Institute of Molecular Biology and Biotechnology

Part I: **“Crosstalk between autophagy and neurotransmission”**

Part II**: “Coordination between Mitophagy and UPR^{mt} through CHN-1/CHIP E3 ubiquitin ligase”**

Evrydiki Asimaki

Lab of Neurogenetics and Aging

Scientific Supervisor: **Nektarios Tavernarakis**

Scientific Committee: **Iannis Talianidis, George Zachos**

Contents

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Prof. Nektarios Tavernarakis, Head Director of the Foundatiob for Research and Technology. The door to Prof. Tavernarakis office was always open whenever I ran into a trouble spot or had a question about my research, writing and provided me with feedback for my professional life and future goals and aims. I would also like to thank my committee members Ascociate Professor G. Zachos and Scientific director for accepting to be review my thesis and support my presentation.

I would also like to thank my direct supervisor Dr. Konstantinos Palikaras, who involved me in these two very interesting projects, supported me, and taught me everything I needed to know. He was my mentor and during this year of my master thesis I enjoyed the interesting conversations we had!

I would like to thank particularly Christina Ploumi, since she was the first one to guide me through the basics of *C.elegans* and Konstantinos Kounakis, my desk "neighbor", for his support on my questions and concerns and for the very interesting scientific discussions we had. I would also like to thank Dr. Maria Markaki, for her constant support and help during my master thesis as well as Angela Pasparaki our "superwoman" lab manager that provided us with everything we needed for the experiments and for the imaging work. I am very grateful to Andrea Princz for her support in Biochemical experiments and for the troubleshooting and advices that she provided me with whenever I needed it! Furthermore, I would like to thank all the lab members: Nikolaos Charmpilas, Manos Chaniotakis, Ioanna Daskalaki, Ilias Gkikas ,Kostas Kounakis, Eirini Lionaki, Thanos Metaxakis ,Margarita Papandreou, Dionysia Petratou, for the continuous support, feedback during the lab meetings and discussions! They were a perfect team, always friendly and available for help!

Finally, I would like to dedicate this work to my beloved father, whom I lost in the beginning of my master studies. I owe to him so much for what I have accomplished so far. Both my mother and my father, were my mentors, my teachers, my encouragers and without their guidance I would not be the person I am today. Also, I would like to express my very profound gratitude to my husband Nikos, who provided me with unfailing support and continuous encouragement throughout the two years of my studies, and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Evrydiki Asimaki

ABSTRACT

An essential process to maintain cellular homeostasis and functions is autophagy, a process responsible for degradation of damaged proteins and organelles. Consequently, misregulation of autophagy can result in a variety of pathological conditions. Recently, autophagy has been linked with the maintenance of meuronal homeostasis. Here, we identify important genes of autophagy like *lgg-2* which is the homolog of the mammalian LC3 autophagosomal protein to play a role in synapses remodelling and neuronal exocytosis. Furthermore we link mitophagy, as well, in the process of neurotransmission, highlighting its post-synaptical function. This knowledge could prove extremely useful as synapses are critical for brain function, with synaptic dysfunction being an early pathological feature in aging and disease. In our second part of the current work we aim to investigate the crosstalk between UPR^{mt} and autophagy specific in mitochondria (known as mitophagy), two of the main mitochondrial quality control processes activated under pathological stress. Activation of the UPR could trigger changes in mitochondrial function or autophagy, which could modulate the UPR, exemplifying crosstalk processes. Many factors are responsible for the regulation of the magnitude , duration and function of these cellular processes. In this study we aim to investigate the role of the ubiquitin ligase CHIP in mitophagy or UPR^{mt} activation. We show that depletion of CHIP leads to decreased mitochondrial bioenergetics (reduced ATP levels, mitochondria membrane potential,increases PDR-1 protein levels (E3 ubiquitin-protein ligase parkin) and leads to a mild reduction in lifespan.

ΠΕΡΙΛΗΨΗ

Μια εξαιρετική σημαντική διαδικασία για τη διατήρηση της κυτταρικής ομοιόστασης και της λειτουργικότητας των κυττάρων είναι η αυτοφαγία, μια διαδικασία υπεύθυνη για την απομάκρυνση/καταστραφή των κατεστραμμένων πρωτεϊνών και οργανιδίων. Κατά συνέπεια, η εσφαλμένη ρύθμιση της αυτοφαγίας μπορεί να οδηγήσει σε μια ποικιλία παθολογικών καταστάσεων. Πρόσφατα, η αυτοφαγία έχει συνδεθεί με τη διατήρηση της ορμονομετρίας. Εδώ, αναγνωρίζουμε σημαντικά γονίδια αυτοφαγίας όπως το *lgg-2*, το οποίο είναι το ομόλογο της αυτοφωσφοσωματικής πρωτεΐνης LC3 των θηλαστικών, να διαδραματίζουν ένα σημαντικό ρόλο στην ανασύνθεση των συνάψεων και την εξωκυττάρωση των νευροδιαβιβαστών στο νευρικό σύστημα. Επιπλέον, συνδέουμε τη μιτοφαγία, με τη διαδικασία της νευροδιαβίβασης, επισημαίνοντας τη μετα-συναπτική λειτουργία της. Αυτή η γνώση θα μπορούσε να αποδειχθεί εξαιρετικά χρήσιμη, καθώς οι συνάψεις είναι κρίσιμες για τη λειτουργία του εγκεφάλου, ενώ η συναπτική δυσλειτουργία είναι ένα πρώιμο παθολογικό χαρακτηριστικό της γήρανσης και διαφόρων ασθενειών. Στο δεύτερο μέρος της εργασίας, επιχειρούμε να μελετήσουμε δύο από τις κύριες διαδικασίες μιτοχονδριακού ελέγχου που ενεργοποιούνται υπό παθολογικό στρες, το UPR^{mt} και την αυτοφαγία των μιτοχονδρίων (γνωστή ως μιτοφαγία). Αυτό που δεν είναι κατανοητό και θεμελιωμένο είναι η συνεργασία αυτών των δύο μηχανισμών. Σε αυτή τη μελέτη στοχεύουμε να διερευνήσουμε τον ρόλο της CHIP λιγάσης ουμπικουϊτίνης στην ενεργοποίηση της μιτοφαγίας ή του UPR^{mt}. Στην παρούσα εργασία επιχειρούμε να δείξουμε ότι η καταστολή της CHIP πρωτείνης οδηγεί σε μειωμένη βιο-ενεργότητα των μιτοχονδρίων, μειωμένα επίπεδα Τριφωσφορικής Αδενοσίνης (ATP), μειωμένο δυναμικό μεμβράνης μιτοχονδρίων, αυξημένα επίπεδα της PDR-1 πρωτείνης η οποία είναι μια E3 λιγάση ουβικουιτίνης ομόλογη της parkin, και ήπια μείωση της διάρκειας ζωής των *C.elegans*.

INTRODUCTION

Introduction to *C.elegans* biology

C.elegans is a free-living transparent nematode, t hat is not parasitic and lives in soil. Its size is about 1 mm in length for adult animals. It is one of the simplest organisms that exist with a nervous system.

The last 44 years, since it was first suggested for biological research by Sydney Brenner, it has been utilized as a model organism and contributed in genetics and physiology. In research laboratories it is grown on solid or liquid growth medium and it is fed with OP50 E.coli. The maintenance of *c.elegans*, thus, is quite inexpensive, easy and not very time-consuming. Furthermore, it is easy to maintain frozen strains in -80oC or nitrogen for several years. [1]. It has proven to be a valuable tool in research, as it contributes to the investigation of cell death mechanisms during development. The advantages of *c.elegans* are its small body size and transparency and the completely deterministic (and always identical) development.

The reproductive circle of *c.elegans* lasts about 2.5 days at 25^{o}C whereas the median lifespan of the animals lasts 2 to 3 weeks in total. The advantageous small lifespan permits aging studies. [2]

During reproduction, the oocyte of the hermaphrodite is fertilized by sperm produced by the same animal. Part of the embryonic development is realized inside the gonad of the adult animal. The first larval stage after egg hatching is referred to as larva 1 (L1). Three more developmental stages follow (L2, L3, L4) before the creation of adult fertile animals, sexually capable to produce and lay eggs. Every adult animal can lay approximately 300 eggs during 3 days.[3] The development is a very sensitive procedure, since in higher temperatures, or inadequate food, L1 animals stop develop, whereas L2 animals are transformed in a resistant form that is called type of stasis.[4] This form is referred to as “dauer” and it can survive harsh conditions. Dauers have a more elongated and slim body morphology, high storage of fat and can survive without the presence of food. When dauers are exposed to physiological conditions again, they reenter the developmental circle in the larva 4 stage. [5]

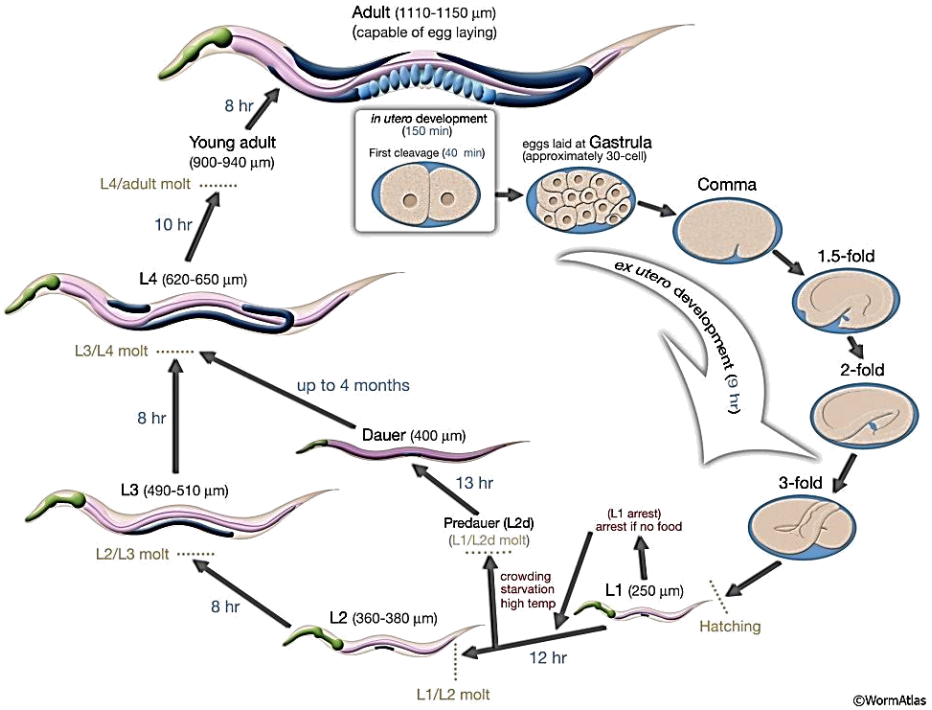


Figure 1.: The life cycle of *C.elegans* at 22°C. The figure demonstrates the life cycle of *C.elegans* form 0 minutes which is the fertilization till Adulthood. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. Adapted from ©WormAtlas

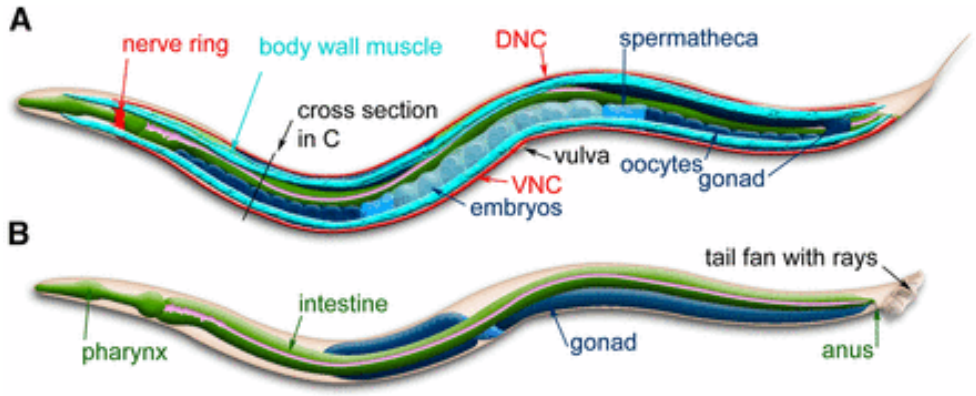


Figure 2.: The *C.elegans* anatomy. The anatomical differences between the hermaphrodite (A) and male (B) are illustrated. Images are modified from those found at www.wormatlas.org .[2]

C.elegans are hermaphrodites and are producing progeny with self-fertilization. However, abnormal separation of the matched pair of sex chromosomes (XX) during meiosis is possible to lead to male population that have only one sex chromosome (X0). [6]Although the presence of male animals is particularly rare in nature, heat shock can facilitate the abnormal separation of the X chromosomes and can eventually lead to male

animals. The male animals can be used for genetic crosses. The populations that are widely used in research come from a common ancestor referred to as Bristol N2. This fact results in genetically identical populations and serves as a valuable tool for comparable results between large populations. [7]

The genome of *c.elegans* has already been sequenced and consists of approximately 20000 open reading frames (ORFs). Genetic research is facilitated with the production of transgenic animals with the technology of micro-injections of DNA sequences directly in the animal's gonad. [2]

Part I: Autophagy role in Neurotransmission

A. AUTOPHAGY, A MAJOR REGULATOR

Autophagy is a process in which during development, at critical times when energy is needed or under nutrient stress, a mechanism of self-degradation is activated. This mechanism plays also a role in removing damaged proteins, clearing and removing damaged organelles [8]. There are different types of autophagy ***(fig.3)*** and they all serve the role of proteolytic degradation of cytosolic components at the lysosome. The first type is called Macro-autophagy which is responsible for the delivery of cytoplasmic cargo to the lysosome through the autophagosome that fuses with the lysosome to form an autolysosome [9]. The second type is micro-autophagy, which is responsible for the degradation of cytosolic components by direct invagination of the lysosomal membrane. Last but not least, the chaperone mediated autophagy, where a complex between targeted proteins and chaperone proteins is formed and leads to the translocation of the proteins across the lysosomal membrane resulting in their unfolding and degradation[10] .

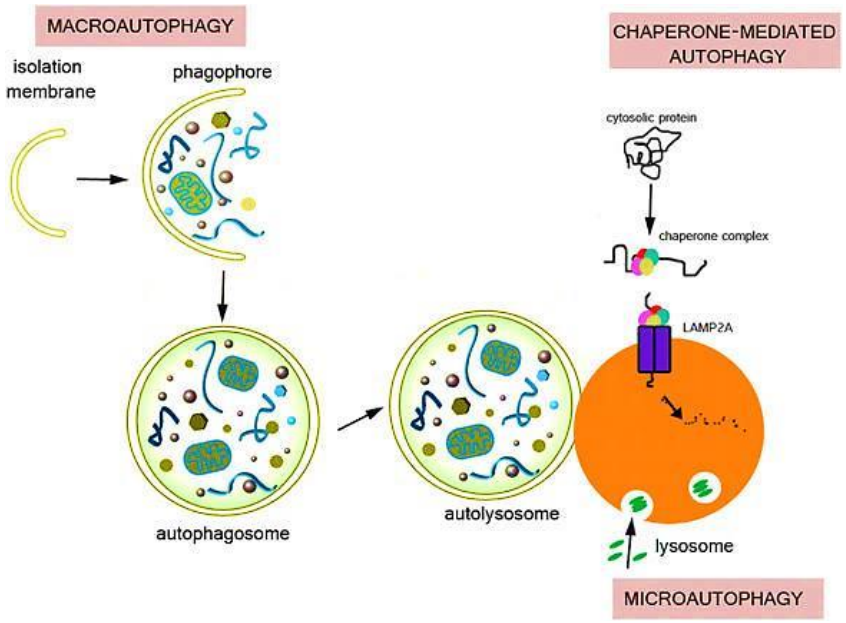


Figure 3.: The different types of autophagy. The figure demonstrates the different types of autophagy which are devided in three categories: Macroautophagy, Chaperone-Mediated autophagy and Microautophagy. V Nikoletopoulou, M-E Papandreou & N Tavernarakis ,Cell Death and Differentiation volume22, pages398–407(2015)

One of the major regulators of autophagy in the TOR kinase, which is the target of rapamycin. The TOR kinase is responsible for the inhibition of autophagy in the presence of growth factors and abundant nutrients[11]. This is accomplished by the linkage between receptor tyrosine kinases and TOR activation, by PI3K/Akt signaling molecules. Other factors that regulate autophagy include Elf2α (the eukaryotic initiation factor 2α) in response to nutrient deprivation, AMPK (5′-AMP-activated protein kinase) in response to limited energy, double-stranded RNA, and endoplasmic reticulum (ER) stress; BH3-only proteins that contain a Bcl-2 homology-3 (BH3) domain inhibition of the Beclin 1/class III PI3K and disrupt Bcl-2/Bcl-X_L complex; the tumor suppressor protein, p53; death-associated protein kinases (DAPk); the ER-membrane-associated protein, Ire-1; the stress-activated kinase, c-Jun-N-terminal kinase; the inositoltrisphosphate (IP_3) receptor (IP_3R); GTPases; Erk1/2; ceramide; and calcium [12]. The importance of autophagy is underlined by the fact that 32 different autophagy-related genes (Atg) have been identified by genetic screening in yeast and, many of these are conserved among different species [13]. These genes encode for proteins that respond to upstream signals such as Tor kinase (Atg1, Atg13, Atg17), proteins that mediate vesicle nucleation (Atg6, Atg14, Vps34, and Vps15), vesicle expansion (the Atg8 and Atg12 systems), proteins involved in the recycling pathway and disassembly of Atg proteins from mature autophagosomes (Atg2, Atg9, Atg18), and those that allow the efflux of amino acids from the degradative compartment (Atg22)[14]. Phosphatidylethanolamine (PE) connection with yeast Atg8 or mammalian LC3 during autophagy results in a non soluble form of Atg8 (Atg8-PE) or LC3 (LC3-II) that stably associates with the autophagosomal membrane. Consequently, autophagy can be detected biochemically (by assessing the generation of Atg8-PE or LC3-II) or microscopically (by observing the localization pattern of fluorescently tagged Atg8 or LC3).

In the current study, important genes in the autophagy process have been used including: Atg-18 protein which is thought to promote the formation and elongation of the phagophore through its binding to phosphoinositides. This protein is a β-propeller, formed by seven WD40 repeats, that contains a conserved FRRG motif [15]. Another important protein which is associated with the membrane of the autophagosomes is Atg-

8. This protein is highly conserved and is present on autophagosome membranes as a phosphatidylethanolamine (PE) conjugated form. In C. elegan's genome, there are two Atg-8 homologous genes: LGG-1 and LGG-2. According to Adriana Albert et al, LGG-2 localizes to autophagosomes and displays an expression pattern overlapping with LGG-1. The expression pattern of LGG-2 is modified in autophagy-induced conditions, namely dauer formation, starvation and aging.. Recent reports suggested that the transcription factor HLH-30 regulates autophagy activation in nutrient availability and is involved in lifespan regulation. Last but not least, another important protein in the autophagy pathway in *C.elegans* is the protein EPG-8. Mutations in *epg-8* cause defects in degradation of various autophagy substrates and also affect survival of animals under nutrient-depletion conditions [16].

B. CELLULAR, MOLECULAR, AND PHYSIOLOGICAL ASPECTS OF AUTOPHAGY

There are four major steps that need to occur for the activation of autophagy. The first step includes vesicle nucleation, which includes the formation of the isolation membrane/phagophore. The second stage includes the vesicle elongation and completion (growth and closure). The third stage includes the fusion of the double-membraned autophagosome with the lysosome to form an autolysosome, and lysis of the autophagosome inner membrane and breakdown of its contents inside the autolysosome

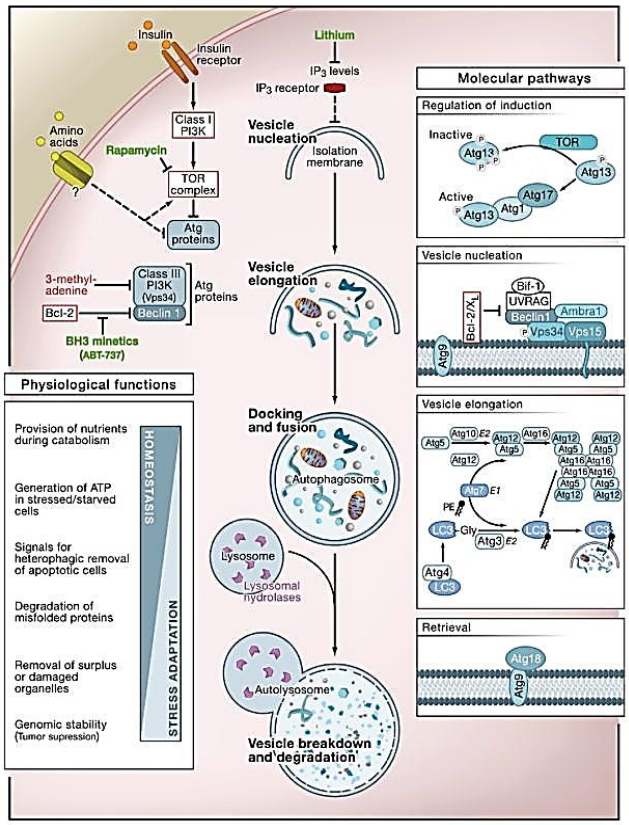


Figure 4.: Machinery of autophagy. The figure illustrates the different steps of autophagy activation. [17]

C. AUTOPHAGY AND DISEASE

During the recent years, research has revealed that autophagy plays an important role in critical times, such as during starvation, general stress, cellular differentiation and development. Genetic deletion of *Atg* genes has revealed a major connection between impairment of autophagy and several diseases such as cancer, infectious and immune system diseases.[18]

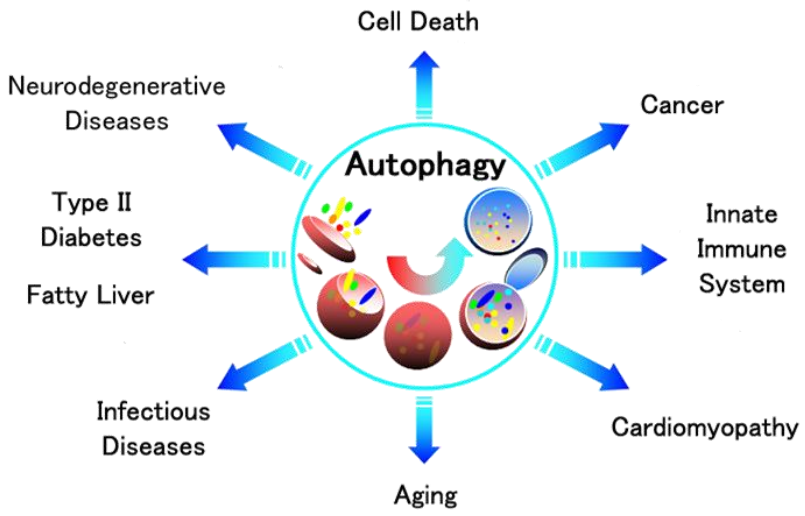


Figure 5.: The pathological functions of autophagy. The figure demonstrates illustrates the pathological consequences of Autophagy impairment such as Neurodegenerative Diseases, Cell Death, Cancer, Innate Imune System diseases, Cardiomyopathy, Aging, Infectious Diseases, Fatty Liver, Type II Diabetes.

Autophagy plays a protective role against various pathologies concerning age-related illnesses as well, like cardiovascular and neurodegenerative diseases [19]. Inhibition of autophagy can lead to accumulation of protein aggregates which mimics pathologies associated with age [20]. This can prove extremely catastrophic for postmitotic or slowly dividing cells like hepatocytes and neurons, where protein aggregate formation leads to cellular degeneration, especially, during aging. Although there is a high need for increased autophagy since the challenges and requirements for the organism are elevated, autophagic activity often decreases during ageing [21].

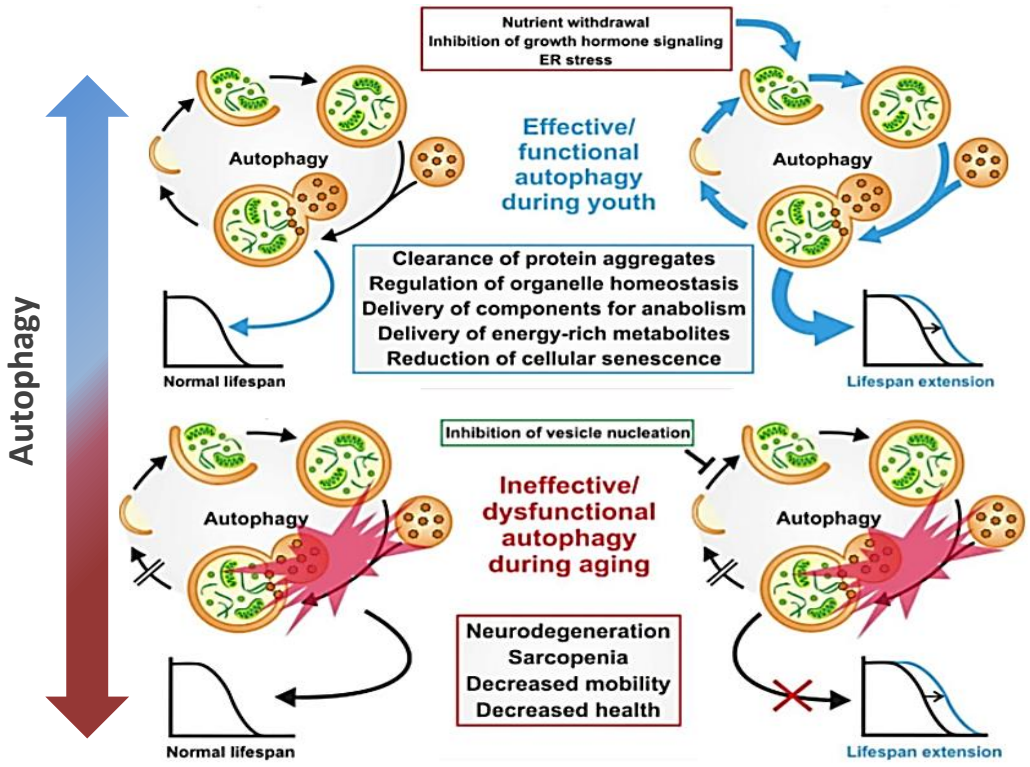


Figure 6.: Consequences of autophagy function and malfunction. The image illustrates the role of autophagy in lifespan extension, clearance of protein aggregates, regulation of organelle homeostasis etsc. When autophagy is impaired, this leads to neurodegeneration, sarcopenia, decreased mobility and decreased healthspan in general. (adapted by [21])

D. AUTOPHAGY AND THE NERVOUS SYSTEM

In the nervous system, autophagy plays a crucial role since it regulates protein homeostasis, In the nervous system, neurons contact each other to form neuronal circuits and drive behavior, relying heavily on synaptic connections. The proper development and growth of synapses allows functional transmission of electrical information between neurons or between neurons and muscle fibers [20]. Defects in synapse-formation or development lead to many diseases. Autophagy, a major determinant of protein turnover, is an essential process that takes place in developing synapses. At the onset of autophagy, proteins and cytoplasmic components are encapsulated in autophagosomes, which are double membrane organoids. The autophagosomes, then, fuse with lysosomes to form autolysosomes. Subsequently, the cargoes are degraded. Perturbations of the autophagic pathway, from its initiation with the autophagosome formation until the degradation in

the autolysosomes, have been linked with several neurodegenerative diseases. The disease-associated proteins and the key points in the pathway are represented in the following figure (fig.7). Some of the diseases demonstrated in the following figure include the Alzheimer disease, amyotrophic lateral sclerosis, β-propeller protein-associated neurodegeneration, Huntington disease, Parkinson disease [10].

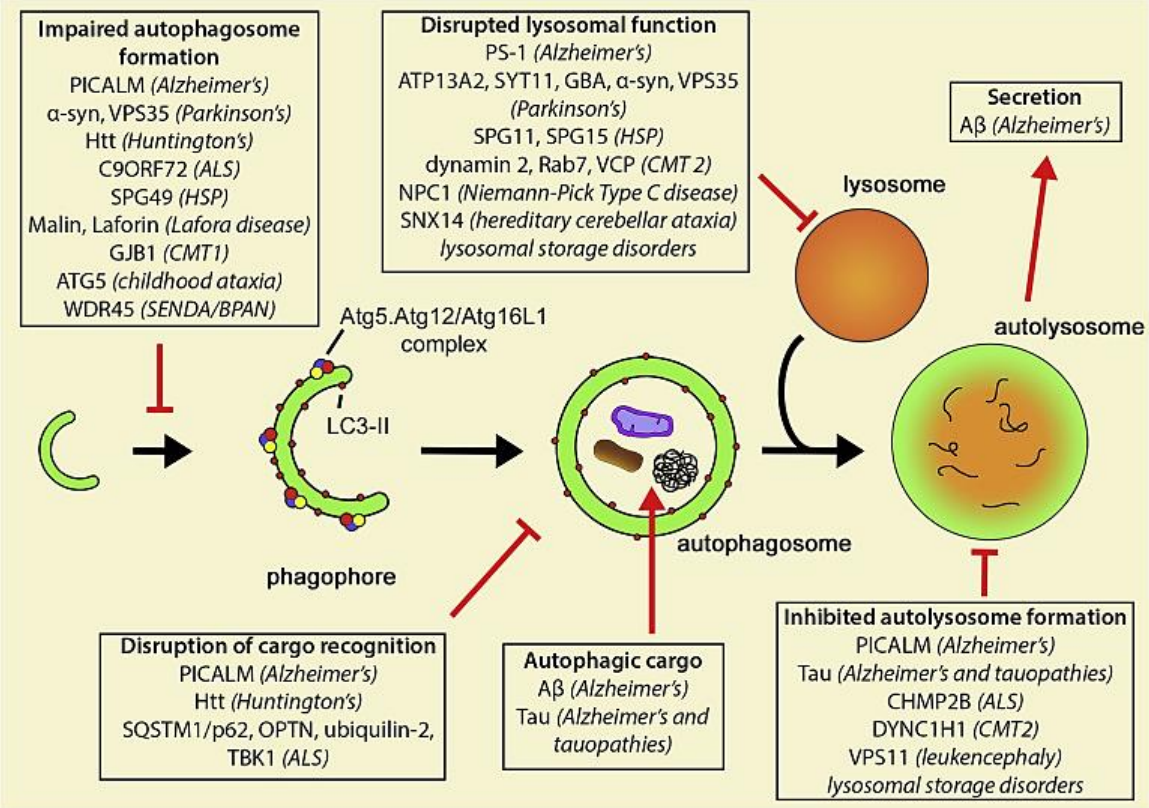


Figure 7.: Autophagy impairment results in neurodegenerative diseases. The figure illustrates the diseases linked with defects in the formation or activation of different steps in the autophagy machinery. [22]

E. NEUROTRANSMISSION AND SYNAPSES

The process responsible for the transmission of information between neurons and their targets is called Neurotransmission. This process is responsible for the regulation of

excitatory and inhibitory functions in central and autonomic nervous system. The place where neurotransmission between a presynaptic and a postsynaptic cell takes place is called the synapse [23]. Consequently, the neurotransmission onsets with the exocytosis of neurotransmitters and the following endocytosis of synaptic vesicles at nerve terminals. Neuronal exocytosis is the final step in a cycle that leads to information transfer across synapses. Acetylcholine is the major neurotransmitter during brain development and the first to be discovered. It is synthesized from choline and acetyl-CoA through the action of choline acetyltransferase (ChAT) [24]. Neurotransmitter release is mediated by a process in which the synaptic vesicles are being transferred from the presynaptic compartment to the active zone. This process is called exocytosis. The necessity for many cycles of exocytosis and release of neurotransmitter demands many cycles of synaptic vesicle trafficking as well [25]. The initiation step of the synaptic vesicle cycle is the import of Ca2+ in the presynaptic neuron that triggers exocytosis that is then followed by different routes of endocytosis and recycling. For the neurotransmitter release, the synaptic vesicle should dock, prime and fuse with the presynaptic membrane. The protein Munc-18-1 plays an important role for the fusion of synaptic vesicle through the collaboratation with SNARE proteins [26].

Other important proteins include the synaptotagmin which acts as a Ca2+ sensor for the fast release of the neurotransmitter and the Rab3 protein which regulates release by binding to the active zone proteins RIM1α and RIM2α.

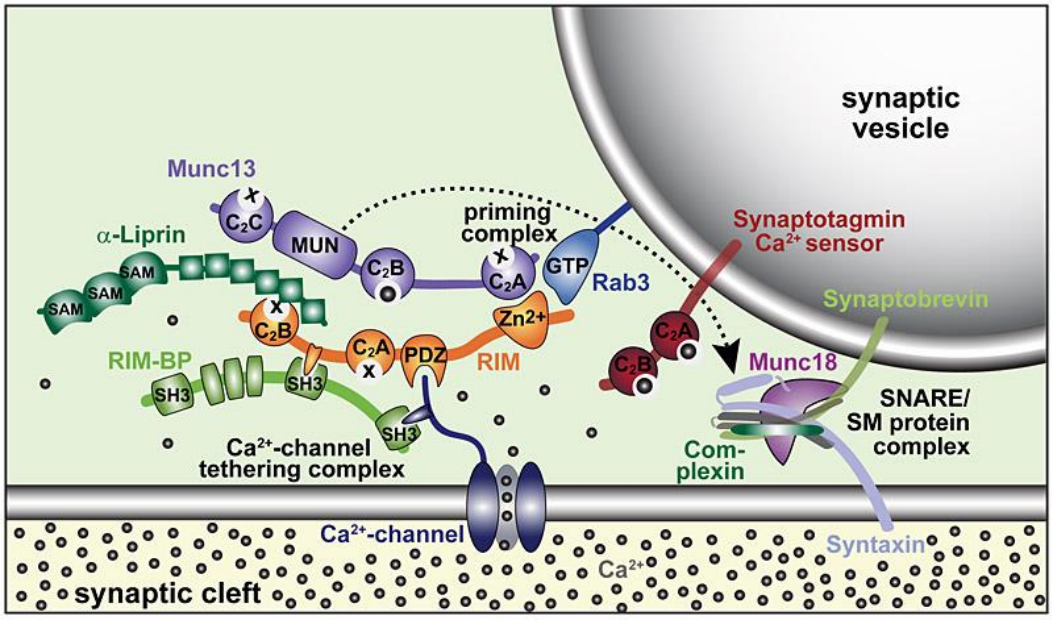


Figure 8.: Neuronal exocytosis pathway. The figure illustrates the proteins involved in the priming, docking and fusion of the synaptic vesicles with the presynaptic compartment in order to release the neurotransmitter in the synaptic cleft [27].

F. AUTOPHAGY IN THE SYNAPSE

Numerous studies have indicated a role of autophagy in the neurotransmission. More specifically, basal and induced autophagy can reduce the kinetics of neurotransmitter release and the density of synaptic vesicles within the presynaptic region of dopaminergic neurons (Hernandez et al., 2012). Consequently, autophagy in the presynaptic compartment can contribute to mechanisms involved in synaptic plasticity or synaptic potentiation and depression. Autophagy has also been linked in the processes of the degradation of postsynaptic receptors leading to long-term synaptic depression. Examples of degradation of post synaptic receptors are inhibitory GABAA receptors and AMPAR receptors[28].

Part II: Coordination between mitophagy and UPR^{mt}

A. THE MITOCHONDRION

Mitochondria is a double-membrane-bound organelle which are located in the cytoplasm of most eukaryotic organisms and play a major role in the organismal evolution [29]. They are responsible for the generation of the cell's supply of ATP (adenosine triphosphate) [30] which is used as a source of chemical energy. The word mitochondrion comes from the Greek μίτος, mitos, "thread", and χονδρίον, chondrion, "granule". Mitochondria are between 0.75 and 3 μm in diameter. By visualization of mitochondria using live microscopy techniques, have demonstrated that mitochondria are highly mobile and active and their shape and size is constantly changing [31].

Every mitochondrion consists of two membranes, the external mitochondrial membrane and the internal mitochondrial membrane which forms multiple cristae. These membranes are forming two distinct compartments: the internal mitochondrial matrix and the transmembranic area which is important for the communication of the two membranes. The external mitochondrial membrane consistes mainly of porins whereas the internal mitochondrial membrane acts as a barrier with high selectivity [32].

Mitochondria contain their one genetic information which encodes for rRNA, tRNA and mRNAs for the synthesis of proteins in ribosomes. However, this synthesis is inadequate for the functional and structural needs of the organisms[33]. For this reason molecules are often introduced from the cytoplasm. Usually the number of molecules required for the proper function of the mitochondria is insufficient and as a result these organisms are not fully autonomous and are characterized as semi-autonomous. The DNA of mitochondria is circular and varies in size from 6000 bp up to 300,000 bp for mitochondria of some plant cells. The mitochondria DNA has been found in many identical copies in each mitochondria.

A. MITOPHAGY

Autophagy is categorized in two different forms: non-specific autophagy and specialized autophagy. The first one is observed in the cell in cases of nutrient absence. Through this process the cells are provided with basic structural units and substances for metabolism and energy demands until nutrients are again available to the cell in the extracellular environment[34]. On the contrary, specialized autophagy is observed when there is plenty of nutrients and occurs to remove unnecessary / damaged organelles or protein aggregates that may prove toxic and detrimental for the cell. Some forms of specialized autophagy that have been observed in the processes of the degradation of peroxisomes, ribosomes, in parts of the endoplasmic network and in cases of invasion of pathogens in the organism.

A very defined form of specialized autophagy is mitophagy (mitophagy), which mediates the selective removal of mitochondria. Mitophagy was first observed in mammalian cells by electron microscopy[11].

Mitophagy has been studied during the last years and has been implicated in the control of Mitochondria number, in a way to compensate for the metabolic needs of the cells and has been also a part of the mitochondria quality control mechanisms since it serves a role in removing damaged mitochondria. In Saccharomyce cerevisiae. [35] and in mammalian cells [36] it has been observed that mitophagy occurs before fission (Fission) of mitochondria [36]. It acts as a quality mechanism control where it removes the damaged mitochondria before they are duplicated. In addition to this quality control, mitophagy has been shown to be required for one continuous control that regulates the number of mitochondria depending on the metabolic activity of cells [37], but also during specialized developmental stages in mammalian cells.

In the current study, important genes in the mitophagy process have been used including: *pink-1* gene, which is important in Parkinson's disease, since mutations in the gene have been linked with increased sensitivity to oxidative stress and mitochondrial dysfunction and *Parkin*, which is the mammalian homolog of the C. *elegans pdr-1*[38]. *Parkin* encodes a protein with E3 ligase activity and loss of function of the E3 ligase leads to autosomal recessive-juvenile parkinsonism. It also plays a role as major regulator gene of

mitophagy. Finally, in the current study we use the *dct-1* gene which is a key mediator of mitophagy and longevity [39]

B. UPR^{mt}

Mitochondrial dysfunction is the primary cause of numerous heterogenous disorders known as mitochondrial diseases. Furthermore, during aging, due to accumulation of damaged mtDNA and increased levels of ROS (reactive oxygen species) the mitochondrial function declines. This dysfunction is implicated with several neurodegenerative diseases such as Parkinson disease and Alzheimer disease due to the loss of muscle strength and neuronal function [40]. The mitochondrial unfolded protein response (UPR^{mt}) is a transcriptional response that is activated by multiple forms of mitochondrial dysfunction. When mitochondrial function declines, the UPR^{mt} is activated to promote the repair and recovery of this network and maintain cellular function [41]. Mitophagy and UPR^{mt}, consequently, belong to a family of mechanisms called Mitochondria quality control mechanisms. It has not been already known, however, how these two systems cooperate and which is the stress signal that leads to the suppression of one and activation of the other. In the current study, we aim to investigate the role of E3 ubiquitin ligase CHIP in this process.

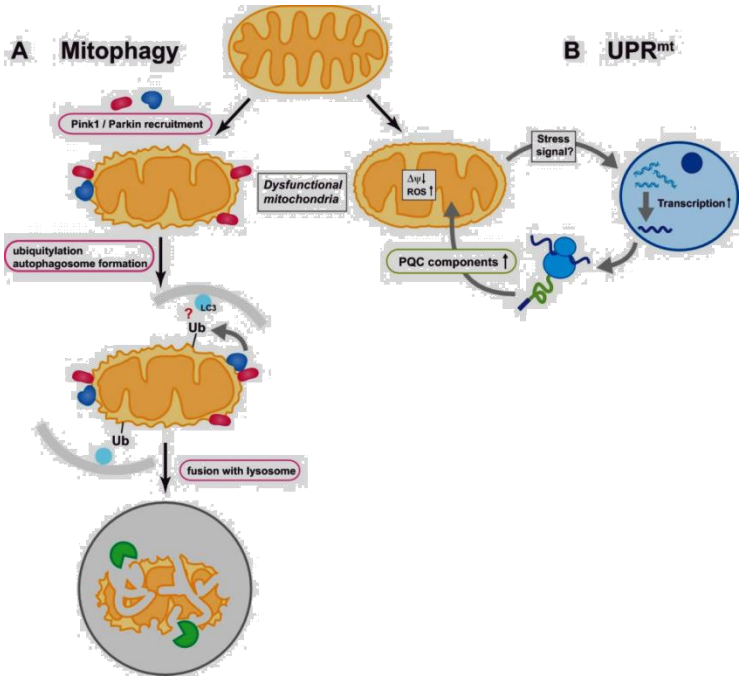


Figure 9.: A. Mechanism of Mitophagy. The figure illustrates the mechanism of function of Mitophagy . In mitochondria, which lose the membrane potential or accumulate unfolded proteins, PINK1 is stabilized on the outer

mitochondrial membrane (OMM) .PINK1 recruits Parkin and activates latent E3 ligase activity of Parkin .Parkin activation leads to proteasomal degradation of OMM proteins and to selective autophagy of damaged mitochondria.

B. UPR^{mt} mechanism. Oxidative phosphorylation perturbation, excessive reactive oxygen species (ROS), impaired complex assembly and accumulation of misfolded proteins impair mitochondrial protein import efficiency. Activation of a transcription factor ATFS-1 is then occurred in order for its translocation to the nucleous and the activation of UPR^{mt}. ATFS-1 also induces genes that play a role in re-establishment of the mitochondrial function.

C. CHIP, A QUALITY-CONTROL E3 UBIQUITIN LIGASE

CHIP is a quality-control E3 ubiquitin ligase that plays a role in keeping cellular balance between folding and degradation [42]. It acts along with other control mechanisms such as chaperones and degradation machineries ubiquitylating damaged proteins and leading them to disposal by the lysosome, proteosomal degradation and autophagy. Interestingly, it has been shown that deletion of CHIP accelerates aging [43]. This is achieved by the mono-ubiquitilation of the Insulin like receptor which leads to reduced insulin signaling and as a result lifespan extension. Another role of CHIP is the interaction with Parkin, a E3-ubiquitin-protein ligase that ubiquitinates itself and specific substrate proteins playing a protective role by sequestering misfolded proteins. Together, parkin, chip and heat-shock protein 70 form a complex enhancing the E3 enzymatic activity of parkin and thus its capability of inhibiting cell death caused by unfolded proteins stress [44]. Based on this knowledge, in our current study we aim to elucidate the role of CHIP ligase in the threshold of the activation of mitpohagy or UPR^{mt}.

MATERIALS AND METHODS

A. Molecular Biology

Bacteria culture

Recipes:

LB broth (Luria-Bertani medium):

- 10g bacto-tryptone
- 5g yeast extract
- 10g NaCl
- (15g bacto-agar for solid culture plates)

LB + ampicillin:

- The antibiotic ampicillin is added in LB medium in a final concentration of 100μg/ml.

LB + tetracycline:

- The antibiotic tetracycline is added in LB, in a final concentration of 10μg/ml.

Transformation of competent bacteria *E. coli*

Procedure:

1. Retrieval of competent cells from -80^{o}C
2. Incubation on ice (4^{o}C) for almost 3 minutes in order for the cells to defrostκύτταρα
3. Addition of 5-10ul plasmid DNA and soft mix
4. Incubation on ice (4°C) for 30 minutes (soft mixing every 10 minutes)
5. Incubation at 42°C for 45 seconds (heat shock)
6. Incubation for 2 minutes on ice (4°C)
7. Addition of 1ml LB medium (or SOC)

8. Incubation at 1 hour at 37°C shaking
9. Centrifuging for 2 minutes at 3000rpm in room temperature
10. Discard the supernatant and keep ~100-150μl in the eppendorf
11. Careful suspension of the pellet with the pipette
12. Plating of cells on plate with a glass rod
13. Incubation of the plates at 37°C overnight

DNA extraction and purification for plasmid DNA

Minipreparation of plasmid DNA is a rapid, small-scale isolation of plasmid DNA from bacteria. It is based on the alkaline lysis method as described (Sambrook et al., 2001). The extracted plasmid DNA resulting from performing a miniprep is itself often called a "miniprep". Minipreps are used in the process of molecular cloning to analyze bacterial clones. A typical plasmid DNA yield of a miniprep is 50 to 100 μg depending on the cell strain.

Procedure:

1. Inoculate 2ml LB medium (containing antibiotic) with a bacterial clone, culture with vigorous shaking at 37 degree for 14-16 hrs.
2. Aliquot 1.5ml culture into a 1.5 ml LABELED microcentrifuge tube
3. Harvest the bacteria by spinning at 12000rpm (~11000g) for 1 min.
4. Remove (aspirate) supernatant and keep pellet.
5. Resuspend bacterial pellet by complete vortexing in 150ul solution P1 resuspension buffer containing RNAse in a final dilution of 1 to 10. (, 50mM glucose, 25mM Tris-HCl, pH8.0, 10mM EDTA)
6. Add 150ul of P2 buffer (0.2M NaOH, 1% SDS, ddH20) and mix gently by inverting 5-6 times at room temperature. To make lysis buffer mix equal volumes of 800mM NaOH and 4% SDS solutions and make enough for the number of preps you are processing plus

7. Incubate for2 minutes at room temperature
8. Add 150ul of P3 (3M potassium acetate,11.5 % glacial acetic acid ddH20), soft vortex, and incubate on ice for 2 min
9. Centrifuge for 5 minutes at 14000rpm
10. Transfer the supernatant in new Eppendorf and add 0.6x volume of isopropanol in the supernatant
11. Mix well and centrifuge for 15min at 4oC full speed.
12. Keep the pellet and discard supernatant
13. Add 500ul EtOH and centrifuge for 15min at 4oC full speed.
14. Centrifuge 15min AT 4oC full speed
15. Remove completely all ethanol and add 30ul of ddH20.

The final concentration of DNA should be 0.5 – 2.0 μg /μl and the 260/280 value should be above

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) *is a technique used in molecular biology to amplify a single copy or a few copies of a specific segment of DNA, generating thousands to millions of copies. For performing a PCR a DNA template is needed, primers that include the DNA sequence needed to be amplified, the buffer of the enzyme, dNTPs,*
The PCR process includes three basic steps. To begin with, the DNA template is heated at 92-95°C in order for the DNA to denaturate. The, the temperature goes down at 55-65°C for the primers to anneal to the monoclonal DNA template. Afterwards, the temperature is increased at 72oC in order for the DNA polymerase to start synthesizing the DNA sequence included in between the primers. This process is repeated for several cycles until the generation of thousand to millions copies.[45]

B. Biochemical assays

Immunoprecipitation preparation for gel loading

Recipe

LUSIS BUFFER 1x buffer (keep at 4oC for maximum 2 weeks)	
Tris-HCl pH 7.3	50mM
Triton x-100	1%
NaCl	50mM
Coctail inhibitors	1x **Add just before use** Prepare one tablet in 700ul ddH20 and this will be 10x. Keep aliquots of the inhibitors in -20oC and thaw only once

WASH BUFFER 1x buffer (keep at 4oC for maximum 2 weeks)	
Tris-HCl pH 7.3	50mM
Triton x-100	0.1%
NaCl	50mM

6X Laemlli Sample Buffer	
Na-Trizine pH 7.8	300mM
DTT or beta-mercaptoethanol	300mM
Glycerol	42%
SDS	12%
Triton x-100	0.15%
Bromophenol Blue	0.006%

Procedure

1. Add 2ul of antibody per 25ul beads + 73ul lysis buffer without inhibitors
2. Rotate at $4^{o}C$ overnight

3. Collect the worms with M9 buffer in 15ml falcon*
4. Wash the animals three times to remove bacteria and remove the supernatant
5. Freeze in -80^{o}C for 30 minutes at least or until you perform the Immunoprecipitation lysis
6. Add lysis buffer containing protease inhibitors (the volume of the lysis buffer to be added depends on the pellet, but it should be always more than 200ul)
7. Sonicate worms (4 rounds, 20seconds each and one minute rest between the rounds)
8. Centrifuge at 14.0000 rpm for fifteen minutes, at 4^{o}C
9. Keep 25ul/well of lysate for input (or more according to the experiment)
10. Split the beads (100ul per condition) after careful resuspension
11. Transfer the rest supernatant in the tubes containing the beads and antibody
12. Incubate for three hours at 4^{o}C, rotating
13. Centrifuge at 2000rpm for five minutes at 4^{o}C
14. Remove supernatant which is the unbound and keep it in new Eppendorf tubes
15. Wash beads with 1ml Washing buffer without inhibitors for three times (each time centrifuge 2000rpm, five minutes, 4^{o}C)
16. Remove carefully the washing buffer and add 6x laemmli buffer (80ul in each condition)
17. Add 6x laemmli sample buffer in the eppendorfs containing the protein input and the unbound protein
18. Boil the samples for ten minutes at 95 ^{o}C
19. Load the samples in a sds-page gel to perform either western or blue-silver staining

Immunoprecipitation of the UNC-18 protein fused with FLAG peptide

1. 35 ul beads were incubated with 5ul of flag antibody plus 60ul of lysis buffer without the cocktail inhibitors O/N.
2. *elegans* worms expressing the UNC-18 protein fused with FLAG peptide were sonicated in lysis buffer containing cocktail inhibitors (40 big plates full of animals).
3. The supernatant was incubated for 3 hours with the beads+antibody and then it was immunoprecipitated and eluted from beads with laemmli buffer.
4. The input, unbound and immunoprecipitated proteins were run in a 12% acrylamide gel and detected with anti-flag antibody diluted 1:1000 in 5% milk and detected by secondary HRP Rabbit antibody diluted 1:10000 in 3%milk).
5. The size of the UNC-18 protein fused with FLAG peptide is expected to be at 68,5kDa

Immunoprecipitation followed by Filter Aided Sample Preparation (FASP) Method

Recipe

LUSIS BUFFER 1x buffer (keep at 4oC for maximum 2 weeks)	
Tris-HCl pH 7.3	50mM
Triton x-100	1%
NaCl	50mM
Coctail inhibitors	1x **Add just before use** Prepare one tablet in 700ul ddH20 and this will be 10x. Keep aliquots of the inhibitors in -20oC and thaw only once

WASH BUFFER 1x buffer (keep at 4oC for maximum 2 weeks)	
Tris-HCl pH 7.3	50mM
Triton x-100	0.1%
NaCl	50mM

6X Laemlli Sample Buffer	
Na-Trizine pH 7.8	300mM
DTT or beta-mercaptoethanol	300mM
Glycerol	42%
SDS	12%
Triton x-100	0.15%
Bromophenol Blue	0.006%

UA solution (freshly prepared)	
Urea Sigma, U5128	8M
Tris-HCl pH 8.5	0.1 M

IIA solution (freshly prepared)	
UA solution	add.
iodoacetamide	0.05 M

ABC solution	
NH4HCO3	0.05M
ddH20	add.

Elution buffer	
SDS	4%
DTT	0.1 M
Tris-HCl	100mM
ddH20	add.

Trypsin stock: 0.4μg/μl

Equipment

- Microcon YM-30 (Millipore, Cat. MRCF0R030)
- Refrigenerated Bench-top centrifuge (Eppendorf 5415R), temperature 20Oc
- Wet chamber with a racj of Eppendorf tubes
- Thermo-mixer set to 20oC
- UV-Spectrometer, Quartz-cuvette
- Sonication bath
- Homemade C18 columns

Procedure

Sample preparation for FASP

1. Add 2ul of antibody per 25ul beads + 73ul lysis buffer without inhibitors
2. Rotate at 4^{o}C overnight
3. Collect the worms with M9 buffer in 15ml falcon*
4. Wash the animals three times to remove bacteria and remove the supernatant
5. Freeze in -80^{o}C for 30 minutes at least or until you perform the Immunoprecipitation lysis
6. Add lysis buffer containing protease inhibitors (the volume of the lysis buffer to be added depends on the pellet, but it should be always more than 200ul)
7. Sonicate worms (4 rounds, 20seconds each and one minute rest between the rounds)

8. Centrifuge at 14.0000 rpm for fifteen minutes, at 4^{o}C
9. Keep 25ul/well of lysate for input (or more according to the experiment)
10. Split the beads (100ul per condition) after careful resuspension
11. Transfer the rest supernatant in the tubes containing the beads and antibody
12. Incubate for three hours at 4^{o}C, rotating
13. Centrifuge at 2000rpm for five minutes at 4^{o}C
14. Remove supernatant which is the unbound and keep it in new Eppendorf tubes
15. Wash beads with 1ml Washing buffer without inhibitors for three times (each time centrifuge 2000rpm, five minutes, 4^{o}C)
16. Remove the washing buffer
17. Add in the beads the elution buffer (50ul per condition)
18. Boil for 15 minutes at 95oC
19. Centrifuge at 14000 rpm for ten minutes at room temperature
20. Transfer 30ul of the supernatant in a new-clean Eppendorf tube

Sample Processing for FASP

21. Mix up to 30µl of a protein extract with 200µl of UA in the filter unit and centrifuge at 14,000 x g for 15 min.
22. Add 200µlof UA to the filter unit and centrifuge at 14,000 x g for 15 min.
23. Discard the flow-through form the collection tube.
24. Add 100 µl IAA solution and mix at 600 rpm in a thermo-mixer for 1 min and incubate without mixing for 20 min.
25. Centrifuge the filter units at 14,000 x g for 10 min.
26. Add 100 µl of UA to the filter unit and centrifuge at 14,000 x g for 15 min. Repeat this step twice.

27. Add 100 µl of ABC to the filter unit and centrifuge at 14,000 x g for 10 min. Repeat this step twice.

28. Concentration of the peptides can be estimated by UV spectrometer assuming that 0.1% solution of vertebrate proteins has at 280 nm an extinction of 1.1 absorbance units. (1mg/ml solution has 1.1 au). Always record a spectrum from 240-340 nm. It should have a distinct peak with a maximum at 270-280 nm and the extinction at 320 nm has to be 0.

29. Add 50 µl ABC with trypsin (enzyme to protein ratio 1:100) and mix at 600 rpm in thermo-mixer for 1 min.

30. Incubate the units in a wet chamber at 37°C for 4 -18 h.

Desalting protocol Homemade C18 columns

1. Pre-treat Sample

a) Dilute sample with a 5% FA (Formic Acid) solution to adjust the pH to less than 4 (usually 20µl of 5% FA per sample).

2. Conditioning - Preparing the C18 column

a) Wet the C18 columns by aspirating 20µl of a 90% acetonitrile (ACN) / 5% FA solution into the tip. Dispense this solution into a container.

b) Repeat with a second 20µl aliquot of the 5% FA/water solution.

3. Sample Application

a) Aspirate up to 20µl of the pre-treated peptide or protein sample onto the conditioned C18 column. (To improve binding of peptides and proteins, dispense sample into a clean vial or well plate).
b) Aspirate and dispense the same sample up to 2 times and discard the sample.

4. Rinse the C18 tip

a) Aspirate 20 µl of 5 % FA into the C18 tip, dispense into a waste container.
b) Repeat with a second 20µl aliquot.

5. Elution

For **LC/MS or LC/MS/MS ANALYSIS**:
a) Aspirate 40 - 60 µl of a 90% acetonitrile (ACN) / 5% FA solution and dispense into a vial.

Western blot

Recipe

RESOLVING GELS (7,5ml)					
Ingredients	**%**	**7.5%**	**10%**	**12%**	**15%**
1.5M Tris pH 8.8		2.5ml	2.5ml	2.5ml	2.5ml
30% acrylamide		1.875ml	2ml	3ml	3.75ml
Water		3.075ml	2.95ml	1.95ml	1.2ml
10%SDS		75ul	75ul	37.5ul	75ul
10%APS		37.5ul	37.5ul	37.5ul	37.5ul
TEMED		12.5ul	12.5ul	12.5ul	12.5ul

STACKING GELS (5ml)	
0.5M Tris pH 6.8	620ul
30% acrylamide	833ul
Water	3.817ml
10%SDS	50ul
10%APS	50ul
TEMED	5ul

Procedure

Make the separating gel:

1. Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
2. Prepare the gel solution (as described above) in a separate small beaker.
3. Swirl the solution gently but thoroughly.
4. Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates.
5. To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until a overflow.
6. Wait for 20-30min to let it gelate.

Make the stacking gel:

7. Discard the water and you can see separating gel left.

8. Pipet in stacking gel untill a overflow.

9. Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.

10. Load samples on the gel into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.

11. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

12. Set an appropriate volt and run the electrophoresis when everything's done.

13. As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker almost reaches the foot line of the glass plate.

Blotting Transfer:

14. Fill the Bio-Ice cooling unit with water and store it in –20C until ready to use. After use, return the cooling unit to the freezer for storage.

15. Cut the membrane and filter paper to the dimensions of the gel.

16. Equilibrate the gel and soak the member, filter paper, and fiber pads in transfer buffer for 15 min.

17. Place the cassette, with the gray side down, on a lean surface.

18. Place one pre-wetted fiber pad on the gray side of cassette.

19. Place a sheet of filter paper on the fiber pad.

20. Place the equilibrated gel on the filter paper.

21. Place the pre-wetted membrane on the gel

22. Complete the sandwich by placing a piece of filter paper on the membrane.

(Removing any air bubbles, which may have formed; it is very important for good results. Using a glass tube to gently roll air bubbles out)

23. Add the last fiber pad.

24. Close the cassette firmly, being careful not to move the gel and filter paper sandwich.

25. Lock the cassette closed with the white latch.

26. Place the cassette in module. Repeat for the other cassette if you have one more.

27. Add the frozen Bio-Ice cooling unit. Place in tank and completely fill the rank with buffer.

28. Put on the lip; plug the cable into the power supply

29. Transfer conditions:1 Hour transfer, 100V , 350mA

30. After the transfer, unclamp the blot sandwich and remove the sheets of blotting paper, exposing the blot membrane. Mark the side of the membrane that was facing the gel. Mark the position of the pre-stained markers, since they may fade away during detection.

Detecting the protein specific antibodies

31. Blocking the membrane with 5% nonfat dried milk in PBS with 0.1%Tween20, for 1 hour

32. Incubate with Primary Antibody in 5% milk overnight at 4oC by shaking.

33. Wash in PBS with 0.1%Tween20, 3 X, and 10 min of each time

34. Incubate with secondary antibody (peroxidase-conjugated goat anti-mouse IgG, etc.) following the manufacturer's instruction, usually 1:200 to 1:2000; 1 hour at room temperature

35. Wash in PBS with 0.1%Tween20, 4 X, and 15 min of each time

Developing the membrane

36. Transfer the membranes in a tray and add 2ml approximately of ECL in dark

37. The membrane is ready for development in Chemidoc system

Western blot for the detection of the endogenous proteins SYNAPTOBREVIN-1 and UNC-10

For the Western blot for the detection of the endogenous proteins SYNAPTOBREVIN-1 and UNC-10 in wild type animals both in the supernatant of the sonicated worms and the pellet we used the following antibodies and buffers:

The dilution for the antibody of SNB-1 protein (Hybridoma) was 1 to 50 in 5% milk and the antibody was detected with anti-mouse HRP secondary antibody diluted 1:10000 in 3% milk. The protein levels were detected by α-tubulin (raised in mouse) using a dilution of 1:10000 in 5% milk and detected by anti-mouse HRP secondary antibody diluted 1:10000 in 3% milk. The gel used contained 15% acrylamide. The dilution for the antibody of UNC-10 protein (Hybridoma) was 1 to 100 in 5% milk and the antibody was detected with anti-mouse HRP secondary antibody diluted 1:10000 in 3% milk. The protein levels were determined by α-tubulin (raised in mouse) using a dilution of 1:10000 in 5% milk and detected by anti-mouse HRP secondary antibody diluted 1:10000 in 3% milk. The gel used containes 7.5% acrylamide.

C. *C.elegans* techniques

Preperation of NGM (Nematode Growth Media) plates for *c.elegans* growth

Recipe:

NGM (Nematode Growth Media) (1lt)

- 3g NaCl
- 2.5g bactopeptone
- 0.2g streptomycin
- 17g agar
- 1 ml CaCl2 1M
- 1 ml MgSO4 1M
- 1 ml cholesterol (5mg/ml σε 100% αιθανόλη)
- 1 ml nystatin (100mg/ml σε 70% αιθανόλη)
- 25 ml K·PO4* 1M, pH 6

The solutions CaCl2, MgSO4, K·PO4, cholesterol and nystatin are added after autoclaving the NGM medium.

Bleaching of *c.elegans* strains to synchronize the population

Recipe:

Bleaching solution

- 0.5M NaOH
- 1% NaOCl
- ddH2O ad.

Procedure:

For the bleaching procedure, petri dishes containing many gravid hermaphrodites *c.elegans* were washed with M9 medium and transferred in a sterile Eppendorf tube. After centrifuge for 30 seconds at 13000 xg, the superenatant was removed without disturbing the worm's pellet letting a final volume of supernatant of approximately 200ul. A 2.5 x volume of freshly prepared bleaching solution was added. After observation of the worms under the steresoscope and vortexing for a total of 4 minutes the worms were

centrifuged for 1 min at 13000 xg to pellet released eggs. The supernatant was aspirated and the worms were washed with 1ml of M9 buffer twice. Finally, after removing the supernatant, a 100ul amount of liquid was left in the Eppendorf tube and the eggs were resuspended using a Pasteur pipette and transferred in a fresh NGM plate seeded with an E.coli OP50 lawn.

The bleaching solution is dissolving the whole animal without penetrating however in the egg. The final supernatant obtained after the bleaching does not contain a completely synchronized population, since the eggs are in different stages..

Freezing of *c.elegans* strains

For freezing *c.elegans* strains/lines, the medium that is used contains the following:

Recipe:

Freezing Buffer

- 0.05M KPO4 (pH=6.0),
- 20% Glycerol
- 3 mM MgSO4
- 0.585 %w/v NaCl

Procedure:

In 5 NGM plates add 5 L4 stage worms. After about 2 generations (appr. 6 days). The descedants from F1 give descendants (F2) in the stage L1-L2 (robust stage for freezing). Prepare 10 cryvials 2 for the liquid N2 and 8 suitable for -80 freezer) and one 15ml falcon tube and place them on ice. Obtain the worms from the plates with the use of the M9 buffer (9ml) and by mobbing the plates gently in order for worms to detach from the plates and place them in the falcon tube. To this volume (approximately 7.5ml) we add the same amount of freezing solution and mix thoroughly. Fill up the cryotubes and place them in the -80 freezer. Two weeks late measure and check whether freezing was correctly performed, by retrieving big amounts of L1-L2 worms.

Isolation of RNA from *C.elegans*

Procedure

1. Pick 40 worms into 40ul M9 in a RNase-free Eppendorf tube
2. Pellet worms by spinning 14000 rpm
3. In the hood add 200ul Trizol
4. Vortex by hand for about 40seconds and then 4 minute vortex
5. 1 cycle a. -80oC for 10minutes
6. 65oC for 5 minutes
7. In the hood add 50ul of chloroform (CHCL3)
8. Vortex for 15 seconds and let them at RT for 3 minutes
9. Centrifuge at 12000rpm for 15 min at 4oC
10. Transfer the clean layer (-125ul_ into a new RNA-free eppendorf tube Repeat steps 6-9
11. Add 125ul 2-propanol and invert to mix
12. Let it at RT for several minutes (-3min)
13. Spin down at 12000rpm for 15 min at 4oC Keep pellet.
14. Leave a few uls at the bottom so as not to disturb the pellet
15. Add 500ul 70% EtOH (use RNase-free ddH20)
16. Spin down at 14000rpm for 15minutes at 4oC
17. Remove as much as supernatant and air-dry pellet (in the hood
18. Dissolve the pellet in 20ul of RNase-free ddH20
19. Quantification: OD260/OD280: 2.0 for pure RNA
20. Autoclave 2 times (blue and yellow) tips and ddH20

Single Worm PCR

Recipe:

Worm PCR Lysis Buffer * Common stock in -20°C stock freezer.

- 50mM KCl
- 10mM Tris (pH 8.3)
- 2.5mM MgCl2
- 0.45% NP-40 (IGEPAL)
- 0.45% Tween-20
- 0.01% Gelatin

Add 0.1mg/mL of proteinase K before use.

Precedure:

1. Add proteinase K to lysis buffer (90µL lysis buffer + 10µL 10mg/mL proteinase K).
2. Place 3-10µL of lysis buffer in top of 1.5mL Eppendorf tube.
3. Pick single worm into lysis buffer.
4. Spin worm to the bottom of tube by spinning in centrifuge for 15 seconds at 14,000 rpm.
5. Lyse the worm and release the genomic DNA by heating tube to 65°C for 60-90 minutes.
6. Inactivate the proteinase K by heating to 95°C for 15 minutes.

Generation of male *c.elegans*

Males (5AA; X0) arise from fusion of nullo-X gametes (gametes that lack an X chromosome) and normal X-bearing gametes. Nullo-X gametes are generated by spontaneous non-disjunction of the X chromosome during meiosis in the germ line. This, however, occurs rarely in the hermaphrodite germ line. Consequently, males occur infrequently in cultures propagated by hermaphrodite self-fertilization (ca. 0.1-0.2%; Ward and Carrel,1979; Hodgkin and Doniach, 1997). However, it is crucial in our

studies, to generate males. For this reason, it is frequently used a procedure named heat shock which is described below.

Procedure:

1. 8 to 10 hermaphrodites in L4 stage are transferred in a petri dish containing NGM and OP50 lawn
2. They are incubated for 50 min in 37^{o}C
3. Then they are transferred in 20oC
4. The descedants are then examined for the presence of male worms.

Crossing hermaphrodites with males

Procedure:

After the production of males from the heat shock process, a sufficient number of male animals was placed in a 30mm petri dish plate containing NGM medium and a very tiny OP50 lawn of approximately 20ul. In the plates were added hermaphrodites (from the strain we wanted to cross) in an analogy of 1:4 (hermaphrodites:males). The plates were placed in 20oC and incubated for 4 days. On the 3rd day of incubation, the male animals were removed and killed. On the 4th day, the plates were checked for the presence of male animals which is an indicator of a successful cross.

After the cross is generated, L4 animals were chosen depending on a specific phenotype that one of the two animals or both have, or they were chosen randomly. These L4 animals were placed in NGM plates containing an OP50 lawn separately. (One animal per plate). This was characterized as the F1 generation. The plates containing the L4 animals were placed in 20oC and when they laid their eggs, their genotype was tested by PCR. If an animal was found to be eterozygous for the gene of interest, the petri dishes containing the eggs of this specific animals were left to grow until L4. Then, L4 animals from this specific petri dish were chosen and were placed in NGM plates containing an

OP50 lawn separately. (One animal per plate). This was characterized as the F2 generation. The plates containing the L4 animals were placed in 20oC and when they laid their eggs, their genotype was tested by PCR for homozygous animals.

Lifespan assays with RNAi treatment

L4 animals were placed in NGM (RNAi) petri dishes that contained 1mM IPTG and were seeded with HT115(DE3) bacteria transformed with either an empty pl4440 vector as a control or with pl4440 vector containing the gene of interest. For the synchronization of the population, the descendants were grown in NGM(RNAi) petri dishes that were previously seeded with HT115(DE3) bacteria transformed with an empty vector. In this way, we excluded any possible defects that the silencing of the target-gene may have during development. All lifespan experiments were realized under 20oC. Furhtermore, in each petri dish NGM(RNAi) were placed groups of 20 animals and in total number of 100 animals for each experiment. t=0 was the first day of adulthood. The animals were transferred in new petri dishes every 2 days in order to prevent contamination by progeny, deprivation of food and possible contaminations. The animals were monitored every day for their viability until their end of their lives.

Every lifespan assay was realized at least 3 times and the graph is a representative image of every experiment.

Measuring Oxygen Consumption Rate in *Caenorhabditis elegans*

*Reference: Konstantinos Palikaras1 and Nektarios Tavernarakis1, 2, **
1Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Crete, Greece; 2Department of Basic Sciences, Faculty of Medicine, University of Crete, Crete, Greece [47]

"The rate of oxygen consumption is a vital marker indicating cellular function during lifetime under normal or metabolically challenged conditions. It is used broadly to study mitochondrial function (Artal-Sanz and Tavernarakis, 2009; Palikaras et al., 2015; Ryu et al., 2016) or investigate factors mediating the switch from oxidative phosphorylation to aerobic glycolysis (Chen et al., 2015; Vander Heiden et al., 2009). In this protocol, we describe a method for the determination of oxygen consumption
rates in the nematode Caenorhabditis elegans."

Recipe & Equipment

- Commercial cigarette paper
- Polytetrafluorethylene (PTFE) membrane (provided by Hansatech, King's Lynn, England)
- 15 ml tube (STARSTEDT, catalog number: 62.554.016)
- 1.5 ml tube (Sigma-Aldrich, catalog number: Z606340)
- Paper towel
- Greiner Petri dishes (60 x 15 mm) (Greiner Bio One, catalog number: 628161)
- Maintenance kit (Hansatech, King's Lynn, England)
- *C. elegans strains*
- *Escherichia coli* OP50 strain (obtained from the Caenorhabditis Genetics Center)
- Distilled water
- Nitrogen gas provided in a tank
- PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Thermo ScientificTM, catalog number:23225)
- Potassium dihydrogen phosphate (KH2PO4) (EMD Millipore, catalog number: 1048731000)
- K2HPO4
- Sodium chloride (NaCl) (EMD Millipore, catalog number: 1064041000)
- BactoTM peptone (BD, BactoTM, catalog number: 211677)
- Streptomycin (Sigma-Aldrich, catalog number: S-6501)
- Agar (Sigma-Aldrich, catalog number: 05040)
- Cholesterol stock solution (SERVA Electrophoresis, catalog number: 17101.01)
- Calcium chloride (CaCl2) (Sigma-Aldrich, catalog number: C-5080)
- Magnesium sulfate (MgSO4) (Sigma-Aldrich, catalog number: M-7506)
- Nystatin stock solution (Sigma-Aldrich, catalog number: N-3503)

- Na2HPO4 (EMD Millipore, catalog number: 1065860500)
- Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P-5405)
- Potassium chloride (KCl) buffer (see Recipes)
- Nematode growth medium (NGM) agar plates (see Recipes)
- M9 buffer (sterile; see Recipes)
- Phosphate buffer (sterile; see Recipes)

Equipment

- Dissecting stereomicroscope (Olympus, model: SMZ645)
- Incubators for stable temperature (AQUA®LYTIC incubator 20 °C)
- DW1/AD clark-type polarographic oxygen sensor (Hansatech Instruments, model: Oxygraph Plus System)
- Water bath at 20 °C
- Tabletop centrifuge (Eppendorf, model: 5424)
- Sonicator (Sonics & Material, model: VC 130PB)

Software

- Oxygraph Plus software (Hansatech, King's Lynn, England)
- Microsoft Office 2011 Excel

Procedure

1. Growth and synchronization of nematode population
2. A mix population of nematodes observed through the dissecting stereomicroscope.
3. Incubate the worms at the standard temperature of 20 °C.
4. Four days later the plates contain mixed nematode population
5. Synchronize nematodes by picking L4 larvae of each strain under a dissecting stereomicroscope and transfer them onto separate plates.
6. Add 20-25 L4 larvae per plate. For each experimental condition, use at least five plates.
7. Set up the electrode disc and the electrode chamber
8. Cut a 1.5 cm2 paper spacer (cigarette paper) and a similar size piece of polytetrafluorethylene(PTFE) membrane

9. Place a small droplet of potassium chloride (KCl) buffer on the top of the dome of the electrode disc.
10. Place the cigarette paper on the center of the dome and cover it with the similar size piece of PTFE membrane
11. Place the small O-ring over the dome and then place the large O-ring around (If the large O-ring is not placed, when the disc is installed in the electrode chamber, the measurements will be affected by ambient air due to impaired sealing)
12. Once the electrode disc has been successfully prepared, check the response of the disc prior to any experiment. Connect the electrode disc to the control unit.
13. Open the software and start recording. A few minutes later the signal will be stabilized and would be around 2,000 mV in air.
14. Test the electrode disc by breathing on the electrode. A steep drop of the signal should be observed due to decreased oxygen levels. Then, the signal should be return to the original levels as the ambient oxygen equilibrates around the electrode disc.
15. If the signal does not respond, it may be caused by an inadequate electrode preparation. Then,disassemble the electrode disc, clean the electrode and repeat the procedure described above
16. Install the prepared electrode disc into the electrode chamber
17. Place and connect the electrode chamber on to the rear of the control unit
18. Place 2 ml sterilized distilled water into the reaction vessel of the chamber.
19. Water should be equilibrated to the assay temperature before calibration process. Set up the water bath at desired temperature and connect it with the electrode chamber to maintain stable temperature during experiments

20. Open Oxygraph Plus software and set up the appropriate temperature and atmospheric pressure.
21. Turn on stirrer to provide smooth stirring of the sample avoiding bubbles generation, which could cause noisy signals.
22. Wait until a plateau of the signal has been reached.
23. Establish zero oxygen conditions. Bubble nitrogen gas into the reaction vessel of the chamber to get rid all the oxygen out of the sample. Wait until the signal has reached a plateau.
24. Add 1 ml M9 buffer into the reaction vessel of the chamber and wait until system equilibration.
25. Assess oxygen consumption rate of the samples
26. Enable stirring throughout the duration of the experimental process.
27. Wash off the NGM plates using M9 buffer and collect nematodes in a 15 ml tube.
28. Let the animals to settle with gravity for few minutes. Use adult worms. Remove most of the supernatant, which contains bacteria, eggs and larvae (L1, L2, L3 and L4). Repeat this step two more times. After the last wash, reduce the volume to ~1.5 ml.
29. Remove M9 buffer from the reaction vessel of the camber
30. Add 1 ml nematode suspension into the reaction vessel of the chamber.
31. Monitor oxygen consumption for 5-10 min.
32. Prepare the next sample. Wash off other plates containing different strains or treated animals
33. Repeat steps 28-31

34. Recover the animals thoroughly from the reaction vessel and place them in a 1.5 ml tube
35. Place the tube on ice. The tubes should be kept on ice until protein determination.
36. Measure the next sample.
37. Save the recorded data from Oxygraph Plus software
38. Collect the samples from ice and proceed to worm lysis and protein determination.
39. Insert sonicator tip into sample and sonicate each sample using ten (10) pulses at 70% power each time. Immediately place the samples on ice
40. Spin down samples at 14,800 *x g* for 10 min at 4 °C.
41. Transfer supernatants (solubilized proteins) into new 1.5 ml tubes and measure protein concentration using a standard protein kit, such as PierceTM BCA Protein Assay Kit, following the instructions of the manufacturer (Estimated protein concentration range: 0.05-0.9 μg).
42. Open and review the data in Microsoft Office 2011 Excel
43. Divide the rate of the negative slope (oxygen consumption; obtained during step D6) with the total protein amount (nmol/min/ml/mg) in Microsoft Office 2011 Excel software
44. Subject the data to further and more advanced statistical analysis

    Electrode maintenance and storage
45. Clean the electrode disc after use.
46. Use the small cotton bud and the polishing paste from the maintenance kit.

47. Grip gently and remove the brown/black deposition of silver chloride and oxidized salt (KCl) that normally generated on silver electrode . Repeat polishing until all brown/black deposits on the electrode surface are removed.
48. Gently polish the center of the electrode dome avoiding scratching.
49. Rinse the electrode disc with distilled water to remove all traces of the polishing paste
50. Dry the electrode disc by using a paper towel.
51. Store the electrode disc in an air-tight vessel.

Intracellular Assessment of ATP Levels in *Caenorhabditis elegans*

*Konstantinos Palikaras1 and Nektarios Tavernarakis1, 2, **
1Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, Crete, Greece; 2Department of Basic Sciences, Faculty of Medicine, University of Crete, Crete, Greece
(Palikaras & Tavernarakis, 2016)

"*Eukaryotic cells heavily depend on adenosine triphosphate (ATP) generated by oxidative phosphorylation (OXPHOS) within mitochondria. ATP is the major energy currency molecule, which fuels cell to carry out numerous processes, including growth, differentiation, transportation and cell death among others (Khakh and Burnstock, 2009). Therefore, ATP levels can serve as a metabolic gauge for cellular homeostasis and survival (Artal-Sanz and Tavernarakis, 2009; Gomes et al., 2011; Palikaras et al., 2015). In this protocol, we describe a method for the determination of intracellular ATP levels using a bioluminescence approach in the nematode Caenorhabditis elegans."*

Materials and Reagents

- 1.5 ml tube
- Greiner Petri dishes (60 x 15 mm) (Greiner Bio One, catalog number: 628161)
- Toothpick
- L4 larvae
- *C. elegans* strains
- *Escherichia coli* OP50 strain (obtained from the Caenorhabditis Genetics Center)
- Liquid nitrogen
- Lyophilized ATP (provided with ATP bioluminescence assay kit CLS II) (Roche Diagnostics, catalog number: 11699695001)

- Distilled water
- PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Thermo ScientificTM, catalog number: 23225)
- Glue
- 70% of EtOH
- ATP bioluminescence assay kit CLS II (Roche Diagnostics, catalog number: 11699695001)
- Sodium chloride (NaCl) (EMD Millipore, catalog number: 1064041000)
- BactoTM peptone (BD, catalog number: 211677)
- Streptomycin (Sigma-Aldrich, catalog number: S-6501)
- Agar (Sigma-Aldrich, catalog number: 05040)
- Cholesterol stock solution (SERVA Electrophoresis, catalog number: 17101.01)
- Calcium chloride (CaCl2) (Sigma-Aldrich, catalog number: C-5080)
- Magnesium sulfate (MgSO4) (Sigma-Aldrich, catalog number: M-7506)
- Nystatin stock solution (Sigma-Aldrich, catalog number: N-3503)
- Potassium dihydrogen phosphate (KH2PO4) (EMD Millipore, catalog number: 1048731000)
- Na2HPO4 (EMD Millipore, catalog number: 1065860500)
- K2HPO4
- Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P-5405)
- Phosphate buffer (1 M; sterile, see Recipes)
- Nematode growth medium (NGM) agar plates (see Recipes)
- M9 buffer (see Recipes)

Equipment

- Dissecting stereomicroscope (Olympus, model: SMZ645)
- Incubators for stable temperature (AQUA®LYTIC incubator 20 °C)
- TD-20/20 luminometer (Turner Designs, model: 2020-000)
- Tabletop centrifuge (Eppendorf, model: 5424)
- Freezers (Siemens, -20 °C; So-Low Environmental Euipment, model: So-Low Ultra C85-22
- horizontal -80 °C freezer)
- Heat plate
- Hot pot

Software

Microsoft Office 2011 Excel (Microsoft Corporation, Redmond, USA

Procedure

1. Incubate the nematodes at the standard temperature of 20 °C.

2. Four days later the plates contain mixed animals population.
3. Synchronize nematodes by picking L4 larvae of each strain under a dissecting stereomicroscope and transfer them onto separate freshly *E. coli* (OP50) seeded plates
4. Add 20-25 L4 larvae per plate. For each experimental condition, use at least five plates.
5. Use an eyebrow/eyelash hair and collect 50-100 adult animals in 50 μl of M9 buffer in a 1.5 ml tube
6. Use the same number of animals of each strain during sample preparation.
7. Freeze the samples in liquid nitrogen.
8. Store the samples at -80 °C until further analysis.
9. Immerse frozen worms in boiling water for 15 min.
10. Place the samples on ice for 5 min.
11. Spin down samples at 14,800 *x g* for 10 min at 4 °C.
12. Transfer sample supernatants into new 1.5 ml tubes.
13. Dilute sample supernatants tenfold by adding water before measurement.
14. Keep the samples on ice or store them at -20 °C.
15. Prepare ATP standards
16. Dissolve lyophilized ATP by adding the appropriate volume of distilled water to get final concentration of 10 mg/ml or 16.5 mM (see Note 2).
17. Store ATP standard solution at -20 °C. ATP standard solutions are stable for at least one week.
18. Dilute ATP standards by serial dilutions in the range of 10 to 1 x 10-4 μM

19. Prepare serial dilutions of one ATP standard solution using distilled water to generate the ATP standard curve.
20. Dissolve lyophilized luciferase by adding 10 ml of distilled water.
21. Incubate dissolved luciferase for 5 min at 4 °C.
22. Gentle swirl the bottle and mix for a homogeneous solution. Avoid shaking.
23. Store luciferase reagent at -20 °C
24. Add 100 μl of each sample or ATP standard in a 1.5 ml tube. Use a sample containing M9 buffer as blank (no ATP). For each sample or ATP standard, use at least 3 replicates.
25. Add 100 μl luciferase reagent to the sample/standard
26. Incubate for 10 sec at room temperature.
27. Measure and record ATP levels of the sample/standard by using TD-20/20 luminometer.
28. Prepare and measure the next sample/standard. Repeat steps D1-D4.
29. Transfer supernatants (solubilized proteins; obtained during step B8) of each sample into new 1.5 ml tubes and measure protein concentration using a standard protein kit, such as PierceTM
30. Open and review the recorded data (obtained during steps D4 and D6) in Microsoft Office 2011 Excel.
31. Subtract the value of the ATP blank sample form the raw data.
32. Prepare ATP standard curve by using the concentrations of ATP standards and their respective bioluminescence values.

33. Typical standard curve of bovine serum albumin (BSA) by using BCA protein determination assay.
34. Calculate the ATP content of each sample by using a log-log plot of the ATP standard curve
35. Divided the ATP levels with the total protein amount (nM/mg)
36. Subject the data to further and more advanced statistical analysis

.

D. Pharmacological techniques

Swimming assay using levamisole

Procedure:

1. 13ul of levamisole diluted in M9 in a final concentration of 400 uM were placed on a slide
2. In this drop, 1 animal was placed and was observed under the stereoscope
3. The time from the placement of the worm till the end of its movement was recorded.

Aldicarb and levamisole resistance assay

Aldicarb is an acetylcholinesterase inhibitor and it is widely used to monitor the neurotransmission function. When wild type c.elegans animals are exposed to aldicarb for an extensive period of time, they end up demonstrating spastic paralysis due to the excess acetylocholine neurotransmitter that is accumulated in the synaptic cleft. The severity of the effect is dependent on the amount of acetylcholine built up in the synaptic cleft and hence the speed of paralysis relies on the rate of acetylcholine release from the presynapse. For the paralytic effect to occur, the muscle should have the ability to contract in response to the translated cholinergic signal from the postsynaptic neuron.

Levamisole in a selective agonist of the cholinergic receptor of the postsynaptic neuron. In the presence of levamisole, animals display spastic paralysis. Using this assay it is possible to have a view of the function of the post-synaptic compartment.

Procedure

Drug plates were made by adding 240 μl levamisole in 60ml medium (Sigma-Aldrich) or 120ul, 60ul and 30ul aldicarb (Sigma-Aldrich) to unseeded NGM plates, giving a final concentration of 400uM for levamisole and 1mM, 0.5mM and 0.25mM for aldicarb respectively. [levamisole was dissolved in distilled water (dH_2O) and aldicarb was

dissolved in 70% ethanol]. These plates were left overnight at room temperature to allow for the drugs to equilibrate into the agar. The plates were seeded with an OP50 lawn. For the assay, 15 worms in the L4 stage were transferred to the drug plate. They movement and paralysis was monitored and recorded every 5 or 10 minutes in response to the picking. Results then were analyzed with Prism software package (GraphPad Software Inc., San Diego, USA).

TMRE staining (Mitochondrial Membrane Potential Assay Kit)

TMRE is used for the labeling of active mitochondria. TMRE is a cell permeant, positively-charged, red-orange dye accumulates in active mitochondria due to their relative negative charge.

Procedure

1. Use small NGM plates
2. UV killing bacteria for 15minutes
3. Put 40ul of TMRE stain in final concentration of 100uM
4. let the plates dry and then place the worms
5. Wait for 24 hours until the observation of the animals under the microscope

Antimycin treatment protocol

Antimycin is a drug used to induce UPR^{mt} since it inhibits the electron transport chain. When c.elegans animals are exposed to antimycin, it is possible to monitor whether specific genes can impair or further induce the UPR^{mt} .

Procedure

1) Synchronize your animals from L4, grow them and one day prior to the day you want to observe them
2) The day before the day you wish to do the experiment, take small NGM plates (with OP50 approx.80ul) and UV kill the bacteria
3) After you take them out of the UV machine, put 40ul of antimycin 0.5mM for a final concentration of 500nM (small plates have 4 ml NGM, this is the final volume)
4) Let them dry for about 20 min (check if they are dry)
5) Place carefully your animals
6) 24 hours later, you can observe them under the microscope

E. Microscopy techniques

For the observance of the animals under the microscope M9 buffer was used and the aminals were anaesthetized in Levamisole with 13mM concentration in M9 buffer. The photos were taken in Room temperature (25°C).

For every individual experiment, all photos were taken with the same magnitude under the same conditions. The microscope used was the Zeiss Axio. Imager Z2 Epifluorescence/DIC Microscope. The analysis of the pictures was realized by the program ImageJ 1.41.

E. Statistical analysis of the results

The statistical analysis was performed in GraphPad Prism 4 (GraphPad Software, USA).

F. Additional Information

The restriction enzymes used in the experiments came from New England Biolabs (NEB) and Minotech.

The chemicals and reagents were produced by the following companies: Sigma-

Aldrich (St. Louis, MO, USA), Qiagen (California, CA, USA), Invitrogen (Carlsbad, USA) and Roche.

The reagents used in the experiments were produced by the companies:

PCRIITOPO Cloning (Invitrogen), Genomic DNA purification kit (Macherey-Nagel, NucleoSpin Tissue), PCR Purification Kit (Qiagen-QIAquick), SSoFast EvaGreen Supermix (Bio-Rad).

RESULTS PART I

Aging is a major risk factor for most common neurodegenerative diseases, including mild cognitive impairment, dementias including Alzheimer's disease, cerebrovascular disease, Parkinson's disease and Lou Gehrig's disease. While much research has focused on diseases of aging, there are few informative studies on the molecular biology of the aging brain (usually spelled ageing brain in British English) in the absence of neurodegenerative disease or the neuropsychological profile of healthy older adults.

Recent studies had suggested that presynaptic autophagy has a role in the regulation of neurotransmission in striatal dopamine neurons. However, little is still known about the mechanism by which autophagy or autophagy proteins regulate neurotransmission. In the present study we focused on the study of autophagy and autophagic proteins in neurotransmission and synaptic function.

1. Role of autophagy mutants defective synaptic transmission

According to literature, the proteins ATG-18, LGG-2, HLH-30, EPG-8 in *c.elegans* are important for autophagy. In order to identify what is the effect of the depletion of the above proteins in neurotransmission, we used two drugs: levamisole and aldicarb. Levamisole is a nicotinic acetylocholine receptor agonist which causes continued stimulation, muscle contraction that leads to paralysis of wild type *C.elegans*. On the other hand, aldicarb, is a cholinesterase inhibitor which prevents the breakdown of acetylcholine in the synapse leading to hypercontracted paralysis. Generating mutants of our genes of interest, we aimed to investigate whether these genes play any role in cholinergic function. We, indeed, discovered, that all four autophagy mutants (*lgg-2, hlh-30, atg-18, epg-8*) were resistant to levamisole drug (400uM) and aldicarb drug (0.5uM, 0.25uM) as well. (fig.10). This experiment was repeated four times and the most efficient concentration for aldicarb assay was found to be 0.25uM, whereas in the presence of 1mM aldicarb concentration, there was not observed any difference between the wild type and the mutants.

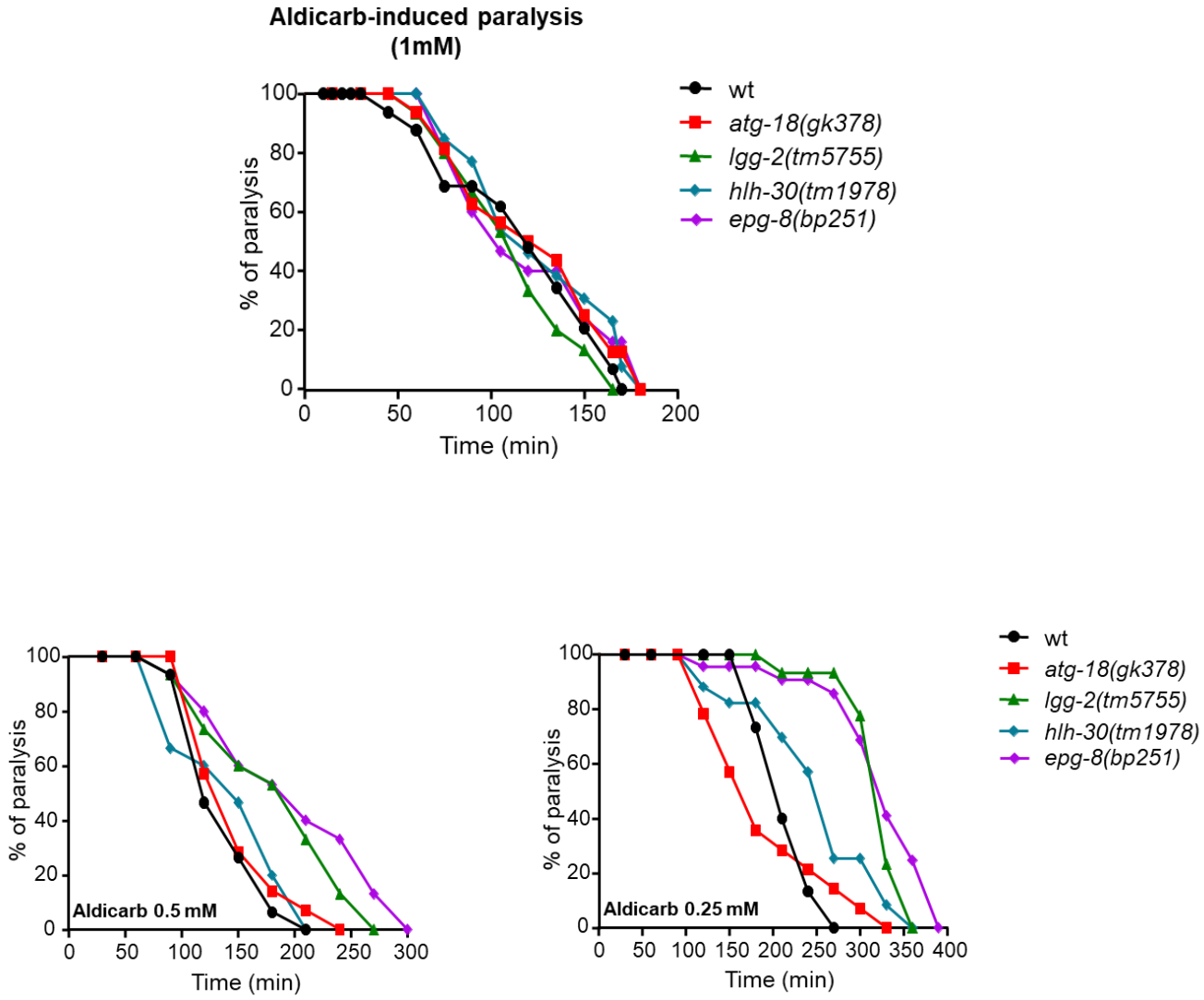


Figure 10.: Pharmacological assays. Graphs displaying percent of paralysis of 15 worms under the presence of levamisole or aldicarb in the plates. All autophagy mutants (*lgg-2*, *hlh-30*, *atg-18*, *epg-8*) display resistance to levamisole drug (400uM) and aldicarb drug (0.5, 0.25uM). No resistance was observed when placed in the plate containing aldicarb 1mM .

2. Role of autophagy impairment in synaptic vesicle generation

Based on the previous results, when autophagy proteins are depleted, in the presence of both aldicarb (0.25mM) and levamisole (0.4mM) drugs, the animals are resistant compared with the wild type (N2). This could indicate that when autophagy proteins are absent, neurotransmitter release in reduced. Consequently, there is probably a presynaptic and/or a post-synaptic defect. Subsequently, we hypothesized that autophagy proteins might play a role on the synaptic vesicle exocytosis.

We aimed to investigate whether autophagy proteins play a role in the formation of synaptic vesicles. For this reason, we used synapto-pHluorin, a genetically encoded optical indicator of vesicle release and recycling. It is used in neuroscience to study neurotransmitter release. It consists of a pH-sensitive form of green fluorescent protein (GFP) fused to the luminal side of a vesicle-associated membrane protein (VAMP). At the acidic pH inside transmitter vesicles, synapto-pHluorin is non-fluorescent. When vesicles get released, synapto-pHluorin is exposed to the neutral extracellular space and the presynaptic terminal becomes brightly fluorescent. Following endocytosis, vesicles become re-acidified and the cycle can start again. Chemical alkalinization of all vesicles is often used for normalization of the synapto-pHluorin signals.

Using an animal expressing synaptobrevin fused with pHluorin (see materials and methods), we monitored whether synaptic vesicles are fused with the plasma membrane in the presynaptic compartment. Using the autophagy mutants (*lgg-2(tm5755), hlh-30(tm1978), atg-18(gk378)*) crossed with a *c.elegans* strain expressing pHluorin, and wild type animals N2 expressing pHluorin, we measured the GFP signal in three different aging conditions (Day 1, Day 3, Day 8). In this way we aimed to assess whether synaptic vesicles fusion is altered during aging as well in these different conditions. Interestingly, we did not observe any statistically difference in the levels of pHluorin in dopaminergic neurons between the autophagy mutants and the wild type condition. This indicated that autophagy proteins do not participate in the process of synaptic fusion with the plasma membrane.

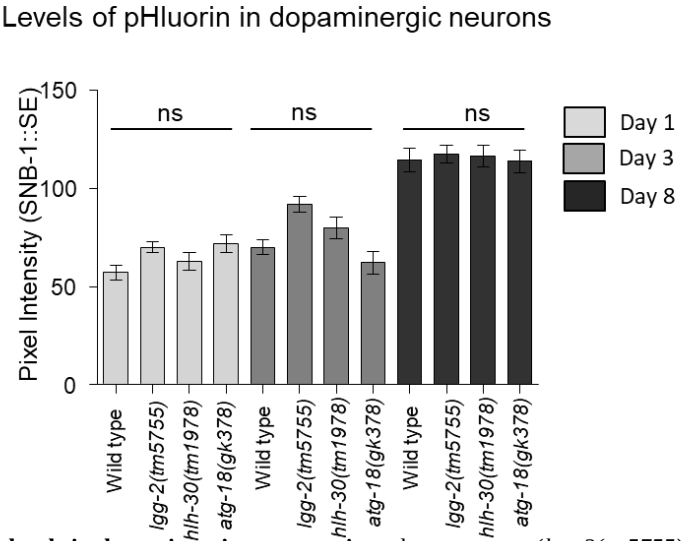


Figure 11.: pHluorin levels in dopaminergic neurons. Autophagy mutants (*lgg-2(tm5755), hlh-30(tm1978), atg-18(gk378))*crossed with a *c.elegans* strain expressing pHluorin, and wild type animals N2 expressing pHluorin,

were used for measurement of GFP signal in three different aging conditions (Day 1, Day 3, Day 8). No significant difference in the GFP levels was observed between wild type and autophagy mutants.

3. Autophagy proteins participate on the same pathway as Synaptotagmin

To further investigate whether autophagy proteins are implicated in the exocytosis pathway we used a mutant in *c.elegans* in the gene encoding synaptotagmin *(snt-1(md290).* These mutants are severely uncoordinated and exhibit synaptic transmission defects (Nonet et al., 1993). Generating crosses between the synaptotagmin mutants and the autophagy mutant *lgg-2(tm5755)* which demonstrated the most prominent phenotype in the previous experiments, we could evaluate whether these proteins participate on the same pathway or not for the regulation of neurotransmission. Consequently, the aldicarb and levamisole assays revealed that when both LGG-2 and SYNAPTOTAGMIN proteins are impaired or depleted, they demonstrate the same resistance as synaptotagmin mutant does alone. We can therefore conclude that LGG-2 protein participate on the same pathway as SYNAPTOTAGMIN protein to support the process of neuronal exocytosis.

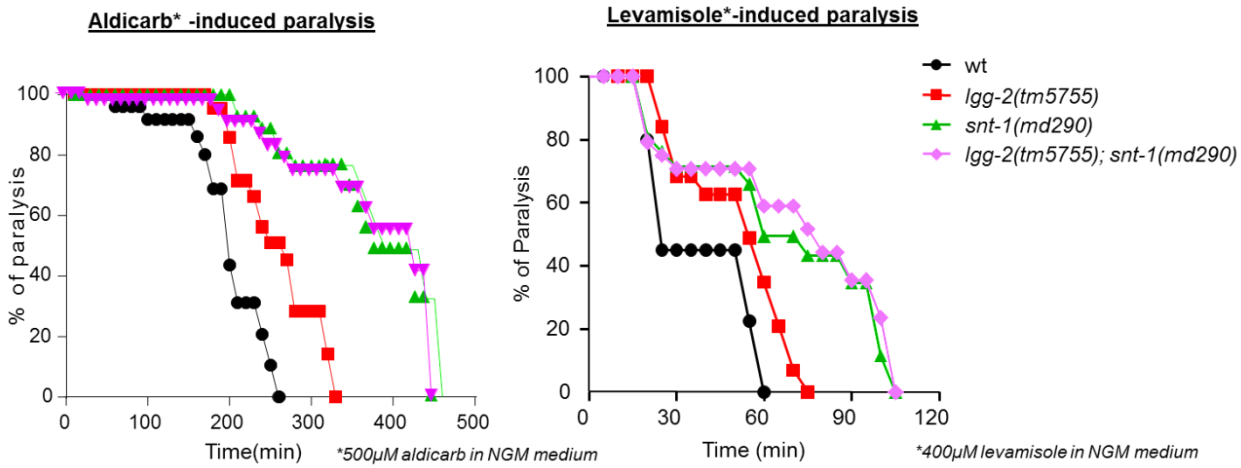


Figure 12.: Aldicarb and Levamisole assays for synaptotagmin mutant animals and autophagy mutant animals. Aldicarb (500μM) and levamisole (400μM) assays revealed that when both LGG-2 and SYNAPTOTAGMIN proteins are impaired or depleted, they demonstrate the same resistance as synaptotagmin mutant does alone.

4. Autophagy proteins participate on the same pathway as Syntaxin

Another important protein in the synaptic vesicle cycle and neuronal exocytosis is Syntaxin. We used a *c.elegans* mutant in *unc-64* (a gene which encodes a mammalian syntaxin 1A homolog, a mutant which results in resistance to inhibitors of acetylcholinesterase and in an abnormal accumulation of ACh, probably due to a defect in synaptic transmission [49]. *Unc-64 mutant* is resistant to aldicarb according to literature. We showed that *Unc-64 mutant* is resistance to levamisole as well and the double mutant for *unc-64* gene and *lgg-2* gene demonstrate the same resistant effect as *unc-64* alone. This result revealed that LGG-2 protein participate on the same pathway as Syntaxin 1A protein.

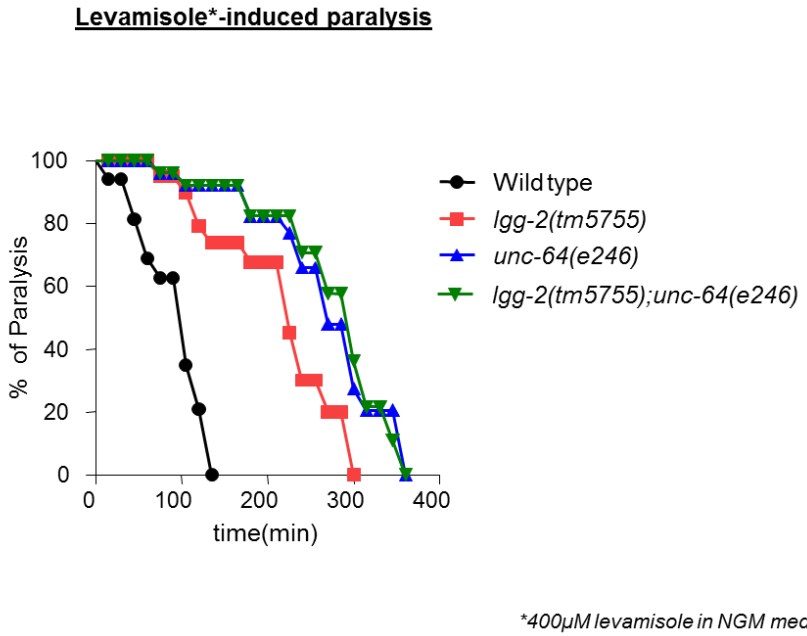


Figure 13.: Levamisole assay (400μM) for unc-64 mutant (syntaxin) mutant animals and autophagy mutant animals. Levamisole assay (400μM) revealed that when both LGG-2 and UNC-64 proteins are impaired or depleted, they demonstrate the same resistance as unc-64 mutant does alone.

5. Autophagy protein depletion rescues the paralytic phenotype of tomosyn-1 mutants

Having the above indications of a role of autophagy proteins in synaptic exocytosis similar to other synaptic proteins such as Synaptotagmin and Syntaxin 1A, we aimed

to investigate whether in conditions of increased neuronal exocytosis, autophagy proteins depletion might rescue the paralysis defect. We utilized a mutant in a gene encoding for *Tomosyn-1*, a protein which inhibits synaptic vesicle priming, in which mutant animals exhibit strong paralytic phenotype compared with wild type animals. However, *c.elegans* mutant animals for both *tomosyn-1* and *lgg-2* genes, demonstrated a rescue of this paralytic phenotype. This indicated that autophagy protein LGG-2 is important and participates in the exocytotic pathway of the neurotransmitter.

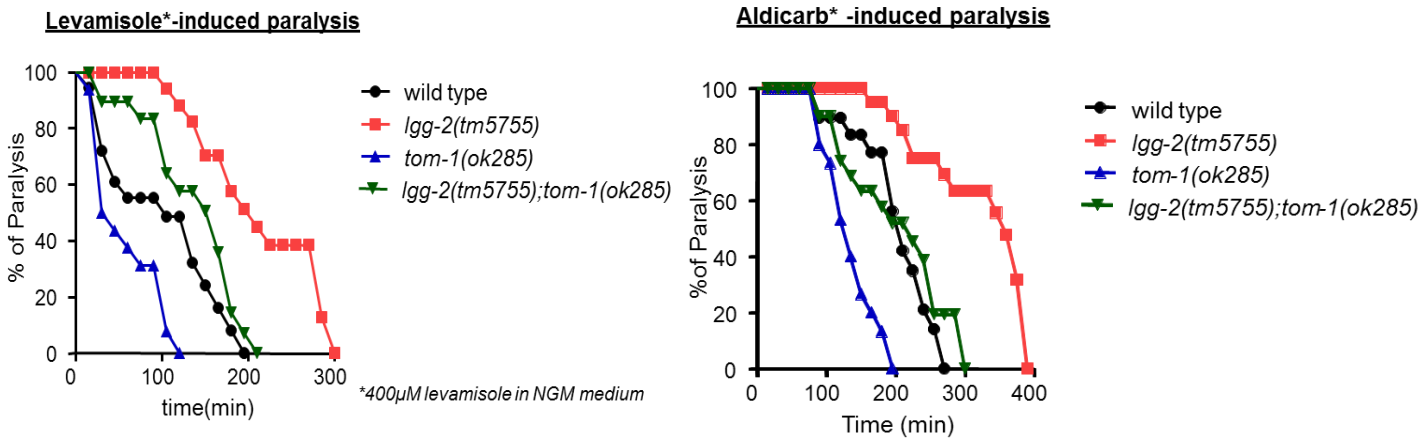


Figure 14.: Aldicarb and Levamisole assays for tom-1 mutant animals and autophagy mutant animals. Aldicarb (500μM) and levamisole (400μM) assays revealed that when LGG-2 protein depletion together with TOM-1 depletion results in the rescue of the paralysis induced by the TOM-1 depletion alone.

6. Interaction between autophagy proteins and synaptic proteins

The above indications for a possible role of autophagy proteins in the process of neurotransmission release and function, raised questions concerning whether there is any interaction between autophagy proteins and proteins important in the neurotransmission pathway. Using antibodies against SYNAPTOBREVIN and UNC-10 proteins, we aimed to identify any possible interactions between these proteins and autophagy proteins. Before testing these interactions with immunoprecipitation, we tried to detect the levels of expression of these proteins in wild type animals and whether the antibodies were detecting these proteins efficiently. Performing a western blot in wild type lysed animals, we were able to detect both proteins.

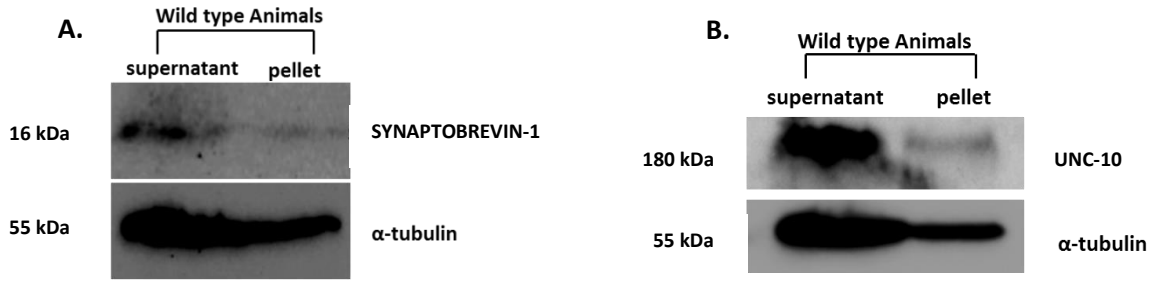


Figure 15.: Western blot for the detection of the endogenous proteins SYNAPTOBREVIN-1 (A) and UNC-10 (B) in wild type animals both in the supernatant of the sonicated worms and the pellet.

After testing the efficiency of the antibodies and whether we could detect the endogenous proteins of UNC-10 and Synaptobrevin, we aimed to test the interaction between these proteins and proteins related to autophagy. For this reason, we tried to immunoprecipitate the LGG-2, LGG-1 and ATG-18 proteins using their GFP tag that was fused with the proteins. We used, therefore, animals from the following strains: p*atg-18*ATG-18::GFP;*rol-6(su1006)*, p*lgg-1*GFP::LGG-1;*rol-6(su1006)*, p*lgg-2*GFP::LGG2 and we immunoprecipitated the proteins using a-GFP antibody (raised in rabbit, diluted 1:2000 in 5%milk). We then tested whether we could detect the synaptic proteins (UNC-10, SYNAPTOBREVIN). Unfortunately we could not detect the proteins by western blot.

For this reason we aimed to detect possible interactions between Munc-18 protein with autophagy proteins. Munc-18 binds syntaxin and forms a complex which plays a critical role in vesicle priming and has multiple roles in exocytosis. Animals expressing UNC-18 protein fused with a flag epitope were immunoprecipitated on FLAG beads and the efficiency of the immunoprecipitation was tested using a FLAG antibody. We indeed managed to immunoprecipitate the UNC-18 protein fused with the flag antibody. However there was still protein detected that was not able to bind on the antibody that is bound on the beads.

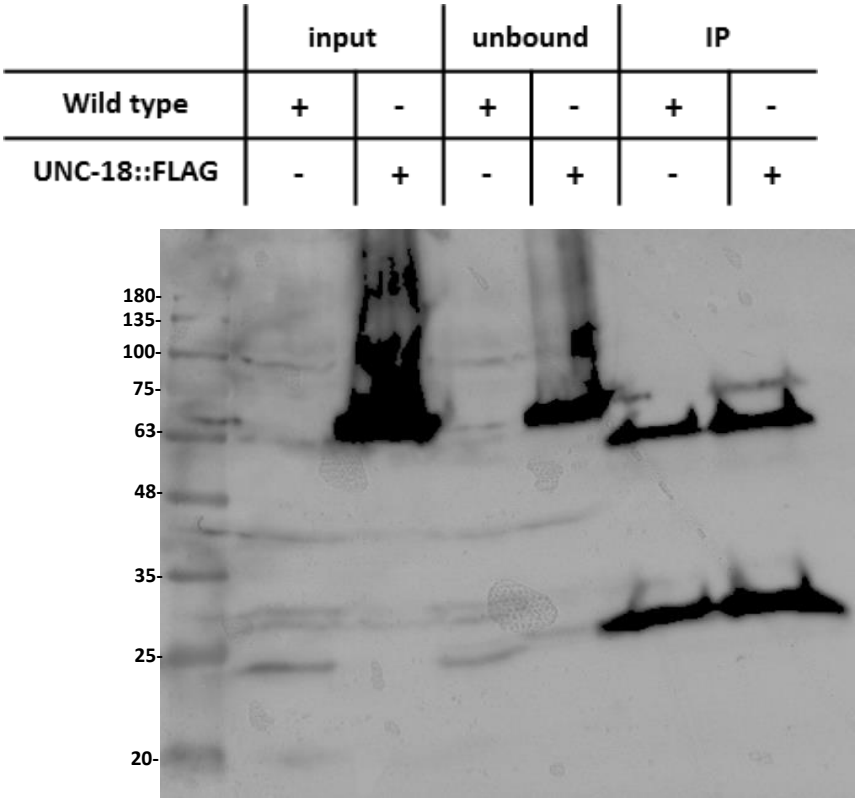


Figure 16.: Immunoprecipitation of the UNC-18::FLAG protein using FLAG antibody. The size of the UNC-18 protein fused with FLAG peptide is expected to be at 68,5kDA.

7. Role of Mitophagy proteins in neuronal exocytosis

Having these indications that autophagy proteins play a crucial role in the neuronal exocytosis, we were interested to investigate whether autophagy proteins specific for mitochondria would play any role in this process. For this reason, using animals that are lacking important proteins for the mitophagy process such as PINK-1, PDR-1 and DCT-1, we tested whether animals, mutants for the genes that encode the mitophagy proteins will demonstrate the same resistant phenotype as autophagy mutants. Indeed, mutant animals treated with 400uM levamisole demonstrated high resistance compared with wild type animals.

Surprisingly, mutant animals treated with Aldicarb in different concentrations (1mM, 0.5Mm, 0.25mM) did not display any significant difference in the time of paralysis compared with the wild type.

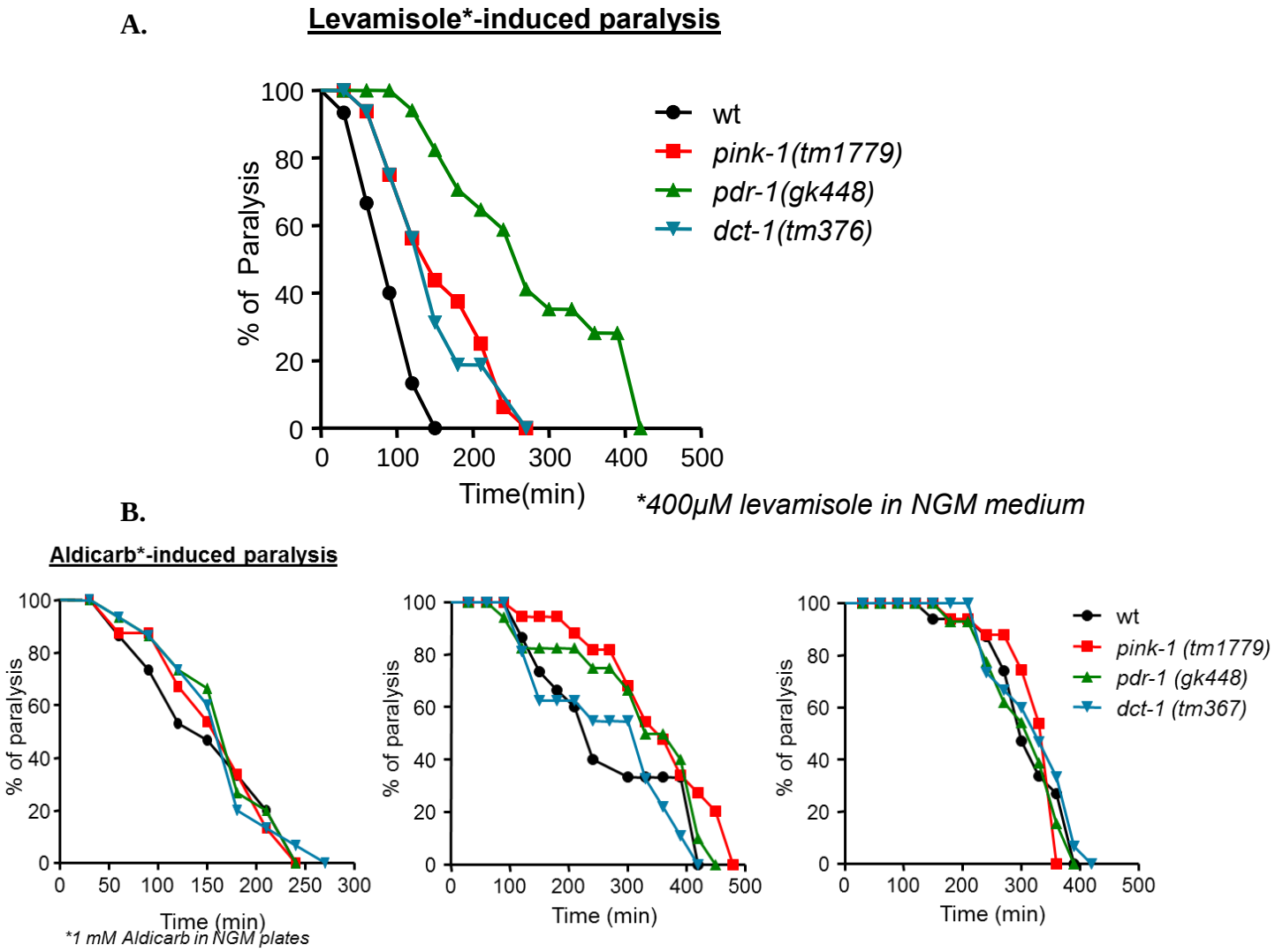


Figure 17.: A. Levamisole assay. Mitophagy mutants are resistant in the presence of levamisole drug (0.4mM) compared with wild type animals**. B. Aldicarb assay.** Mitophagy mutants do not display any difference in the resistance compared with wild-type animals in the presence of the Aldicarb drug (1mM,0.5mM,0.25mM).

DISCUSSION PART I

Although there have been significant advances recently in autophagy and mitophagy processes, what remains still unclear is the role of autophagy in synaptic neurotransmission. In the current study we demonstrate that impairment of significant autophagy genes, leads to defects in neurotransmission in either the pre-synaptic or the post-synaptic compartment. This defect is not related though with the production of synaptic vesicles. In more detail, proteins important in the autophagic pathway (LGG-2 etc) seem to act on the same pathway as other proteins important for the neuroral exocytosis of the neurotransmitter in the synaptic cleft. Consequently, our results indicate a role of autophagy genes either in the synthesis and vesicular transport of neurotransmitter, either regulatory or structural. Finally, mitochondria specific autophagy (mitophagy) proteins seem to play a role in neuronal function, regulating the pathway differently from autophagy proteins.

RESULTS PART II

It is well established, during the last few years, that two of the main mitochondrial quality control processes activated under pathological conditions, are UPR^{mt} and autophagy specific in mitochondria (mitophagy). What is not, however well understood is the coordination between these two mechanisms. Many factors are responsible for the regulation of the magnitude , duration and function of these processes. Among these factors are ubiquitin ligases such as CHIP. CHIP was shown recently to play a role in lifespan extension and proteostasis (Riga Tawo et all, Cell, 2017 Apr 20; 169(3): 470–482.e13.).

1. Role of CHIP in lifespan extension and proteostasis

CHN-1/CHIP E3 ubiquitin ligase plays a major role in lifespan extension and proteostasis. It has been recently shown that under normal conditions, CHIP ubiquitinates the Insulin signaling receptor leading to its suppression. This lead to reduced insulin signaling and as a result to extension of lifespan. Under stress conditions, however, when misfolded proteins accumulate, CHIP relocalizes, freeing the INSR, and ubiquitinates the protein aggregates. This leads to increased insulin signaling and a decrease in lifespan.

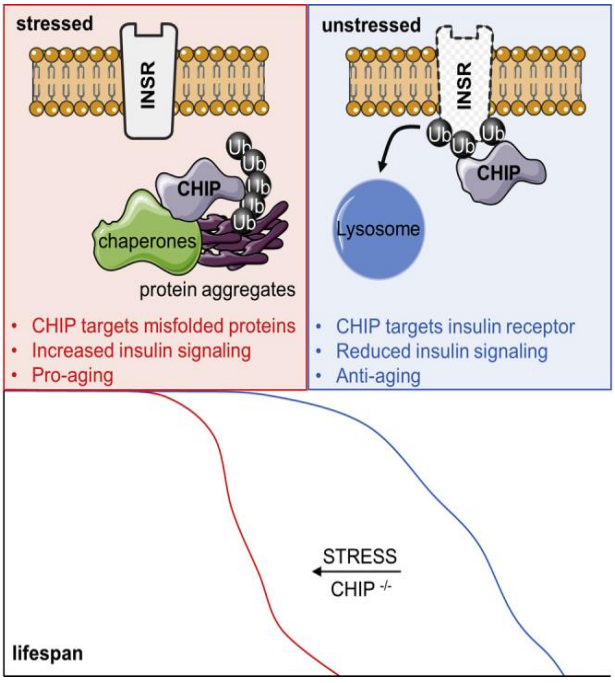


Figure 18.: A. CHN-1/CHIP role in lifespan extension and Proteostasis. CHIP ubiquitinates the Insulin signaling receptor leading to its suppression. This lead to reduced insulin signaling and as a result to extension of lifespan. Under stress conditions, however, when misfolded proteins accumulate, CHIP relocalizes, freeing the INSR, and ubiquitinates the protein aggregates. This leads to increased insulin signaling and a decrese in lifespan.

Indeed, they have shown that upon depletion of CHIP, there is a statistically significant reduction on lifespan of *c. elegans* worms, compared with wild type animals.

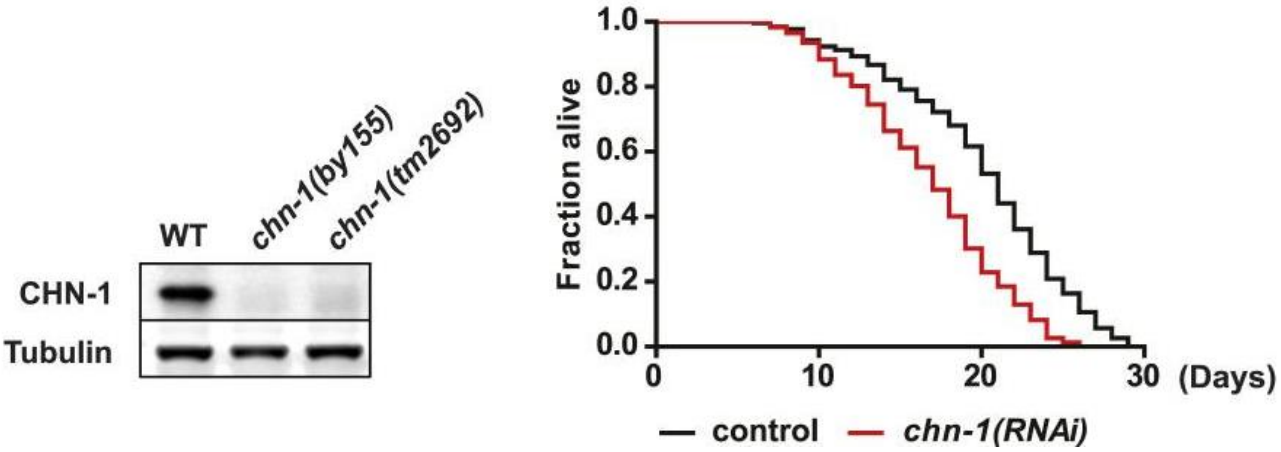


Figure 19.: CHN-1/CHIP loss reduces lifespan. CHIP protein loss by RNAi leads to reduced lifespan.[43]

2. CHN-1/CHIP role in *c.elegans* bioenergetics

For this reason, we aimed to investigate the impact of the loss of CHIP in *c.elegans* bioenergetics. We, therefore, compared the ATP levels of Day 1 adult animals in four different conditions: Wild type, upon CHIP loss (chn-1(by155)), upon overexpression of CHIP (p_{chn-1}CHN-1::FLAG) and upon depletion of a domain which is thought to be responsible for the ubiquitination process (p_{chn-1}CHN-1(UBOX)::FLAG). Interestingly, the ATP levels were significant reduced upon depletion of CHIP. This phenotype was rescued when we overexpressed CHIP in the mutant animal. Animals from which the UBOX domain was depleted show a small reduction in ATP levels compared to the wild type animals. The same experiment was performed using Day 5 adult animals. The same phenotype was observed, however animals not expressing the UBOX domain did not show any significant reduction of ATP.

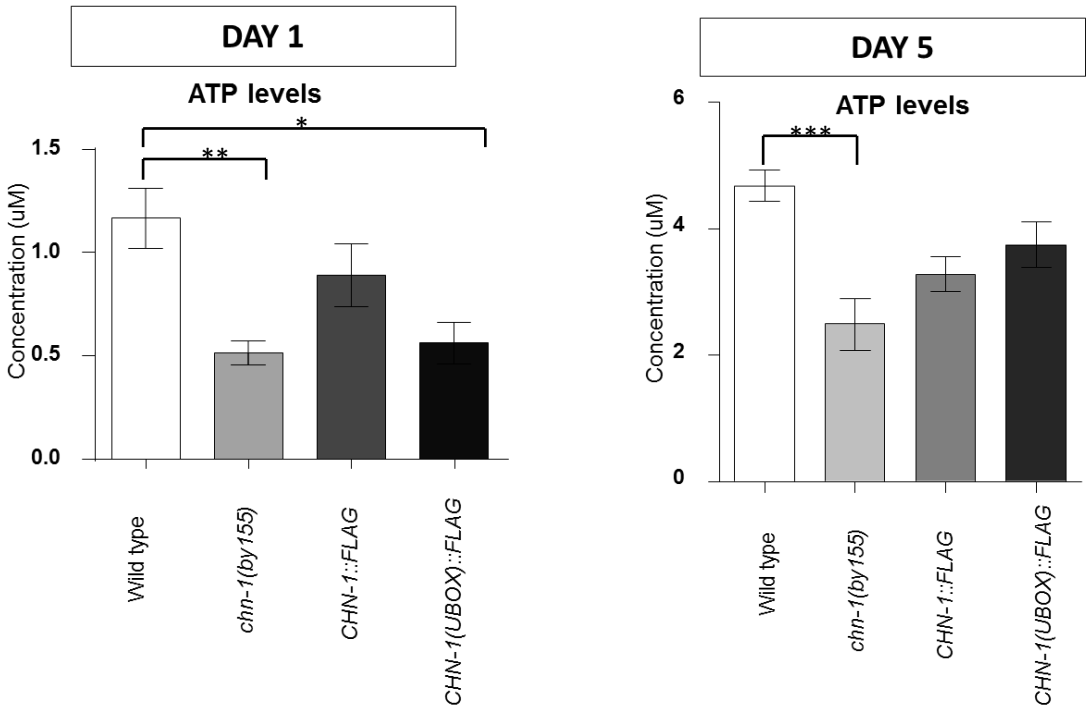


Figure 20.: CHN-1/CHIP depletion leads to reduced *c.elegans* bioenergetics. Day 1 and Day 5 animals were used for the ATP levels measurement. At Day 1, ATP levels were significant reduced upon depletion of CHIP. This phenotype was rescued when we overexpressed CHIP in the mutant animal. Animals from which the UBOX domain was depleted show a small reduction in ATP levels compared to the wild type animals. The same experiment was performed using Day 5 adult animals. The same phenotype was observed, however animals not expressing the UBOX domain did not show any significant reduction of ATP.

To further investigate whether CHIP depletion affects the mitochondrial bioenergetics, we stained the animals with TMRE (tetramethylrhodamine, ethyl ester). This red-orange dye is cell permeant, positively charged and accumulates in active mitochondria due to their negative charge. We used the *chn-1(by155)* mutant animals as well as the overexpression of CHN-1 animals fused with flag peptide and the deletion of the UBOX domain of the CHN-1. We indeed discovered that upon CHN-1/CHIP depletion in day 1 adult animals, the mitochondria were stained significantly less than wild type animals, revealing that upon CHN-1/CHIP depletion, mitochondria are less active. The overexpression of CHN-1/CHIP rescued the observed phenotype whereas the deletion of the UBOX domain displayed the same phenotype as wild type animals. The same experiment was performed using day 5 adult animals and did not reveal any significant difference between the mutants.

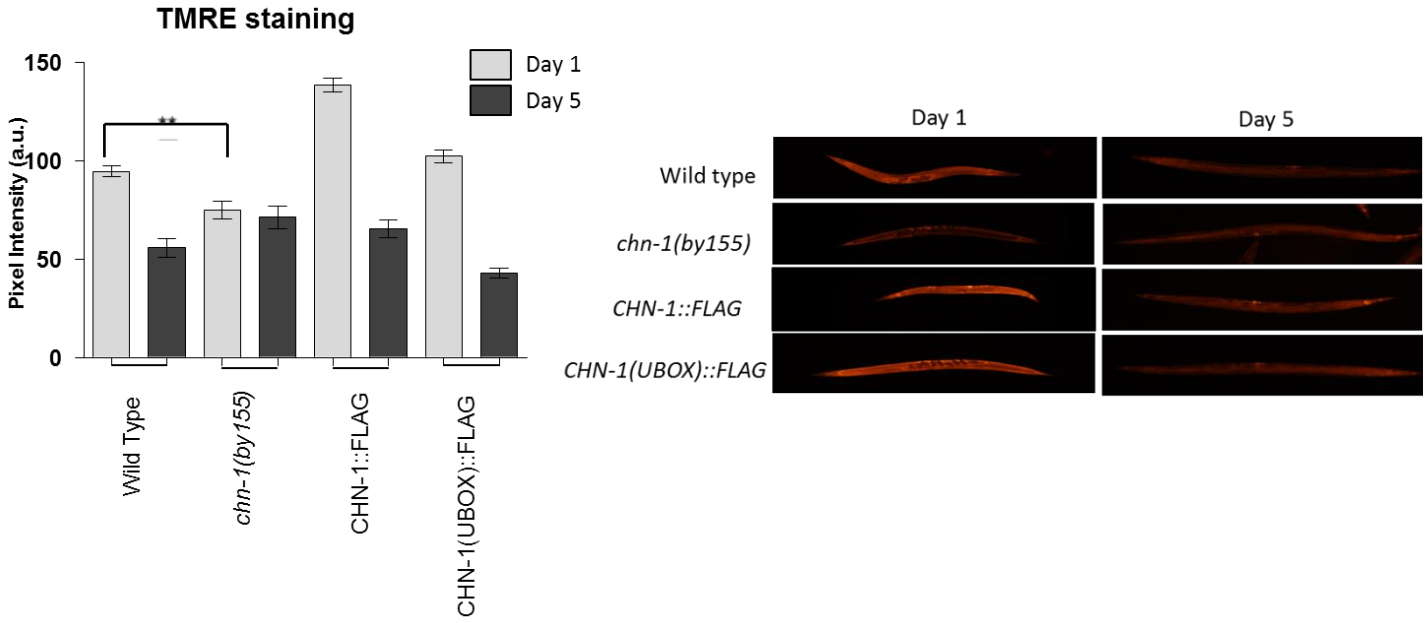


Figure 21.: TMRE staining. CHN-1/CHIP depletion in day 1 adult animals, leads to decreased activity of the mitochondria, a phenotype which is rescued upon overexpression of CHN-1/CHIP. However, the deletion of the UBOX domain of CHN-1 did not cause any decrease in the mitochondrial activity. The same experiment was performed using day 5 adult animals and did not reveal any significant difference between the mutants.

Given these differences in the bioenergetics of mitochondria upon depletion of CHIP, we aimed to measure oxygen consumption rate since mitochondria, in order to drive energy-requiring reactions in eukaryotic organisms require oxygen to produce ATP in sufficient quantities. Using wild type animals, mutant animals for CHIP, mutant animals overexpressing CHIP and mutant animals for the UBOX domain of CHIP, we measured the oxygen consumption rate when animals were day 1 adults. Surprisingly, we did not observe any significant difference between wild type animals and the three different conditions.

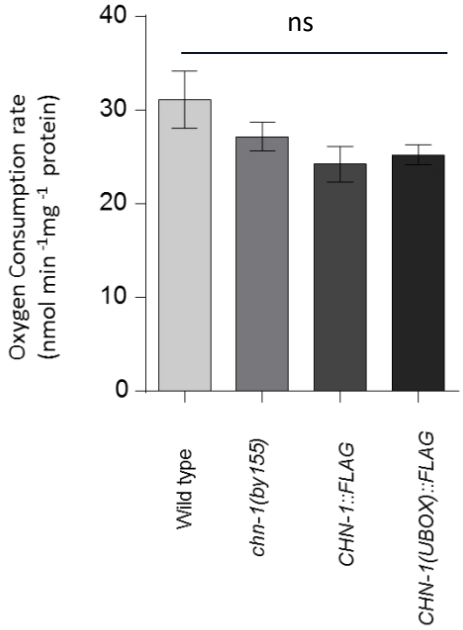
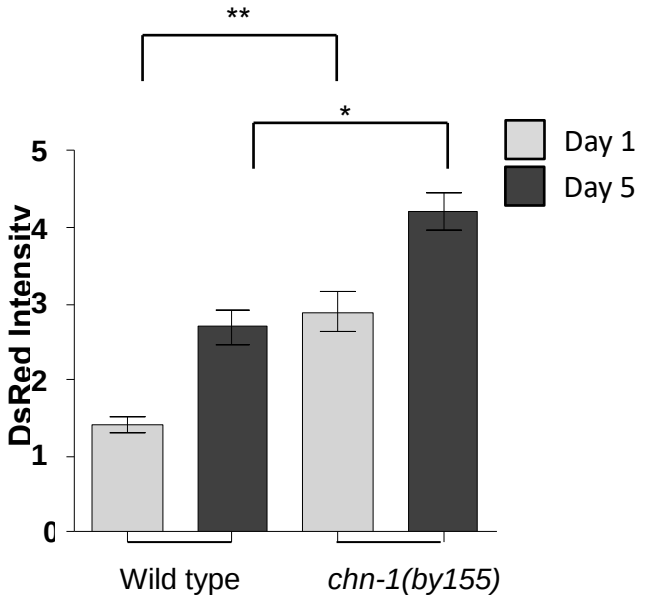


Figure 22.: Oxygen Consumption rate (nmol min $^{-1}$mg^{-1} protein). The oxygen levels were measured in wild type animals, *chn-1(by155)* mutant animals, and in animals overexpessing CHN-1 and having a deletion of the UBOC domain of CHN-1 protein. No significant difference between these conditions was observed.

3. PDR-1 levels are increased after deletion of CHIP ligase

Since one of the roles of CHIP is to enhance the enzymatic activity of parkin (PDR-1) to self-ubiquitinate, we expected that deletion of CHIP, will increase the levels of parkin. Using an animal expressing PDR-1 fused with DsRed, we compared the levels of PDR-1 protein in wild type animals and in animals that were mutants for chn-1 protein. Indeed, after the deletion of chn-1 in worms, the levels of PDR-1 were significantly increased both in Day 1 animals and D3 animals.



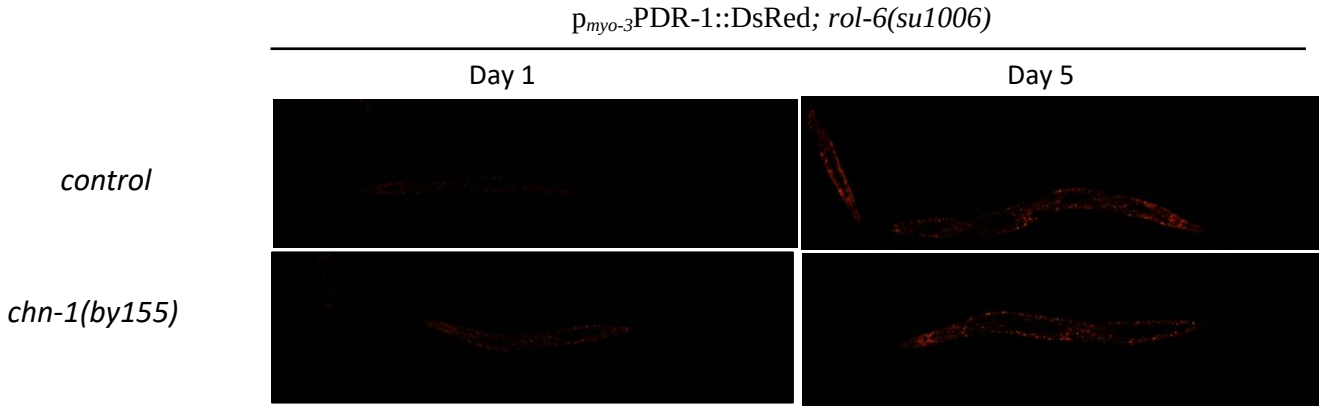


Figure 23.: PDR-1 levels upon CHN-1 depletion. The PDR-1 protein levels were quantified using the DsRed tag which was fused with the protein. Upon CHN-1 depletion the PDR-1 protein levels were increased both in Day 1 animals and in Day 5 animals.

4. CHN-1/CHIP depletion does not alter DCT-1 protein levels

According to literature, the mitochondrial proteins NIX/BNIP3 in mammals play a very important role in the process of mitophagy. Their deletion, however, does not affect the general autophagy machinery of the cell., The homolog of these proteins in *c.elegans* is the proteins DCT-1 (DAF-16 Controlled, germline Tumor affectin). Since DCT-1 protein has a major role in mitophagy, we aimed to investigate, whether loss of CHIP protein would have any impact on the levels of the proteins. We used a *c.elegans* strain expressing DCT-1 fused with GFP under its own promoter (Pdct-1). We checked the GFP levels which corresponded to the DCT-1 levels in wild type worms and chn-1 mutant worms. We did not, however, detect any significant difference between the wild type and the mutant animals. Consequently, *chn-1* loss does not affect the protein levels of DCT-1.

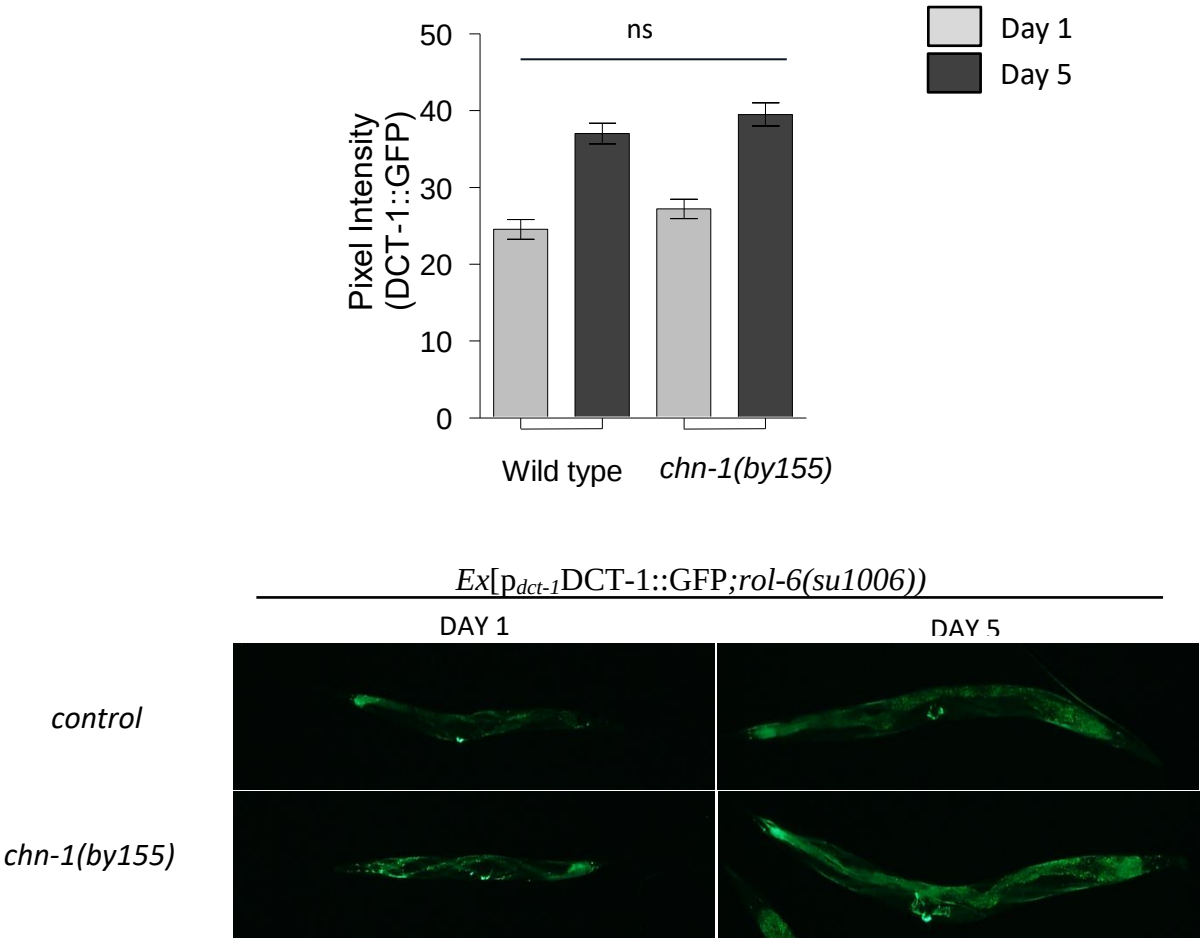


Figure 24.: DCT-1 levels upon CHN-1 depletion. The DCT-1 protein levels were quantified using the GFP tag which was fused with the protein. Upon CHN-1 depletion the DCT-1 protein levels were not altered both in Day 1 animals and in Day 5 animals compared with wild type animals expressing DCT-1::GFP.

5. CHN-1/CHIP deletion does not affect UPR^{mt} activation

Upon induction of the UPR^{mt}, mitochondrial chaperone genes hsp-6 and hsp-60 are transcriptionally upregulated according to literature. In order to assess whether the deletion of chn-1 ligase would have any impact on the induction of UPR^{mt}, we used a drug named Antimycin which induces UPR^{mt} by inhibiting the electron transport chain. Wild type and mutant for chn-1 animals expressing the HSP-6 protein fused with GFP under the promoter of hsp-6 and were synchronized until day 4 and treated with antimycin for 24 hours. Another group of wild type animals and chn-1 mutant animals were raised simultaneously without antimycin treatment. This group was considered as the control. Then, at Day 5, they were observed under the epi-fluorescence microscope and pictures were taken and analyzed. The GFP levels were quantified with Image J. After the quantification, we could observe that after UPR^{mt} induction, the HSP-6 protein levels were increased both in the wild type animals and chn-1 mutant animals. The same experiment was performed with RNAi of the hsp-6 in pL4440.

Subsequently, we hypothesized that CHIP loss does not affect the induction of UPR^{mt}.

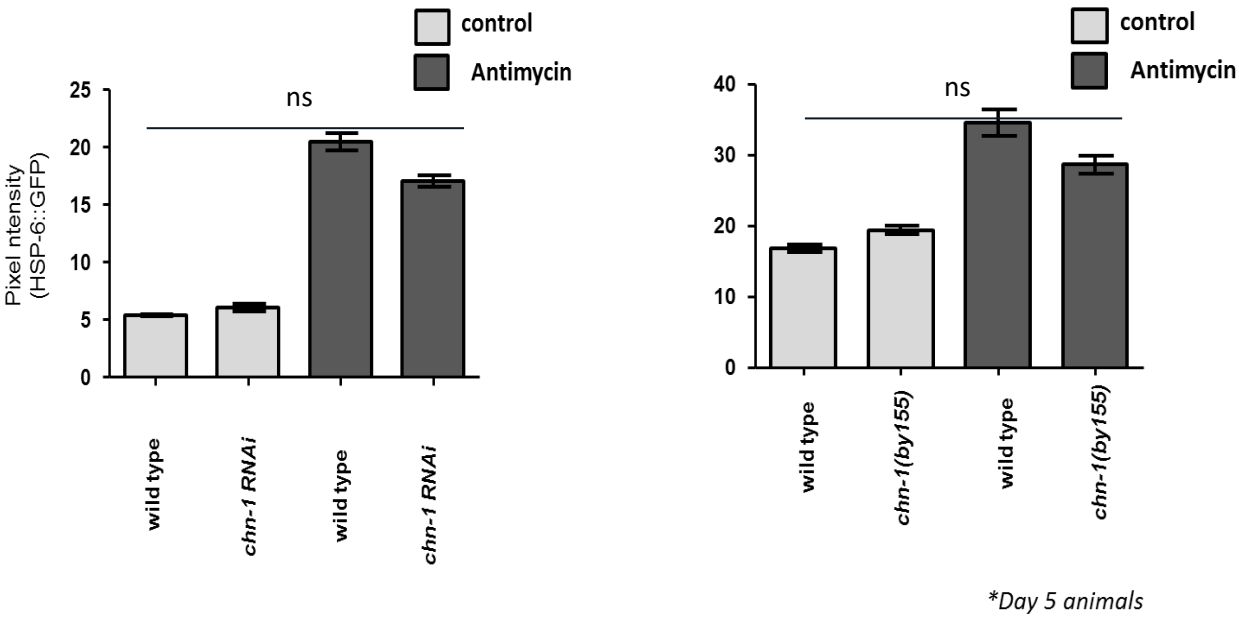
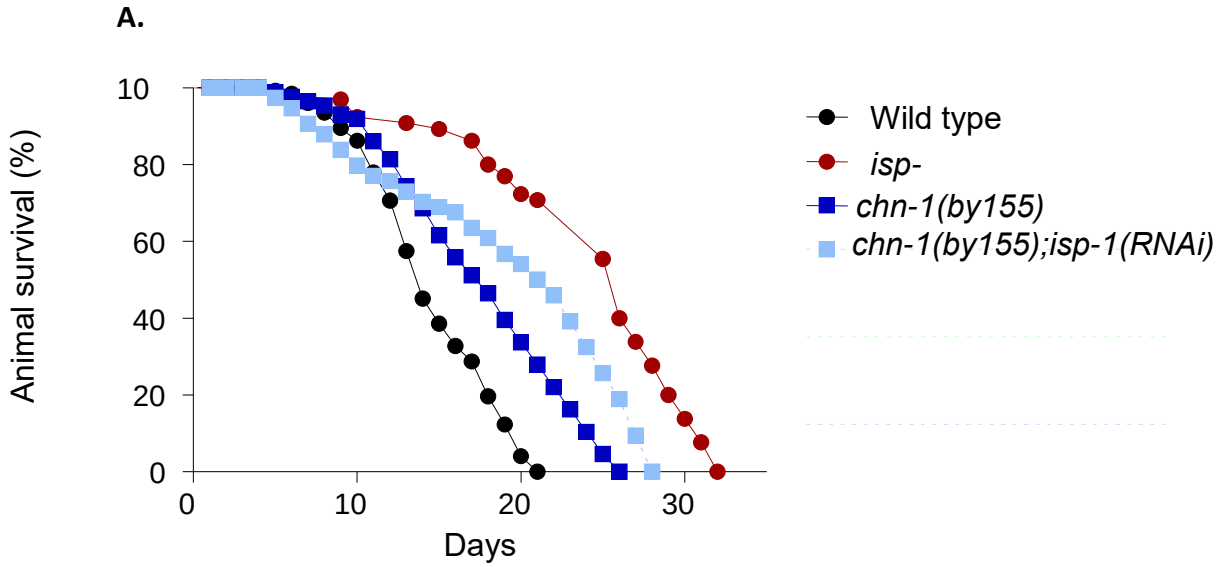


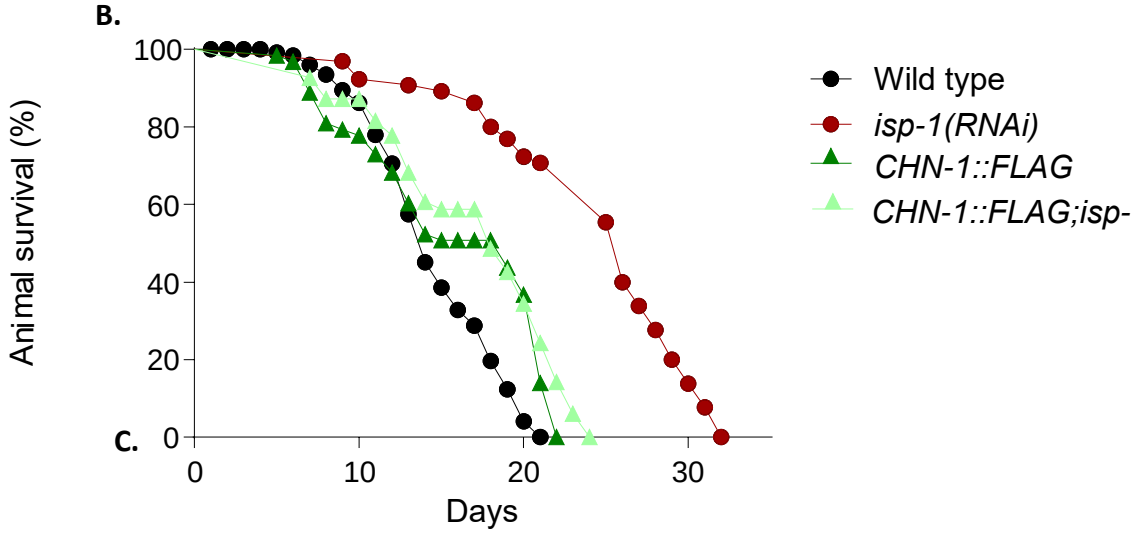
Figure 25.: UPR^{mt} induction with Antimycin A treatment. Wild type and mutant for chn-1 animals expressing the HSP-6 protein fused with GFP under the promoter of hsp-6 and were synchronized until day 4 and treated with antimycin for 24 hours. Another group of wild type animals and chn-1 mutant animals were raised simultaneously without antimycin treatment. This group was considered as the control. Then, at Day 5, they were observed under

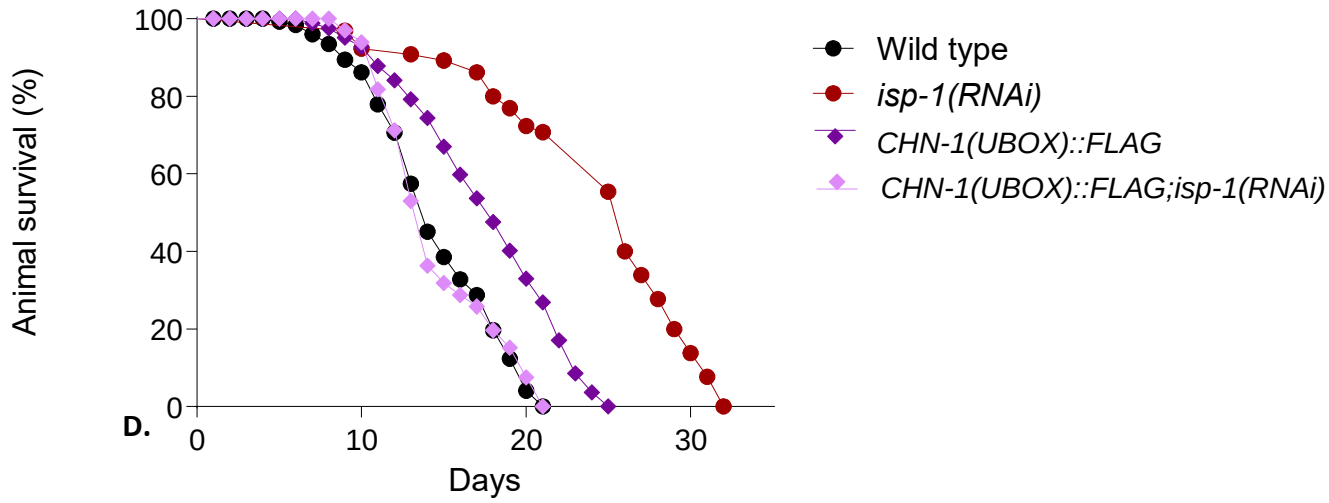
the epifluorescence microscope and pictures were taken and analyzed. The GFP levels were quantified with Image J. After the quantification, we could observe that after UPR^{mt} induction, the HSP-6 protein levels were increased both in the wild type animals and chn-1 mutant animals. The same experiment was performed with RNAi of the hsp-6 in pL4440.

6. CHN-1/CHIP deletion results in mild impairment of longevity

We finally, aimed to investigate whether the loss of CHIP would affect longevity. In previous studies, they have shown that indeed, chn-1 RNAi results in reduction of longevity. We aimed to see whether we would observe the same phenotype with the *chn-1(by155)* mutant. Furthermore, we aimed to investigate whether the UBOX domain loss is the one responsible for the phenotype. Using wild type animals as a control, wild type animals treated with isp-1 RNAi (a mutation in the Caenorhabditis *elegans* iron sulfur protein (isp-1) of mitochondrial complex III, which results in increased lifespan) as a positive control and *chn-1(by155),* p$_{chn-1}$CHN-1::FLAG and p$_{chn-1}$CHN-1(UBOX)::FLAG all treated with the empty pl4440 vector or the isp-1 in pL4440. We indeed observed a decrease in longevity when *chn-1* gene was depleted. The same phenotype was observed in the case of the depletion of the UBOX domain of *chn-1* gene. However, the overexpression of CHN-1 protein did not rescue the effect.







Strains	Median survival	Maximum survival
N2	14	21
N2;isp-1(RNAi)	26	32
chn-1(by155)	18	26
*chn-1(by155);*isp(RNAi)	21.5	28
p_{chn-1}CHN-1::FLAG	19	22
p_{chn-1}CHN-1::FLAG;isp-1(RNAi)	18	24
p_{chn-1}CHN-1(UBOX)::FLAG	18	25
p_{chn-1}CHN-1(UBOX)::FLAG UBOX;isp-1(RNAi)	14	21

Figure 26.: Lifespan assays. A. Lifespan assay between the chn-1(by155) mutant and wild type animals treated both with isp-1 RNAi and empty pL4440 vector. **B.** Lifespan assay between the overexpressing line of CHN-1 protein and wild type animals treated both with isp-1 RNAi and empty pL4440 vector. **C.** Lifespan assay between UBOX depleted animals and wild type animals treated both with isp-1 RNAi and empty pL4440 vector. **D.** Median and Maximum survival for A, B,C lifespan assays

DISCUSSION PART II

Impaired mitochondria quality control can result in detrimental consequences for the cell leading to less ATP production, accumulation of damaged mitochondria, increase in the reactive oxygen species production etc. There is a necessity, as a result, for mitochondria quality control mechanisms which can restore the damage and the serious consequences of this damage for the cell. These mechanisms include UPR^{mt}, mitophagy and apoptosis. The first mechanism activated is UPR^{mt} and when this mechanism is not able to compensate with the size of the existing damage, mitophagy takes its place. If Mitophagy, as well, cannot restore and repair the damage, then the final result is apoptosis. However, what is not known is the dynamic coordination between UPR^{mt} and mitophagy. In this study, we suggest a role of CHIP/CHN-1 E3 ubiquitin ligase in the coordination of these two quality control systems. Our findings indicate that CHIP plays an important role in mitophagy since its absence results in impaired mitochondria bioenergetics, increased pdr-1 levels and reduction in lifespan.

REFERENCES

1. Wormatlas Homepage. http://www.wormatlas.org/. Accessed July 19, 2018.

2. Sifri CD, Begun J, Ausubel FM, Calderwood SB. Caenorhabditis elegans as a model host for Staphylococcus aureus pathogenesis. *Infect Immun*. 2003;71(4):2208-2217. doi:10.1128/IAI.71.4.2208-2217.2003.

3. Mok DZL, Sternberg PW, Inoue T. Morphologically defined sub-stages of C. elegans vulval development in the fourth larval stage. *BMC Dev Biol*. 2015;15:26. doi:10.1186/s12861-015-0076-7.

4. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Caenorhabditis Elegans: Development from the Perspective of the Individual Cell. 2002. https://www.ncbi.nlm.nih.gov/books/NBK26861/. Accessed July 20, 2018.

5. Gilbert SF. Early Development of the Nematode Caenorhabditis elegans. 2000. https://www.ncbi.nlm.nih.gov/books/NBK10011/. Accessed July 20, 2018.

6. Teotónio H, Estes S, Phillips PC, Baer CF. Experimental Evolution with Caenorhabditis Nematodes. *Genetics*. 2017;206(2):691-716. doi:10.1534/genetics.115.186288.

7. Albeg A, Smith CJ, Chatzigeorgiou M, et al. C. elegans multi-dendritic sensory neurons: morphology and function. *Mol Cell Neurosci*. 2011;46(1):308-317. doi:10.1016/j.mcn.2010.10.001.

8. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol*. 2010;221(1):3-12. doi:10.1002/path.2697.

9. Yang Z, Klionsky DJ. An Overview of the Molecular Mechanism of Autophagy. In: *Current Topics in Microbiology and Immunology*. Vol 335. ; 2009:1-32. doi:10.1007/978-3-642-00302-8_1.

10. Nikoletopoulou V, Papandreou M-E, Tavernarakis N. Autophagy in the physiology and pathology of the central nervous system. *Cell Death Differ*. 2015;22(3):398-407. doi:10.1038/cdd.2014.204.

11. Galluzzi L, Baehrecke EH, Ballabio A, et al. Molecular definitions of

autophagy and related processes. *EMBO J*. 2017;36(13):1811-1836. doi:10.15252/embj.201796697.

12. Martinez-Lopez N, Athonvarangkul D, Mishall P, Sahu S, Singh R. Autophagy proteins regulate ERK phosphorylation. *Nat Commun*. 2013;4(1):2799. doi:10.1038/ncomms3799.

13. Obara K, Sekito T, Niimi K, Ohsumi Y. The Atg18-Atg2 complex is recruited to autophagic membranes via phosphatidylinositol 3-phosphate and exerts an essential function. *J Biol Chem*. 2008;283(35):23972-23980. doi:10.1074/jbc.M803180200.

14. Mizushima N, Yoshimori T, Ohsumi Y. The Role of Atg Proteins in Autophagosome Formation. *Annu Rev Cell Dev Biol*. 2011;27(1):107-132. doi:10.1146/annurev-cellbio-092910-154005.

15. Reggiori F, Ungermann C. Autophagosome Maturation and Fusion. *J Mol Biol*. 2017;429(4):486-496. doi:10.1016/j.jmb.2017.01.002.

16. Alberti A, Michelet X, Djeddi A, Legouis R. The autophagosomal protein LGG-2 acts synergistically with LGG-1 in dauer formation and longevity in *C. elegans*. *Autophagy*. 2010;6(5):622-633. doi:10.4161/auto.6.5.12252.

17. Autophagy in Human Disease - mTOR/ Autophagy 2 - Intensive Dietary Management (IDM). https://idmprogram.com/autophagy-in-human-disease-mtor-autophagy-2/. Accessed July 19, 2018.

18. Caltag Medsystems: Autophagy: an Extensive Line Up of Autophagic Targets. http://caltagmedsystems.blogspot.com/2013/09/autophagy-extensive-line-up-of_13.html. Accessed July 19, 2018.

19. Jiang P, Mizushima N. Autophagy and human diseases. *Cell Res*. 2014;24(1):69-79. doi:10.1038/cr.2013.161.

20. Martinez-Lopez N, Athonvarangkul D, Singh R. Autophagy and aging. *Adv Exp Med Biol*. 2015;847:73-87. doi:10.1007/978-1-4939-2404-2_3.

21. Wilhelm T, Richly H, Richly H. Autophagy during ageing-from Dr Jekyll to Mr Hyde. doi:10.1111/febs.14453.

22. Menzies FM, Fleming A, Rubinsztein DC. Compromised autophagy and neurodegenerative diseases. *Nat Rev Neurosci*. 2015;16(6):345-357. doi:10.1038/nrn3961.

23. Chung K, Wallace J, Kim S-Y, et al. Structural and molecular interrogation of intact biological systems. *Nature*. 2013;497(7449):332-337. doi:10.1038/nature12107.

24. Oda Y. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. *Pathol Int*. 1999;49(11):921-937. http://www.ncbi.nlm.nih.gov/pubmed/10594838. Accessed July 19, 2018.

25. Mullen GP, Mathews EA, Vu MH, et al. Choline transport and de novo choline synthesis support acetylcholine biosynthesis in Caenorhabditis elegans cholinergic neurons. *Genetics*. 2007;177(1):195-204. doi:10.1534/genetics.107.074120.

26. Ungar D, Hughson FM. SNARE Protein Structure and Function. *Annu Rev Cell Dev Biol*. 2003;19(1):493-517. doi:10.1146/annurev.cellbio.19.110701.155609.

27. Südhof TC. The Presynaptic Active Zone. *Neuron*. 2012;75(1):11-25. doi:10.1016/j.neuron.2012.06.012.

28. Shen D-N, Zhang L-H, Wei E-Q, Yang Y. Autophagy in synaptic development, function, and pathology. *Neurosci Bull*. 2015;31(4):416-426. doi:10.1007/s12264-015-1536-6.

29. Brand MD, Orr AL, Perevoshchikova I V, Quinlan CL. The role of mitochondrial function and cellular bioenergetics in ageing and disease. *Br J Dermatol*. 2013;169 Suppl 2(0 2):1-8. doi:10.1111/bjd.12208.

30. Jonckheere AI, Smeitink JAM, Rodenburg RJT. Mitochondrial ATP synthase: architecture, function and pathology. *J Inherit Metab Dis*. 2012;35(2):211-225. doi:10.1007/s10545-011-9382-9.

31. Friedman JR, Nunnari J. Mitochondrial form and function. *Nature*. 2014;505(7483):335-343. doi:10.1038/nature12985.

32. Osellame LD, Blacker TS, Duchen MR. Cellular and molecular mechanisms of

mitochondrial function. *Best Pract Res Clin Endocrinol Metab*. 2012;26(6):711-723. doi:10.1016/j.beem.2012.05.003.

33. Linnane AW, Lukins HB, Molloy PL, et al. Biogenesis of mitochondria: molecular mapping of the mitochondrial genome of yeast. *Proc Natl Acad Sci U S A*. 1976;73(6):2082-2085. http://www.ncbi.nlm.nih.gov/pubmed/778855. Accessed July 20, 2018.

34. Tanida I. Autophagy basics. *Microbiol Immunol*. 2011;55(1):1-11. doi:10.1111/j.1348-0421.2010.00271.x.

35. Nowikovsky K, Reipert S, Devenish RJ, Schweyen RJ. Mdm38 protein depletion causes loss of mitochondrial K+/H+ exchange activity, osmotic swelling and mitophagy. *Cell Death Differ*. 2007;14(9):1647-1656. doi:10.1038/sj.cdd.4402167.

36. Twig G, Hyde B, Shirihai OS. Mitochondrial fusion, fission and autophagy as a quality control axis: The bioenergetic view. *Biochim Biophys Acta - Bioenerg*. 2008;1777(9):1092-1097. doi:10.1016/j.bbabio.2008.05.001.

37. Kissová I, Deffieu M, Manon S, Camougrand N. Uth1p Is Involved in the Autophagic Degradation of Mitochondria. *J Biol Chem*. 2004;279(37):39068-39074. doi:10.1074/jbc.M406960200.

38. Whitworth AJ, Pallanck LJ. PINK1/Parkin mitophagy and neurodegeneration—what do we really know in vivo ? *Curr Opin Genet Dev*. 2017;44:47-53. doi:10.1016/j.gde.2017.01.016.

39. Palikaras K, Lionaki E, Tavernarakis N. Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature*. 2015;521(7553):525-528. doi:10.1038/nature14300.

40. Fischer F, Hamann A, Osiewacz HD. Mitochondrial quality control: an integrated network of pathways. *Trends Biochem Sci*. 2012;37:284-292. doi:10.1016/j.tibs.2012.02.004.

41. Shpilka T, Haynes CM. The mitochondrial UPR: mechanisms, physiological functions and implications in ageing. *Nat Rev Mol Cell Biol*. 2017;19(2):109-

120. doi:10.1038/nrm.2017.110.

42. Nyamsuren O, Faggionato D, Loch W, Schulze E, Baumeister R. A mutation in CHN-1/CHIP suppresses muscle degeneration in Caenorhabditis elegans. *Dev Biol*. 2007;312(1):193-202. doi:10.1016/J.YDBIO.2007.09.033.

43. Tawo R, Pokrzywa W, Kevei É, et al. The Ubiquitin Ligase CHIP Integrates Proteostasis and Aging by Regulation of Insulin Receptor Turnover. *Cell*. 2017;169(3):470-482.e13. doi:10.1016/j.cell.2017.04.003.

44. Kumar P, Pradhan K, Karunya R, Ambasta RK, Querfurth HW. Cross-functional E3 ligases Parkin and C-terminus Hsp70-interacting protein in neurodegenerative disorders. *J Neurochem*. 2012;120(3):350-370. doi:10.1111/j.1471-4159.2011.07588.x.

45. Lorenz TC. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J Vis Exp*. 2012;(63):e3998. doi:10.3791/3998.

46. *Single Worm PCR | The Chin-Sang Lab*. http://post.queensu.ca/~chinsang/lab-protocols/single-worm-pcr.html. Accessed July 20, 2018.

47. Palikaras K, Tavernarakis N. Measuring Oxygen Consumption Rate in Caenorhabditis elegans. *Bio-protocol*. 2016;6(23). doi:10.21769/BioProtoc.2049.

48. Palikaras K, Tavernarakis N. Intracellular Assessment of ATP Levels in Caenorhabditis elegans. *BIO-PROTOCOL*. 2016;6(23). doi:10.21769/BioProtoc.2048.

49. Saifee O, Wei L, Nonet ML. The Caenorhabditis elegans unc-64 locus encodes a syntaxin that interacts genetically with synaptobrevin. *Mol Biol Cell*. 1998;9(6):1235-1252. http://www.ncbi.nlm.nih.gov/pubmed/9614171. Accessed July 20, 2018.