and looking for suppressing interventions, the screen will allow the identification of potential drugs to treat HMADs; on the other hand an indirect approach, using wild type animals and looking for compounds leading to the characteristic phenotype associated in

*Mit*mutants to lifespan extension, will allow to identify potential anti-aging drugs.

6.2 Introduction

Screenings for drugs can be nowadays classified in two main categories: high-throughput screening (HTS) and high-content screening (HCS). HTS uses robotics, data processing and control software, liquid handling devices, and sensitive detectors, to allow quick tests of large numbers of chemicals. HTS is characterized by low costs, simplicity, rapidness and high efficiency (Liu et al., 2004). HCS is instead an automated microscope-based screening that measures multiple features in single cells (or whole organisms) following treatments with the drugs under study. In contrast to traditional HTS, which has a single readout of activity, HCS allows to measure many properties at once. The ability to study many features simultaneously is both the strong and the weak point of HCS because of the huge amount of data that need to be handled (Buchser et al., 2004). Both these screenings are relatively easy on mammalian tissue culture cells, bacteria and yeast. Nevertheless, screening for potential beneficial compounds against many pathologies is not desirable in such a limited approach for different reasons:

(i) some diseases affect organs as a whole, and sometimes more than one organ, and most organs cannot be reconstituted *in vitro*. This is particularly important for pathologies that affect the muscular or nervous system. (ii) Cells and organs are physiologically connected, and this interaction may be critical in disease development and progression. This is an aspect that cannot be reconstituted *in vitro*. (iii) The time component of disease development is usually not respected *in vitro*.

On the other hand *C. elegans* is an excellent candidate for HCS strategies analogous to those developed for cell-based systems. The history of *C. elegans*-based screening is quite recent but the progresses reached in the last decade have shown clearly its potential.

C. elegans is a tiny animal model organism with a unique combination of features, which make it a powerful platform for the discovery and characterization of small bioactive molecules. The cell lineage of *C. elegans* is known and invariant and its genome has been completely sequenced and shares a high homology with the human one (Consortium, 1998). The worm body is optically transparent, enabling easy observation

of cells with or without the aid of fluorescent gene expression or use of dyes. All these aspects confer to *C. elegans* the ability to model human diseases and to use it as a versatile and powerful genetic platforms for *in vivo* high-throughput, high-content screening (Artal-Sanz et al., 2006; Burns et al., 2006; Segalat, 2007).

The history of *C. elegans*-based screening started with the first large-scale drug screen reported in 2006 (Kwok et al., 2006). The authors combined automated worm transfer with a semi-automated image acquisition system to screen the effect of small molecules on a selection of phenotypes (scored visually) in wild-type worms. In the same year Lehner and colleagues (Lehner et al., 2006) developed an all-liquid workflow to allow HTS with the nematode in a 96-well format. The use of the workflow permitted the use of automated robotic liquid handlers and the integration of automated imaging platforms. After that other liquid-based screens have been performed and identified compounds promoting the nematode extension of lifespan (Breger et al., 2007; Petrascheck et al., 2007, 2009). Microfluidic devices have been also recently developed as potentially powerful tools for HTS with *C. elegans* (Ben-Yakar et al., 2009) and they allow to isolate animals, deliver drugs and observe worms in a high throughput fashion. But the first automated high-content drug screen integrating automated worm transfer, image acquisition and data analysis was reported only four years ago (Gosai et al., 2010). Gosai and colleagues used the COPAS™ BIOSORT and the Arrayscan VTI (Thermo Scientific Cellomics) for image acquisition. Real-time data analysis was performed using the SpotDetector BioApplication.

Our work aimed at contributing to the effort of exploiting the versatility of *C. elegans* to identify new drugs against HMADs and generally for beneficial pro-longevity compounds, which are indeed desirable in a society where the percentage of the aged population increases more and more and the life expectancy is growing faster in the last decade.

6.3 Results

6.3.1 High-content phenotypic screening

6.3.1.1 Screening Parameters optimization

We want to develop an *in vivo* screening platform to identify compounds that induce the mitochondrial-adaptive beneficial responses and can rescue the detrimental effects associated with severe mitochondrial dysfunction.

The discovery of dietary and pharmacological factors that can delay age-associated detrimental changes is a very promising and attractive strategy as compared to the less feasible approach of genetic manipulation. In the last decade, the use of *C. elegans* as a powerful platform for *in vivo* high-throughput, high-content screenings for the identification of genetic or pharmacological interventions with anti-aging effects grew exponentially (Kolesnick, 2002; Petrascheck et al., 2007). The Cellomics ArrayScan VTI HCS Reader screening technology (http://www.cellomics.com/content/menu/arrayscan/), has been successfully adapted to *C. elegans* (Gosai et al., 2010). Similar to other screening platforms it combines automated bright-field and fluorescence images acquisition in multi-well format, with quantification of multiple information-rich parameters such as size, shape, fluorescence intensity and distribution. The software allows also the quantitative data analysis and the possibility to repeat a scan of the same plate with different protocol.

As Gosai and collegues did, to investigate disorders caused by misfolded and aggregation-prone proteins (Gosai et al., 2010), I adapted the Cellomics ArrayScan VTI HCS Reader to live animals and I optimized a screening to look at a mitochondrial fingerprint. Our purpose is to screen for pharmacological interventions (autophagy modulator drugs, natural compounds, neuroactive compounds) potentially acting

through mitochondria. The screening idea consists of titrating different chemicals looking for those that induce the classical phenotype of animals with mild mitochondrial stress.

C. elegans develops from egg to adult in 3 days at 20ºC through 4 larval stages (L1-L4), which progressively grow in size (eggs= 45 µm to adults =1mm length). Partial mitochondria alteration leads in *C. elegans* to reproducible phenotypic and biochemical changes (i.e. smaller sized adults, reduced fertility, and induction of stress response genes). To detect and quantify these phenotypic alterations, which are associated with increased animal robustness and longevity, I optimized different protocols using the SpotDetector, one of the provided Bioapplications of the Cellomics ArrayScan Reader.

To be able to use the BioApplication SpotDetector with our model organism we needed to optimize several necessary steps, specifically we looked at: number of animals per well, size of the animals and transgene expression quantification.

Population size (96 well): the SpotDetector rejects objects (animals) that touch each other or touch the wall of the well. We established that the optimal number of animals per well to have a reliable count is <100, in a population of mixed stage animals (**Figure 23**).

Figure 23. **Optimized protocol based on object number**. The SpotDetector BioApplication can identify a maximum of 100 animals in a mixed population containing worms from L1 to adults. Using a population of only adults it is preferable to have <50 animals per well.

The higher the number of objects, the higher the probability that they touch each other and that they are therefore excluded from the count.

Using a population of only adults it is thus more advisable to not exceed 50 animals per well.

Animal size and cut-off: to distinguish animals in different phases of their development or of different size, the number of objects detected in each well can be counted by specifying morphological size (width, length, area). The SpotDetector can correctly select objects within, and exclude objects without, the pre-selected size parameters. We quantified the number of detected objects based on their length and area (from embryo to adult). Specifically, at 20ºC wild-type eggs hatch and developed into adults (~1mm in length) in 3 days. L3 larvae are ~0,5mm in length. Animals left untreated or treated with two different doses RNAi against two mitochondrial proteins (NUO-2 (Ventura and Rea, 2007) and SPG-7 respectively) were used to set up two different cut-off: one for objects longer than 0.85 mm and one for objects shorter than 0,55 mm (**Figure 24**). Very similar results were obtained targeting other MRC complexes such as complex III (*isp-1* RNAi) or complex V (*atp-3* RNAi) (not shown).

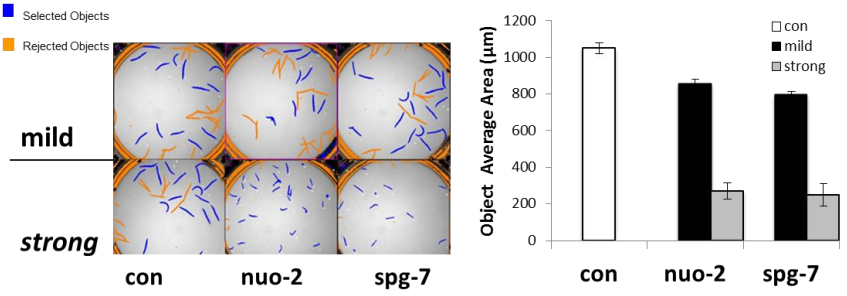


Figure 24. **Optimization of protocol for animal size.** To set up the size parameters we fed the animals with mild or strong concentration of two known RNAi interference (*nuo-2* and *spg-7*) whose effect on the size of the worms was already known from the screening. In this case the mild treatment correspond to the P0 generation and the strong to the F1. We grew a synchronized population of both treatments and then quantified with the Spotdetector at Cellomics. The protocol automatically excludes animals that are touching each other, overlapping or touching the wall of the well (orange labeled). The identified animals (blue labeled) are then distributed according to the size parameters set up to distinguish three population of animals: 0,85 mm = no mitochondria stress - 0,85 mm <

objects < 0,55 mm = mild mitochondrial stress - objects < 0,5 mm = severe mitochondria stress.

This allowed us to distribute detected objects in three different categories of size as surrogate markers for different degrees of mitochondrial stress: - objects > 0,85 mm = no mitochondria stress - 0,85 mm < objects < 0,55 mm = mild mitochondrial stress - objects < 0,5 mm = severe mitochondria stress We will therefore use these cut off references to identify compounds that primarily reduce animal size between 0,85 mm and 0,55 mm as a first indication for those acting through a mild mitochondrial stress.

Transgenes expression/distribution: the BioApplication SpotDetector can detect and quantitate fluorescence as total fluorescence intensity per object, total spot numbers or total spot area per object (Gosai et al., 2010). We set up the threshold parameters using different strains with diffuse or punctate pattern of GFP expression, like CL2166 (*gst-4*::GFP) or *ftn-2*::GFP.

As a control of our screening and to set up the fluorescent parameters we choose to use with *spg-7* interference treatment that, as already described (Yoneda et al., 2004) induce the stress response gene *hsp-6* (**Figure 25**). The GFP intensity was measured at the first day of adulthood after 3 days on RNAi interference plates. Upon mild treatment the worms reach the stage of gravid adult and were then compared with control worms at the same stage (**Figure 25A**). Upon strong RNAi against *spg-7* the animals arrest as L2/L3 larvae, comparisons were therefore made versus control worms at the same larval stage (**Figure 25B**).

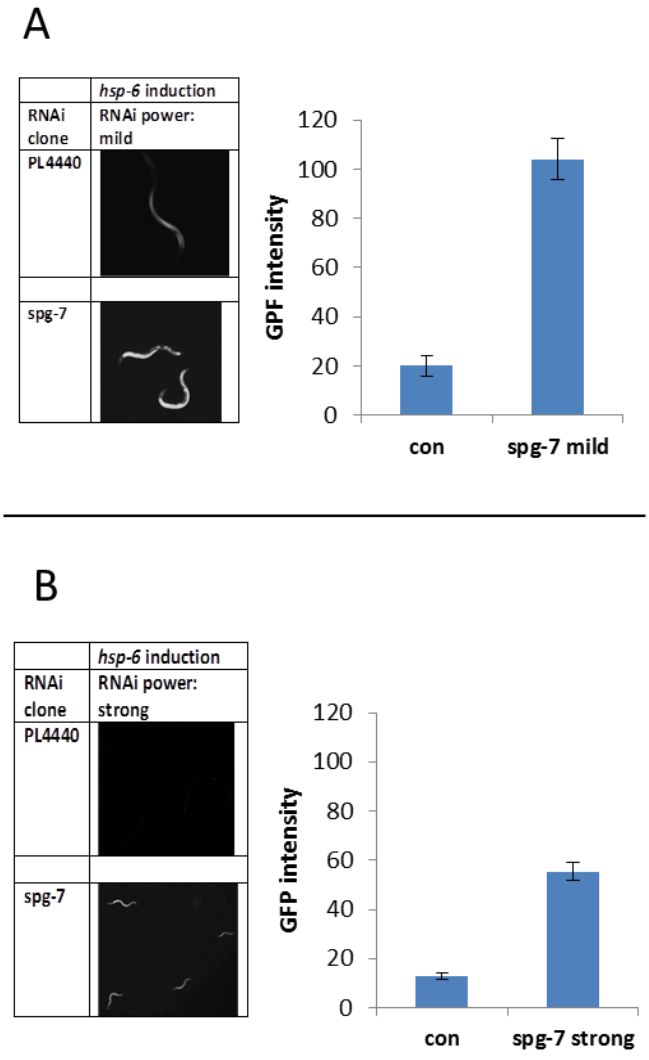


Figure 25. GFP induction in *hsp-6*::GFP reporter strain upon mild (A) or strong (B) RNAi against *spg-7*. Pictures were acquired with a Leica MZ10 F modular stereo microscope, 32× magnification, connected to a color digital camera 0.5x GFP signal quantified with the SpotDetector BioApllication.

We also set up different protocols using the fluorescent channel to be able to check (in the same time with the size and shape) other parameters like the iron uptake into the cells.

To set this we used two reporter strains in which the expression of two proteins is monitored. The iron-storage protein ferritin, *ftn-2* is transcriptionally inhibited during iron deficiency, while on the contrary; *smf-3* (homologous to mammalian divalent metal transporter-1) is transcriptional activated during iron deficiency.

We grew up these two strains on agar supplemented with an iron chelator (BP) and an iron donor (FAC) and we were able to appreciate the increase or the decrease of the fluorescent signal with the Cellomics using the Spot Detector Application that can count the number of the spots inside each animal and give a measure of the their intensity (**Figure 26**).

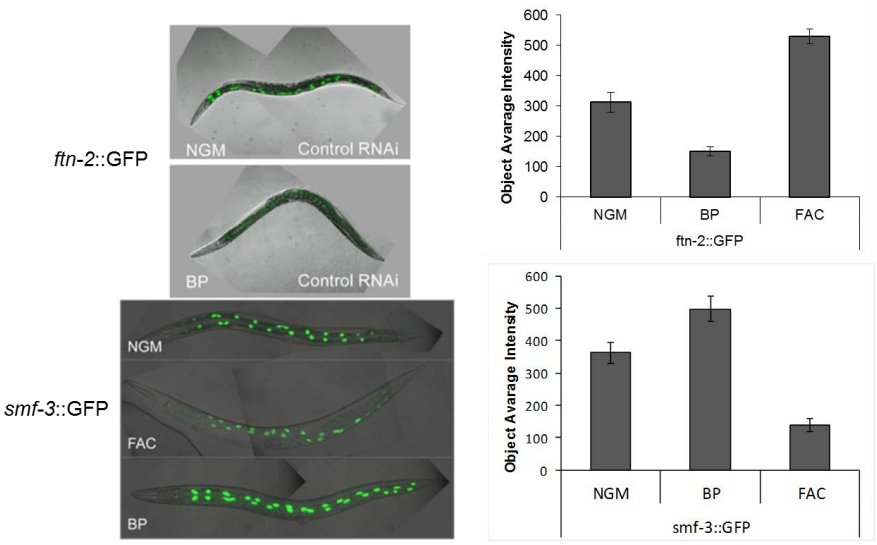


Figure 26. Iron uptake into the cells can be indirectly assessed using *smf-3* reporter strains, picture from (Romney et al., 2011). Thresholds set treating worms with 2,2 Bipyridil (BP) and Ferric Ammonium Citrate (FAC), quantified with the Spotdetector BioApplication.

6.3.1.2 Screening validation

After setting up the software with the screening parameters to be automatically acquired and analyzed the set up continued with the research for positive controls to be used. We proceeded by testing mitochondrial active drugs to find concentrations that were mimicking the effect of mild and strong mitochondrial dysfunctions. We used mitochondrial targeting compounds whose effect on animal´s lifespan has been already tested, and we proceeded testing those doses already shown to have pro-longevity effects. We looked for concentrations affecting the phenotype in a similar manner to the mild (and strong) mitochondrial deficiency.

With the Spot detector Bioapplication it is possible to quantify the GFP intensity in worms that express the fluorescent protein both in a diffuse or a punctate way (where this is not too weak or localized only in few cells). Therefore we are able to detect the increased signal in a vast range of mutants. For our purpose we wanted to monitor the expression of stress reporter genes, like glutation-S-tranferase-4 (*gst-4*) and heat sock protein 6 (*hsp-6*), whose induction is associated, although not always directly correlated, with degrees of lifespan extension (Bennett et al., 2014; Torgovnick et al., 2010) upon treatments with different doses of genetic or pharmacological inhibitors of the MRC.

In *C. elegans*, a mild frataxin suppression extends the longevity and induces the expression of the antioxidant *gst-4* over that of the control animals (Ventura et al., 2009). Concerning *hsp-6*, it has been shown that inhibition of mitochondrial respiration triggers the mitochondrial unfolded protein response (UPR ^{mit}), increasing *hsp-6* expression (Yang and Hekimi, 2010). This response is needed for the longevity in Mit mutants and acts through unidentified signaling molecules, maybe ROS, between different tissues (Durieux et al., 2011).

Using these two strains and looking at animal development, size, fertility and GFP expression we tested chemicals at different doses looking at the 2 concentration that were, at an intermediate dose, reducing size and fertility of the animals, and at a high dose, arresting the development at a larval stage (L2/L3).

Specifically we used: Rotenone, Paraquat, Doxycycline and Oligomycine.

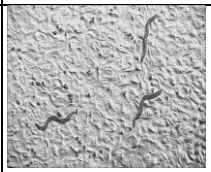
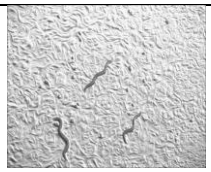
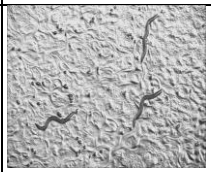
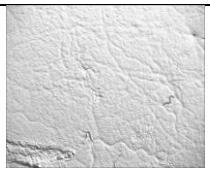
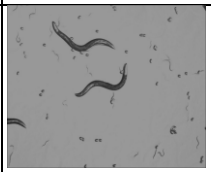
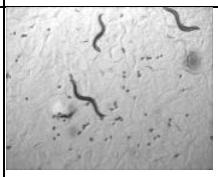
Rotenone is an inhibitor of complex I and at the concentration of 100 nM extends *C. elegans* lifespan (Schmeisser et al., 2013a). Paraquat generates superoxide in mitochondria and showed to be able to increase significantly *C. elegans* lifespan (Lee et al., 2010). Doxycycline is an inhibitor of mitochondrial translation, thus it blocks MRC biogenesis, it has been shown that 15 ng/ml and increasing concentration (till 60 ng/ml) of this chemical extends lifespan of the nematode (Houtkooper et al., 2013). Oligomycine is an inhibitor of complex V-ATP synthase. Treatment with oligomycin, began at the young adult stage, at a dose of 40 μM, increased the mean lifespan of N2 worms by 32.3% (Chin et al., 2014).

We identified for all the drugs tested 2 concentrations that were giving us a "mild" and a "strong phenotype".

All the concentration identified correlate with slow development, reduction of size and progeny number (for mild) and arrest of the development (for strong) (tables VII and VIII).

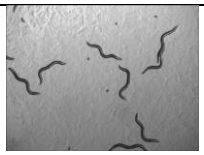
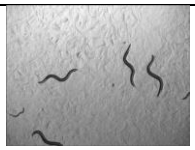
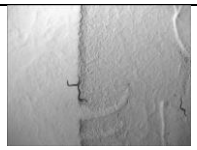
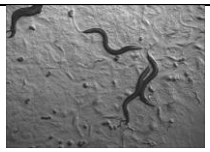
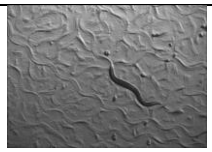
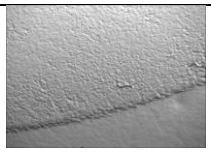
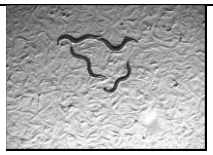
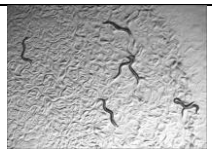
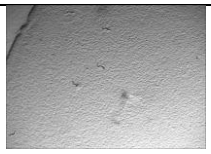
The expression of the two reporter genes was although not always clearly correlating.

Table VII

(*gst-4*::gfp)	**Control**	**Mild**	**Strong**
compound	control	**Rotenone 0.5 nM**	**Rotenone 10 nM**
phenotype			
GFP induction	++	++	-
compound	control	**Oligomycin 2.5 µM**	**Oligomycin 5 µM**
phenotype			
GFP induction	++	++	-
compound	control	**Paraquat 0.1 mM**	**Paraquat 0.4mM**
phenotype			
GFP induction	+	++	+++
compound	control	**Doxycycline 10 ng/ul**	**Doxycycline 100 ng/ul**
phenotype			
GFP induction	+	+	++

Identification of "mild" and "strong" concentrations using mitochondrial specific targeting drugs using the gst-4::GFP reporter strain.

Table VIII

(hsp-6::gfp)	Control	Mild	Strong
compound	Control	**Rotenone 0.5 nM**	**Rotenone 10 nM**
phenotype			
GFP induction	+	+++	+++
compound	control	**Oligomycin 2.5 µM**	**Oligomycin 5 µM**
phenotype			
GFP induction	+	++	+++
compound	control	**Paraquat 0.1 mM**	**Paraquat 0.4mM**
phenotype			
GFP induction	+	++	++++
compound	control	**Doxycycline 10 ng/ul**	**Doxycycline 100 ng/ul**
phenotype			
GFP induction	+	++++	+++

Identification of "mild" and "strong" concentrations using mitochondrial specific targeting drugs using the *hsp-6*::GFP reporter strain.

6.4 Discussion

Most of the high-content instruments and software are, nowadays, designed for cell cultures. To adapt such technology to a multicellular organism, highly mobile, with morphological characteristics that can be very disparate from strain to strain and from different larval stages, is a very challenging intent. Nonetheless, the nematode offers several features that makes it suitable for drug screening. It has a small size and is possible to let it develop in liquid medium in 96 well plate format. The *C. elegans'* body is transparent at all developmental stages, thus, creating transgenic lines expressing fluorescent proteins, allows to follow specific biological processes. Finally, live animal screens appear to be ideal candidates for drug discovery strategies designed to address suppressors of specific phenotypes, to find out molecules that deregulate the expression of specific proteins or physiological processes (Andreux et al., 2013; Andreux et al., 2014; Jansen-Olesen et al., 2013).

Gosai and colleagues already showed that *C. elegans* can be exploited for high-quality drug discovery platform using a combination of transgenic lines expressing fluorescent proteins and a commercially available automated fluorescence microscopy imaging system, the ArrayScan VTI (Gosai et al., 2010).

We here demonstrated the possibility to use this HCS platform to identify genetic and pharmacological interventions recapitulating our HMAD models and most importantly we can now carry out suppressor screening.

In fact, in the specific context of HMAD, after optimizing the screening protocols for the different parameters we want to check in the animals, if the ArrayScan can be exploited for the search of potential drugs suppressing the phenotype that mimic the human symptoms. Specifically we are able to monitor animal development and dysregulation of specific genes using fluorescent reporter strains. We can now identify drugs rescuing the arrested development of the worms modeling HMADs and restoring normal levels of *hsp-6* and *gst-4* expression.

Of note, the potentiality of this screening platform is not limited to this class of diseases. Ideally any characterized model developed in the worms with specific phenotypic readouts, can be used to carry out drug screening, looking for treatments that trigger or suppress that precise phenotype, representing a toxic or therapeutic intervention respectively.

Moreover, the characteristic of the *C. elegans* mitochondrial mutants is that different degrees of mitochondrial stresscan trigger a bimodal response. Upon mild mitochondrial alteration the nematode lives longer and healthier, and this response is associated with clear phenotypic readouts. Therefore, looking for chemicals able to trigger this beneficial response, at the level of mitochondria or downstream, can also importantly lead to the identification of general anti-aging treatments. This strategy represents the second type of screening approach for which our system can be used.

In conclusion, we developed and validate an *in vivo* phenotypic-based drug screening platform exploiting the genetically tractable *C. elegans* as a powerful model organisms. During the setup phase of this screen, we identified the parameters to distinguish the different phenotypic features associated with mild and strong mitochondrial stress; we tested different negative and positive control substances that alter mitochondrial functions and consequentially the phenotype of the worms; and we established the threshold values and algorithms to be able to appreciate GFP expression differences using various report strains.

We envision that such a screening approach is suitable and convenient to identify novel mitochondrial modulators and also new pro-longevity treatments. Such compounds will be beneficial for both rare and common diseases in which mitochondria are dysfunctional and to delay pathophysiological aging (**Figure 27**).

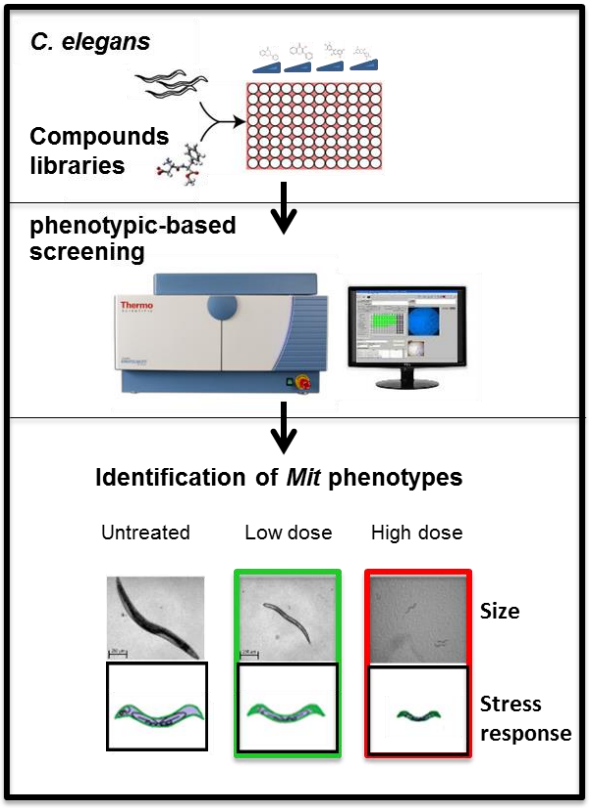


Figure 27. Optimization of an *in vivo*-phenotypic based, automated microscopy screening platform to test library of compounds using the model organism *C. elegans*. Possibilities to carry out direct and indirect screenings. The first approach aims to identify interventions able to rescue the phenotype associated to strong mitochondrial dysfunction, therefore representing potential HMADs treatments. The second approach aims to identify intervention that trigger the phenotype typical for mild mitochondrial alterations (associated with lifespan extension). The compounds identified will represent anti-aging drugs.

Conclusions and future perspectives

Our data clearly indicate the primary role played by sensory neuron modifications in the aging process in our model organism, and confirm the possibility to use *C. elegans* to study and understand the mechanisms underlying these evolutionarily conserved age-associated changes.

Our results also suggest that mild mitochondrial stress could extend lifespan in a hormetic-like manner via alteration of a specific subset of neurons, in a cell-autonomous or non–autonomous manner. We clearly showed a connection between those two pathways (mitochondrial alteration and neuronal hormesis) and additional work is needed to understand how and in which extent the nervous system is playing a role in mitochondrial stress control of longevity. One of the first experiments to carry out will be to use strains in which the effect of the RNA interference is limited or excluded in the nervous system (and other tissues), to test how the mitochondrial alteration would affect sensory functionality and animals' lifespan through specific subset of neurons.

With my PhD work I also characterized new models for human mitochondrial associated diseases. We showed the validity of the nematode in mimicking the main features of these pathologies, e.g. altered nervous system functionality and developmental arrest when the mitochondrial dysfunction rises over a specific threshold. On the other hand we characterized the very interesting opposite response of the organism to a mild mitochondrial alteration. With this characterization we confirmed once more the existence of protective pathways activated to the cells to cope with mitochondrial stress (Maglioni et al., 2014; Rea et al., 2007; Schiavi et al., 2013; Ventura et al., 2009), which can be exploited to develop new therapeutic approaches for these non-curable devastating disorders. In the next future we will continue the characterization of the new models with a major focus on the strong mitochondrial dysfunction to better characterize the features that mimic the human symptoms (e.g. mitochondrial and neuronal dysfunction). We will measure different mitochondrial parameters to compare which are mainly altered compared to wild type animals and in this way we will be

able to identify interventions that rescue the deficits (e.g. ATP and ROS levels). We will look for both pharmacological and genetic intervention able to suppress the arrest development associated with severe mitochondrial Interestingly, although we generally observed that severe mitochondrial stress lead to animal developmental arrest, preliminary study (not show) revealed differences between the different mutants under study: for example loss of function of the *cep-1*,the *C. elegans* homolog of the human p53, rescues the arrest development due to strong *nuo-2* RNAi but not the one due to *spg-7* RNAi (data not shown).Therefore we will try to differentiate our characterization with the aim to categorize and classify the models (e.g. based on the mitochondrial subunit whose functionality is compromised).

Least but not last, we propose in this study a new high-content screening to take advantage of the phenotypic readout described in *C. elegans* to different levels of mitochondrial alterations. After adapting the screening platform to our model and set up all the control conditions we are able to now screen compounds for their potential beneficial action on mitochondria. A small library of compounds widely used in our Institute for environmental medicine studies and containing different food components and contaminants and environmental xenobiotics (total of 85 chemicals) will be screened. Firstly, the compounds will be seeded at four different testing concentrations to initially exclude toxic or non-active ones. Twenty-five L1 larvae will be dispensed in duplicate in 96 wells plates and the effect of the compounds on animal viability and development will be observed for one week. Secondly, selected compounds will be then automatically screened through the Cellomics ArrayScan using bright-field illumination and fluorescence filters to identify those giving the expected phenotypes (reduction in size, increase of stress response genes). Fifty L1 of each transgenic strain will be dispensed in triplicate in 96 wells plates pre-seeded with adjusted compounds concentrations and their effects on animal size and fluorescence expression/distribution will be assessed on day 4 and day 7. Finally, a validation phase will be carried out, the ten compounds that give the most significant results will be further validated for their ability to affect mitochondrial functionality. To characterize mitochondrial bioenergetics, ATP and ROS levels and oxygen consumption will be measured in worms (Houtkooper et al., 2013).

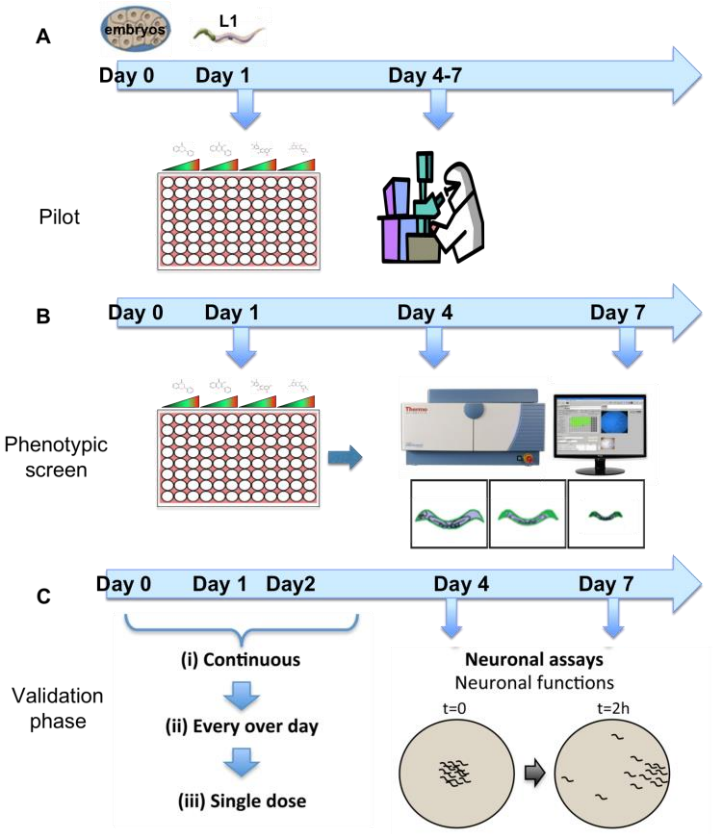


Figure 28. Phenotypic-based screen flow-chart. A) Pilot screen: 85 selected compounds will be seeded at four different testing concentrations to initially exclude toxic or non-active ones. Twenty-five L1 larvae will be dispensed in duplicate in 96 wells plates and the effect of the compounds on animal viability and development will be observed for one week. **B) Phenotypic-based screen:** selected compounds will be then automatically screened through the Cellomics ArrayScan using bright-field illumination and fluorescence filters to identify those giving the expected phenotypes (see text for details). Fifty L1 of each transgenic strain will be dispensed in triplicate in 96 wells plates pre-seeded with adjusted compounds concentrations and their effects on animal size and fluorescence expression/distribution will be assessed on day 4 and day 7. **C) Validation phase:** the ten compounds that give the most significant results will be further validated for their ability to affect mitochondrial functionality and assessed, using well established behavioral assays, for their ability to rescue the detrimental phenotypes associated with our HMAD models.

Out of these ten, the three most promising compounds will be characterized in details through different assays to assess their ability to ameliorate the detrimental phenotypes induce by severe mitochondrial stress in HMAD models (**Figure 28**).

We are now able to test chemical libraries on nematodes with disease-relevant phenotypes (arrest of development) to identify molecules able to reverse the phenotype and thus representing potential therapeutic drugs to fight HMAD (**Figure 29B**). On the other hand our screening platform can be used for a wider purpose, not limited to the HMAD diseases.

Moreover, the screening platform that we set up in this work could be also used to carry out indirect screenings aimed to identify potential pro-longevity intervention (**Figure 29A**). In fact, treating wild type worms with a library of chemicals and screen for the intervention giving the typical mild mitochondrial mutant phenotype, indicating the activation of beneficial pathways, could reveal pro-longevity compounds.

We can therefore screen compounds targeting pathways (MRC, autophagy, neuro-sensory neurons) that we know to be altered in the *Mit* mutants or new interventions.

Any intervention that give us the typical mild mitochondrial stress phenotype is probably activating one or more protective pathways, likely the same activated in our mutants but maybe not working through mitochondria. Identified chemicals will be then investigated for their pro-longevity effect and considered to be further studied in mammalian system.

For these reasons the potential results obtained with this screening system will directly impact on basic research, but could also affect environmental medicine and socio-economic aspects of our society. It is in fact more and more important to understand and identify anti-aging treatment in a society in which the percentage of elderly individuals increases constantly. *C. elegans* is undoubtedly a versatile organism that has great utility for investigation of diseases. Despite the large evolutionary distance between worms and humans, human proteins appear to maintain their functionality when expressed in the nematodes and mutations that cause diseases in humans lead also in *C. elegans* to abnormal phenotypes. Moreover, although it is certainly hard to compare the complexity of the human brain with the simplicity of the nervous

system of *C. elegans*, all neuronal types are very well represented in this organism, which therefore represent an excellent platform for preliminary screening of putative therapeutic compounds, and to understand the biology of neurons *in vivo*.

Above observations certainly make these tiny worms a very good and promising experimental system to gain insight into different biological processes and to find new therapeutic approaches to delay the deterioration of the nervous system during aging and in the context of different diseases.

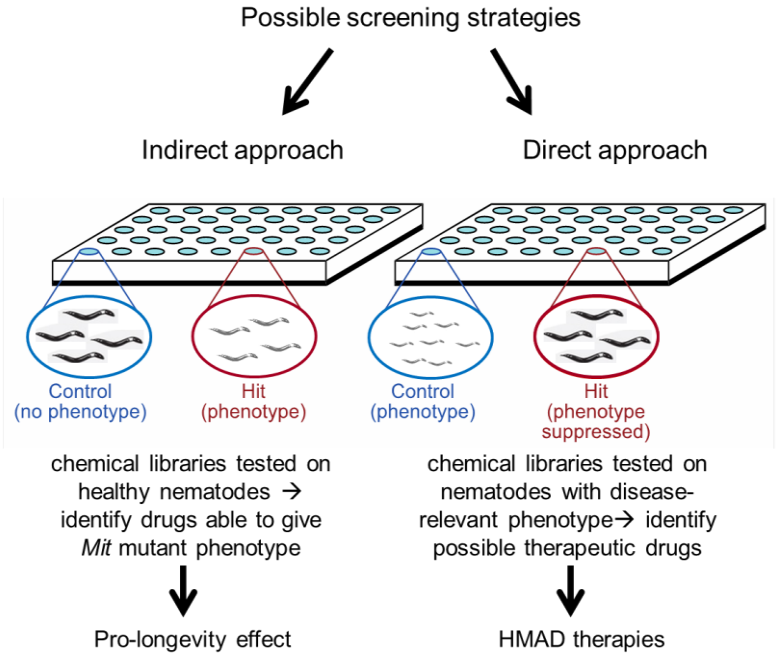


Figure 29. Two possibly to use our screening platform: A) chemical libraries can be tested on healthy nematodes to identify drugs that are able to give the typical mild mitochondrial mutant phenotype, indicating the activation of beneficial pathway, potentially revealing pro-longevity interventions B) chemical libraries can be tested on nematodes with disease-relevant phenotype (arrest of development) to identify molecules able to reverse the phenotype and thus representing potential therapeutic drugs for HMAD. Figure modified from (Segalat, 2007).

List of Publications

Publications:

1. Arczewska KD, Tomazella GG, Lindvall JM, Kassahun H, **Maglioni** S, Torgovnick A, Henriksson J, Matilainen O, Marquis BJ, Nelson BC, Jaruga P, Babaie E, Holmberg CI, Bürglin TR, Ventura N, Thiede B, Nilsen H. "Active transcriptomic and proteomic reprogramming in the *C. elegans* nucleotide excision repair mutant xpa-1." Nucleic Acids Res. 2013 Apr 10
2. Torgovnick A, Schiavi A, **Maglioni S**, Ventura N. "Healthy aging: what can we learn from *C. elegans*?" Journal of Gerontology and Geriatrics. August 2013
3. **Maglioni S**, Schiavi A, Runci A, Shaik A, Ventura N. "Mitochondrial stress extends lifespan in *C. elegans* through neuronal hormesis" Exp Gerontol. 2014 Apr 4

In preparation:

1. **Maglioni S,** Schiavi A, Torgovnick A, Ventura N., "*C. elegans* as a model organism for Human Mitochondria-associated Diseases"
2. **Maglioni S**, Ventura N., "A novel screening strategy to identify pro-longevity intervention in *C. elegans*"
3. Schiavi A, **Maglioni S,** Torgovnick A, Ventura N. "Reduced frataxin expression induces an iron starvation response that extends *C. elegans* lifespan via Parkin/Bnip3-regulated mitophagy " (*under revision*)

4. Herholz M, Cepeda E, Baumann L, Kukat A, Pavlenko V, Pujo C, Bratic-Hench I, Frommolt P, **Maglioni S**, Ventura N, Trifunovic A. "KLF-1 orchestrates a xenobiotic detoxification program essential for multiple longevity pathways" (*submitted*)

5. Torgovnick A, Schiavi A, **Maglioni S**, Ventura N. "Role of DNA Damage Response in the long-lived Mit mutants"

6. Kassahun H, Skjeldam HK, Arczewska KD, **Maglioni S**, Estes S, Schiavi A, SenGupta T, Fensgård Ø, Eide L, Ventura N, Nilsen H. "Bioenergetic imbalance in a *Caenorhabditis elegans* endonuclease III nth-1 mutant affects p38MAPK activation and apoptotic responses to reactive oxigen species"

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Appendix A

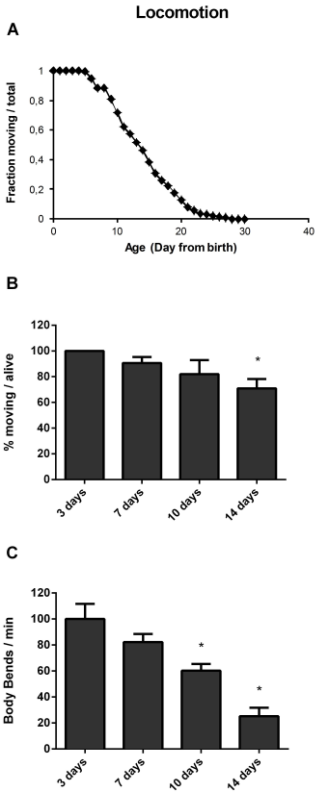


Figure S1. Locomotion decline during aging. A) Fraction of wild-type animals moving spontaneously or upon touch during their lifespan. Shown is a curve from cumulative data obtained from 2 experiments carried out with a population of 80 synchronized animals. **B)** Percentage of animals moving spontaneously or upon touch of the fraction of animals alive. Bars and errors indicate mean and SEM from 2 experiments. **C)** Locomotion activity (body bends per minute) in wild-type animals. Values are normalized to 3 days-old worms. Bars and errors indicate normalized mean and SEM of body bends from 20 animals from one representative experiment out of three.

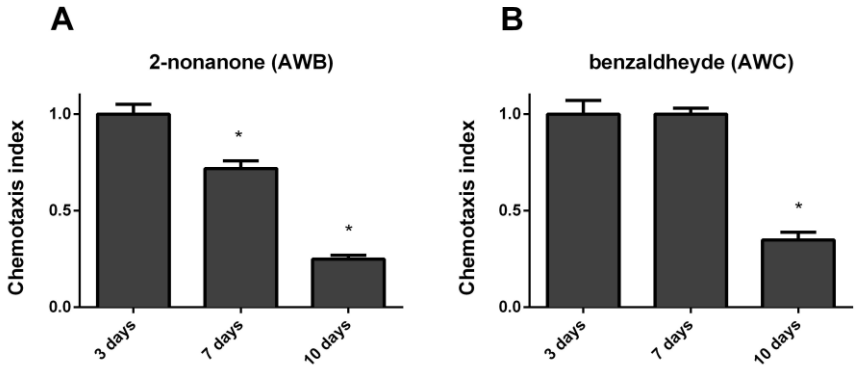
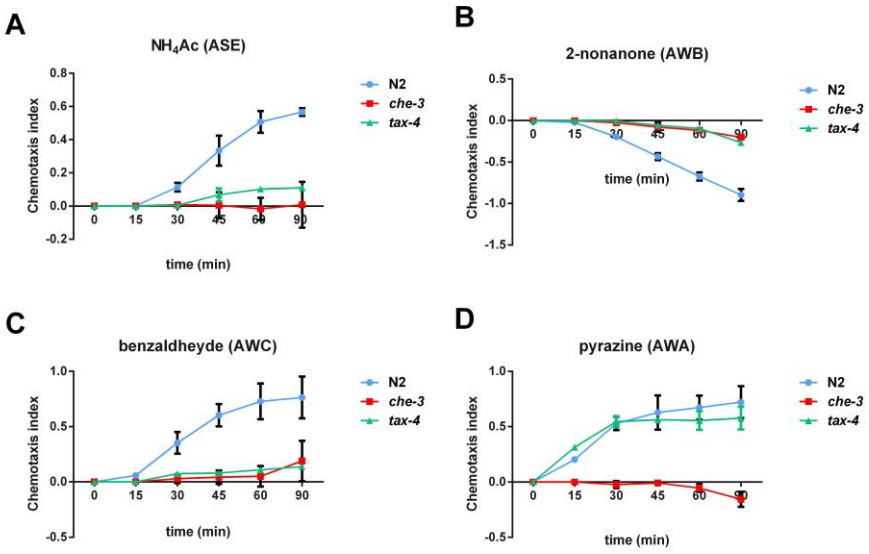


Figure S2. Chemosensory function decline during aging. Chemotaxis Index to 2-nonanone **(A)** and benzaldheyde **(B)** after 2 hours from the beginning of the assay in wild-type animals. Chemotaxis Index is normalized to 3-days-old animals. Bars and errors indicate mean and SEM from two to four independent replicates carried out with 80-100 animals each. *$p < 0.05$ vs control, t-test.


A
NH4Ac (ASE)
Chemotaxis index
time (min)
N2
che-3
tax-4
B
2-nonanone (AWB)
Chemotaxis index
time (min)
N2
che-3
tax-4
C
benzaldheyde (AWC)
Chemotaxis index
time (min)
N2
che-3
tax-4
D
pyrazine (AWA)
Chemotaxis index
time (min)
N2
che-3
tax-4

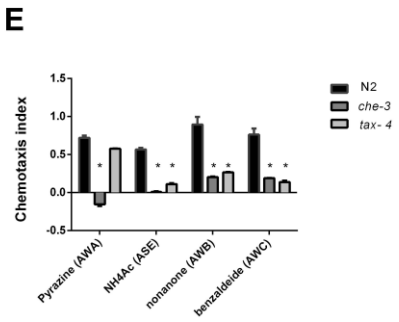


Figure S3. Chemosensory function in sensory mutants. A-D) Representative chemotaxis index curves with raw, not normalized values from wild-type (N2), *che-3(e1124)* and *tax-4(p678)* mutants, 3-day-old animals fed with OP50 bacteria. **E)** Chemotaxis index after 2h from the beginning of the assay in wild-type (N2), *che-3(e1124)* and *tax-4(p678)* mutants, 3-day-old animals fed with OP50 bacteria. Bars and errors indicate mean values and SEM from three independent replicates. *$p < 0.05$ vs control, t-test.

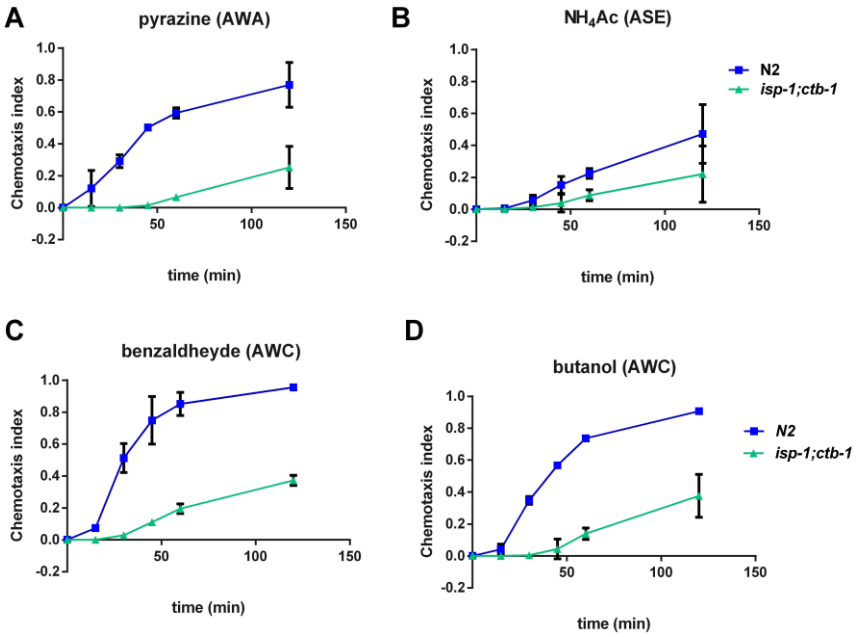


Figure S4. ***isp-1;ctb-1*** **Mit mutant display reduced chemosensory neurons functionality. A-D)** Chemotaxis index curves with raw, not normalized values from wild-type (N2) and *isp-1;ctb-1* mutants, 1st day fertile adults animals fed with OP50 bacteria. Errors bars indicate SEM from two independent replicates.

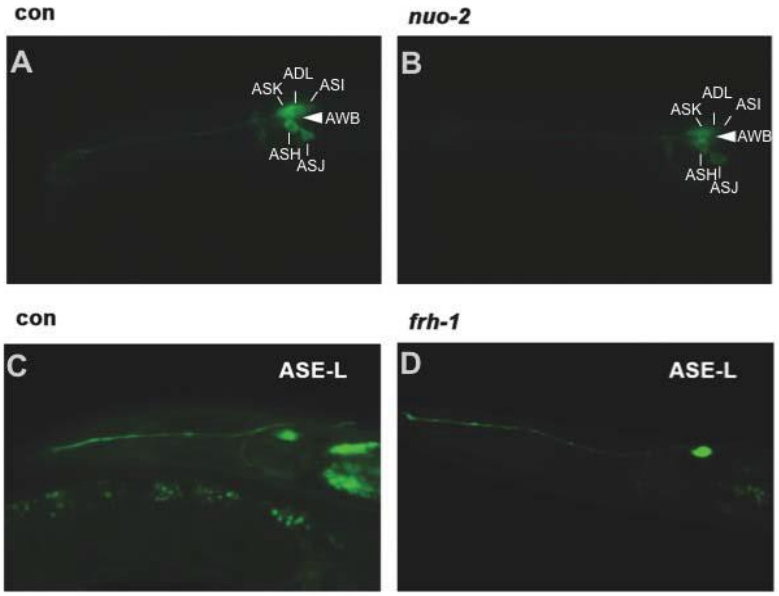


Figure S5. Neuronal structure. Neuronal structure in 3-days-old animals where observed using a Zeiss Imager 2 microscope equipped for fluorescence microscopy at 400× magnification. 20 animals per condition were acquired. **A-B)** Representative pictures of amphid neurons stained with DiO in animals fed bacteria transformed with empty-vector (A) or with *nuo-2* dsRNA *(*B). **C-D)** Representative picture of ASE-L neurons using the reporter strain *gcy-7::gfp* in animals fed bacteria transformed with empty vector (C) or with *frh-1* dsRNA *(*D).

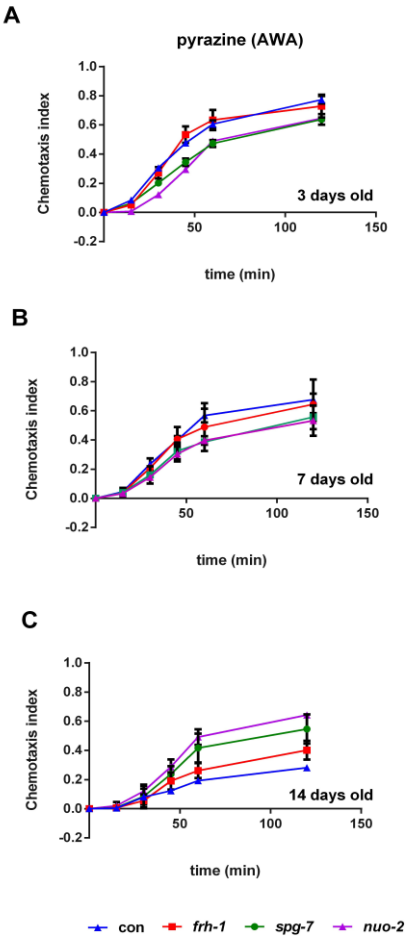


Figure S6. Chemosensory function during aging upon mitochondrial stress. Chemotaxis index in 3-days- (A), 7-days- (B) and 14-days- (C) old wild-type animals fed bacteria expressing empty-vector (con) or the indicated dsRNA. Representative Chemotaxis Index curves with raw, not normalized values.

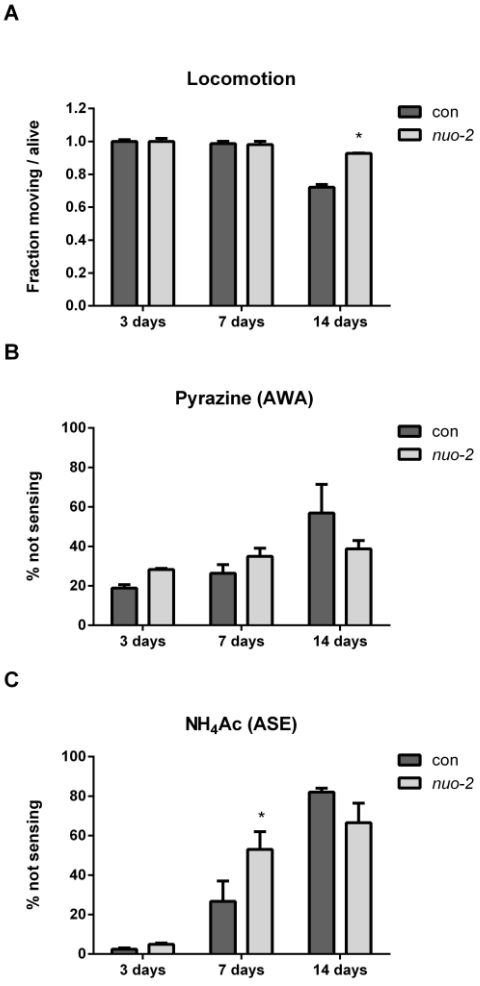


Figure S7. A) Fraction of wild-type animals fed empty-vector (con) or *nuo-2* dsRNA moving spontaneously or upon touch on the number of animal alive during their lifespan. Bars and errors represent mean and SEM from 2 independent experiments carried out with a synchronized population of 80 animals. **B-C)** Fraction of animals treated as in (A) with altered chemotaxis to Pyrazine (B) or Sodium Acetate (C) during their lifespan. Bars and errors represent mean and SEM from 2 independent experiments carried out with synchronized population of 80-100 animals.

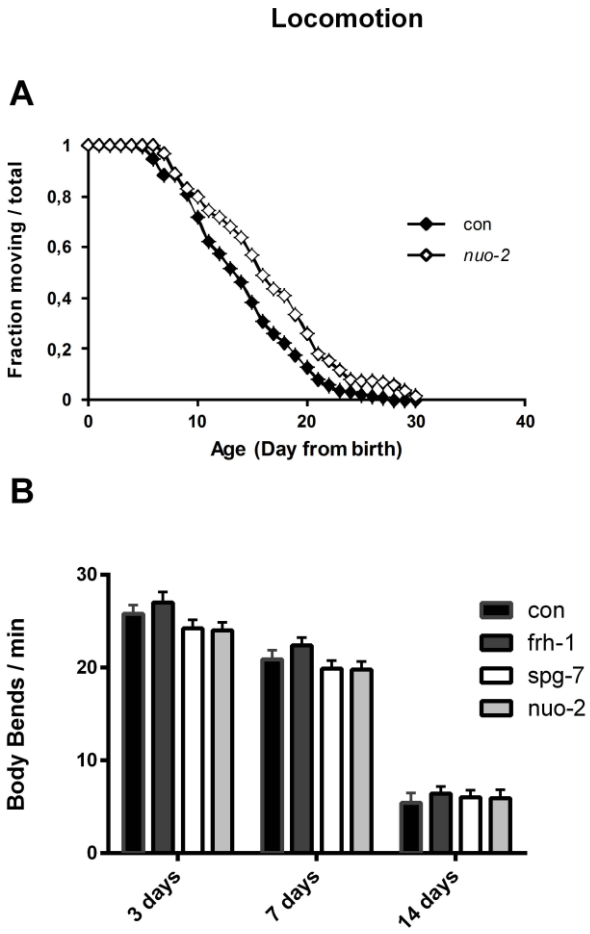
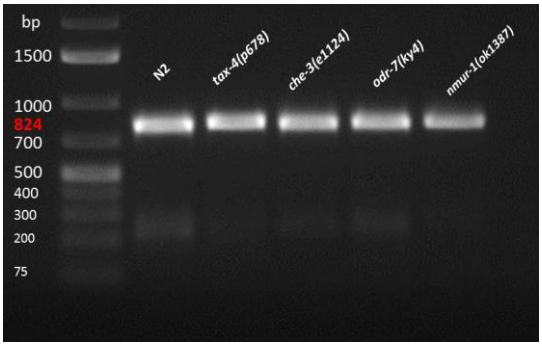


Figure S8. A) Fraction of wild-type animals fed empty-vector (con) or *nuo-2* dsRNA moving spontaneously or upon touch during their lifespan. Shown are curves from cumulative data obtained from 2 experiments carried out with a population of 80 synchronized animals. **B)** Locomotion activity (body bends per minute) in wild-type animals fed either empty-vector (con) or any of the indicated dsRNA. Bars and errors indicate mean and SEM of body bends from 20 animals from one representative experiment out of three.

A



B

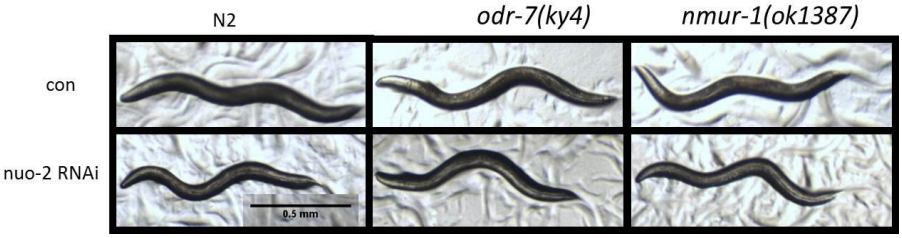


Figure S9. A) Single worm PCR genotyping was used to check the presence of the *mut-16(mg461)* deletion which induces RNAi-defective (Rde) phenotype. Strains were genotyped using the following primers: For-primer CCCGCCGATACAGAAACTAA, Rev-primer AATATTCGATCGGCAAGCAG. Strains that are wild-type at the locus yield a 824bp PCR product, while a 373bp product is observed from strains that contain the *mg461* allele. None of the assessed stains show the 373 bp product. **B)** Morphological phenotype (young adults)

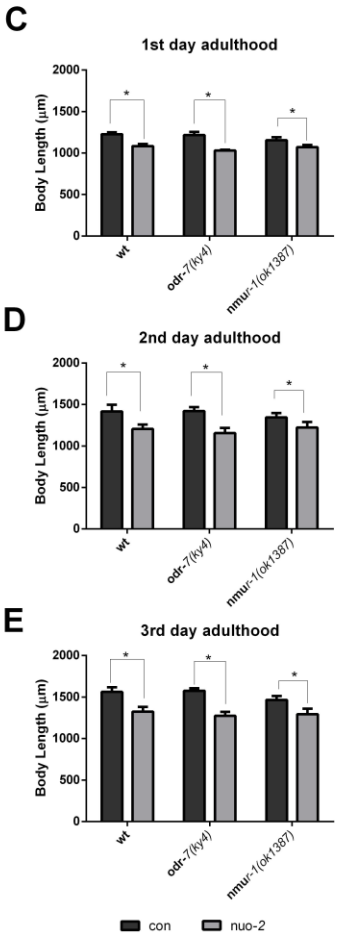


Figure S9. C-E) size quantification (1st- 2nd- 3rd-day adulthood) of N2 (wild-type), *odr7(ky4)* and *nmur-1(ok1387)* mutants fed bacteria transformed with empty vector or *nuo-2* dsRNA. All the strains show the same reduction in body size compared to untreated animals after *nuo-2* RNAi feeding.

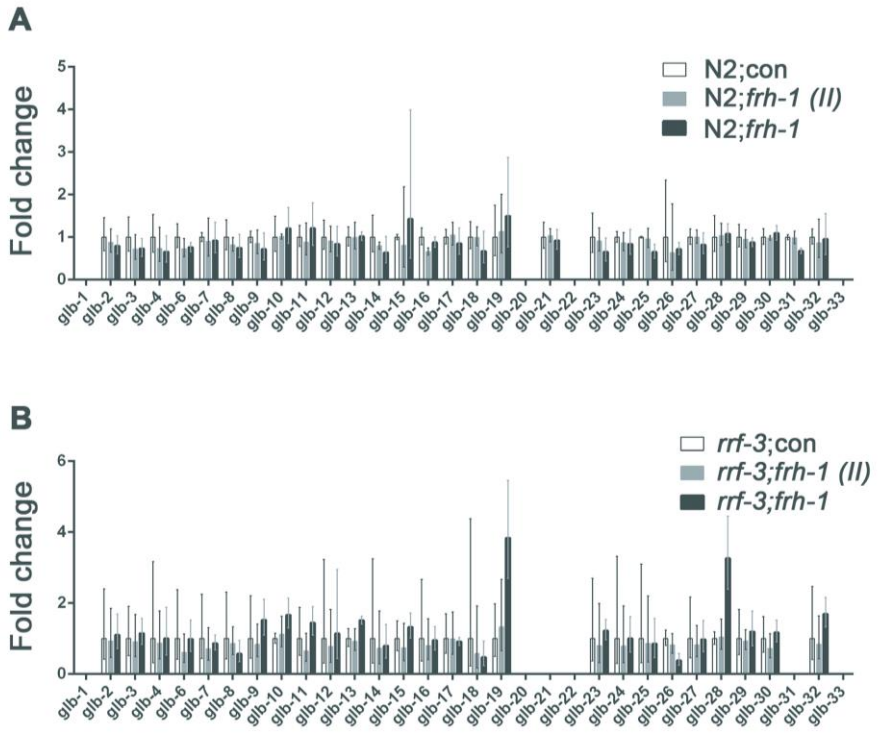


Figure S10.

A-B) Transcript levels of all 33 globins (*glb-1—glb-33)* were measured by qRT-PCR in **A)** wild-type animal (N2) while in **B)** the RNAi hypersensitive mutants *rrf-3(pk1426) (rrf-3)*. Animal in **A)** and **B)** fed bacteria transformed with empty-vector (con) or with vector expressing dsRNA against frh-1 (*frh-1* (IV)) and the *frh-1 dsRNA* (*frh-1 (II)*) that does not extend longevity. All transcript levels were normalized to that of gamma-tubulin (tbg-1) and presented as average ± SEM from at least three independent experiments. *P<0.05, **P<0.01; Student's t-test**.**

Appendix B

Table I

cosmid ID	gene	01:10	P0	F1
ZK973.10	*lpd-5*		few progeny	slow development; few eggs
Y47G6A.10	*spg-7*	pale, thin	pale, small, thin few eggs, few progeny	slow development, arrested
C01F1.2	*sco-1*			slow development, sick, thin, sterile
C15F1.7	*sod-1*		small	
D2013.5	*eat-3**		thin	thin, slow development
C09H10.3	*nuo-1**		pale, thin, sterile	
T20H4.5	T20H4.5 (NDUFS8) *		pale, thin, small , few eggs	arrested L2/L3
H14A12.2	*fum-1*		few eggs	few progeny, slow development
F54H12.1	*aco-2*		few progeny	sterile
M03C11.5	*ymel-1*			
K08E3.7	*pdr-1**	slow moving, pale, few progeny	slow moving, pale, few eggs, protruding vulva	
T27E9.1	*tag-61**	thin, pale	small, pale, sterile, protruding vulva	

Description of the phenotypic alterations observed in the screening described in paragraph 4.3.1 (supplementary information relative to Table III in the text).

Table II. Lifespan summary statistics

Gene/ RNAi clone	RNAi power	Mean Lifespan ± SE (Days)	Lifespan change (%)[a]	P value vs control[b]	P value mild vs strong[c]	Sample size/ n trials
con	mild	19,05 ± 0,28				275/4
	strong	19,98 ± 0,40			0,61	140/3
lpd-5	mild	22,01 ± 0,36	+15,53	< 0,0001		220/3
	strong	23,44 ± 0,65	+17,31	< 0,0001	0,0033	190/3
C01F1.2	mild	20,04 ± 0,41	+4,38	0,062		220/3
	strong	26,36 ± 0,8	+31,8	< 0,0001	< 0,0001	200/3
nuo-5	mild	24,91 ± 0,62	+30,76	< 0,0001		200/3
	strong	26,85 ± 1,00	+34,38	< 0,0001	0,125	200/3
con	mild	20,3 ± 0,27				204/3
	strong	19,46 ± 0,38			0,9	220/3
spg-7	mild	23,69 ± 0,39	+16,69	< 0,0001		230/3
	strong	25,23 ± 0,35	+29,6	< 0,0001	0,0001	220/3
F01F1.12	mild	19,82 ± 0,40	-5,25	0,08		160/2
	strong	18,50 ± 0,66	-6,56	0,70	0,652	140/2
ymel-1	mild	20,92 ± 0,40	0	0,8697		150/2
	strong	18,27± 0,39	-7,72	0,0292	< 0,0001	160/2
con	mild	21,65 ± 0,42			0,73	235/3
	strong	21,31 ± 0,42				235/3
sod-1	mild	19,72 ± 0,31	- 9,91	< 0,0001		240/3
	strong	18,65 ± 0,31	- 12,48	< 0,0001	0.0184	240/3
con	mild	23,60 ± 0,56				160/2
	strong	23,59 ± 0,55			0.83	150/2
Y46G5A.2	mild	21,92 ± 0,47	- 7,11	0,01		160/2
	strong	21,50 ± 0,46	- 8,85	0,0044	0,69	160/2
con	mild	19,99 ± 0,33				235/3
	strong	21,87 ± 0,37			<0,0001	220/3

T20H4.5	mild strong	26,18± 0,58 29,28± 0,65	+30,96 + 33,88	< 0,0001 < 0,0001	<0,0001	250/3 220/3
con	mild strong	18,74 ± 0,34 21,49 ± 0,63			0,012	200/3 120/2
eat-3	mild strong	21,33 ± 0,45 23,77 ± 0,95	+ 13,82 + 10,60	< 0,0001 < 0,0001	0,0027	140/2 120/2
aco-2	mild strong	16,88 ± 0,44 17,46 ± 1,03	- 9,92 - 18,75	0,013 0,027	0,28	140/2 120/2
con	mild strong	21,15 ± 0,42 20,19 ± 0,41			0,05	150/2 160/2
fum-1	mild strong	14,56 ± 0,23 18,62 ± 0,33	- 31,15 - 7,77	< 0,0001 0,0006	< 0,0001	160/2 160/2
con	mild strong	18,93 ± 0,42 18,38 ± 0,45			0,57	150/2 160/2
drp-1	mild strong	18,61 ± 0,40 18,15 ± 0,36	-1,69 -1,25	0,37 0,35	0,425	140/2 140/2
con	mild strong	18,17 ± 0,29 18,76 ± 0,45			1,16	260/3 140/2
T22B11.5	mild strong	18,69 ± 0,38 21,45 ± 0,50	+2,86 +14,33	2,89 0,0003	< 0,0001	200/3 200/3
hmg-5	mild strong	20,69 ± 0,48 18,40 ± 0,33	+9,29 -0,1	0,0005 0,417	< 0,0001	135/2 140/2
tag-316	mild strong	23,68 ± 0,48 20,94 ± 0,33	+25,1 +13,9	< 0,0001 0,0077	0,0003	125/2 140/2
con	mild strong	20,69 ± 0,24 20,29 ± 0,31			0,68	315/4 300/4
F53F4.10	mild strong	23,73 ± 0,48 28,92 ± 0,74	+14,69 +42,53	< 0,0001 < 0,0001	< 0,0001	320/4 300/4
sdha-1	mild strong	14,9 ± 0,23 13,97 ± 0,15	-22,99 -25,53	< 0,0001 < 0,0001	< 0,0001	120/2 140/2

Table III. Lifespan summary statistics

Gene/ RNAi clone	RNAi power	Mean Lifespan ± SE (Days)	Lifespan change (%)[a]	P value vs control[b]	P value mild vs strong[c]	Sample size/ n trials
con		18,61 ± 0,26				330/4
spg-7	mild	22,79 ± 0,34	+22,4	< 0,0001		230/3
	strong	21,69 ± 0,32	+16,5	< 0,0001	0,023	230/3
con		18,13 ± 0,27				250/3
nuo-1	mild	23,91 ± 0,51	+31,8	< 0,0001		200/3
	strong	27,83 ± 0,68	+53,5	< 0,0001	< 0,0001	200/3
T20H4.5	mild	18,74 ± 0,32	+3,36	0,63		160/2
	strong	27,34 ± 0,66	+50,79	< 0,0001	< 0,0001	140/2
W02F12.5	mild	18,49 ± 0,29	+1,98	2,13		200/3
	strong	20,37 ± 048	+12,34	< 0,0001	0,001	200/3
con	mild	17,83 ± 0,56				250/3
pdr-1	mild (1:10)	17,75 ± 0,27	- 0,44	5,32		200/3
	mild (1:50)	20,56 ± 0,47	+15,31	< 0,0001	< 0,0001	80/1
	strong	13,38 ± 0,22	-24,95	< 0,0001	< 0,0001	200/3
F23B12.5	mild	17,59 ± 0,30	-1,34	1,55		200/3
	strong	18,41± 0,26	+3,25	5,34	1,32	200/3
con		17,70 ± 0,28				235/3
tag-61	mild	21,54 ± 0,36	+21,46	< 0,0001		250/3
	strong	22,40± 0,41	+ 26,55	< 0,0001	0,73	220/3
con		17,55 ± 0,34				190/3
F25B4.6	mild (1:10)	11,32 ± 0,45	-35,49	< 0,0001		140/2
	mild (1:50)	14,54 ± 0,95	-17,15	< 0,0001	< 0,0001	80/1
	strong	8,07 ± 0,25	-54,0	< 0,0001	< 0,0001	140/2

Table Legend

[a] % increase normalized mean lifespan compared to control ; [b] Kaplan-Meier survival analysis, Log-rank test against control; [c] Kaplan-Meier survival analysis, Log-rank test between mild and strong treatment;