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Master thesis:

"The role of autophagy in lifespan regulation upon inhibition of protein synthesis in *Caenorhabditis elegans*"

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Μεταπτυχιακή εργασία:

"Η αυτοφαγία ως μηχανισμός ρύθμισης του προσδόκιμου ζωής υπό συνθήκες μειωμένης πρωτεϊνοσύνθεσης στο νηματώδη *Caenorhabditis elegans*"

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SUMMARY

Autophagy is a conserved degradation pathway which regulates several aspects of cellular homeostasis. Based on its key role in recycling and quality control, autophagy is associated with enhanced longevity and healthspan. Under the same concept of cellular homeostasis and energetic balance, conditions that attenuate protein synthesis lead to lifespan extension in several model organisms. Additionally, reduction in ribosomal proteins or genetic inhibition of ribosome biogenesis genes promotes longevity. The mTOR signaling pathway stands as common sensor and modulator of autophagy, protein synthesis and ribosomal biogenesis in lifespan regulation. We used *Caenorhabditis elegans* as a model in order to investigate the implication of autophagy in lifespan extension under conditions of reduced protein synthesis and also examine the possibility of selective degradation of ribosomes through the autophagic pathway. Our results indicate that core components of the autophagic machinery are required, at least in some cases, for the longevity associated with translation inhibition. Survival assays, involving a variety of mutant strains and RNAi treatments against key players of the translational machinery, suggest that autophagy is essential for lifespan extension observed upon reduced protein synthesis. RNAi-mediated knockdown of ribosomal genes leads to an increase of the autophagic flux while the number of autophagosomes is also markedly increased in mutant strains characterized by reduced global protein synthesis. Furthermore, RNAi-mediated knockdown of a LIR-bearing ribosomal protein (RPS-24) ameliorates the polyglutamine aggregation phenotype in a nematode model of Huntington's disease. In conclusion, our results suggest a role of autophagy in lifespan regulation under conditions of protein synthesis inhibition. We anticipate that further work will elucidate the complex interplay between autophagy and protein synthesis and provide evidence concerning its regulation.

ΠΕΡΙΛΗΨΗ

Η αυτοφαγία αποτελεί ένα συντηρημένο μηχανισμό αποδόμησης κυτταρικών συστατικών με σημαντικό ρόλο στη διατήρηση του ενεργειακού ισοζυγίου και της ομοιόστασης του κυττάρου. Βάσει της συνεισφοράς της στην ανακύκλωση και τον ποιοτικό έλεγχο μακρομορίων και κυτταρικών οργανιδίων, η αυτοφαγία ενισχύει τη μακροβιότητα και καθυστερεί τη διαδικασία της γήρανσης καθώς και την εμφάνιση παθολογιών που τη συνοδεύουν. Στο ίδιο πλαίσιο της διατήρησης της ομοιόστασης και ενεργειακής ισορροπίας του κυττάρου, παράγοντες που οδηγούν σε μειωμένη πρωτεϊνοσύνθεση σχετίζονται με επέκταση του προσδόκιμου ζωής πολλών οργανισμών-μοντέλων. Μάλιστα, μείωση στα επίπεδα ριβοσωμικών πρωτεϊνών ή καταστολή γονιδίων υπεύθυνων για τη βιοσύνθεση ριβοσωμάτων προωθεί και ενισχύει τη μακροζωία. Επιπρόσθετα, η αυτοφαγία και η πρωτεϊνοσύνθεση, και ειδικότερα η βιοσύνθεση ριβοσωμάτων, υπόκεινται σε κοινό έλεγχο από το σηματοδοτικό μονοπάτι mTOR για τη ρύθμιση του προσδόκιμου ζωής. Στη μελέτη αυτή χρησιμοποιήσαμε το νηματώδη *Caenorhabditis elegans* προκειμένου να διερευνήσουμε το ρόλο της αυτοφαγίας στην επέκταση του προσδόκιμου ορίου ζωής που παρατηρείται κάτω από συνθήκες μειωμένης πρωτεϊνοσύνθεσης και επιπρόσθετα να εξετάσουμε την πιθανότητα της επιλεκτικής αποδόμησης ριβοσωμάτων μέσω της αυτοφαγίας. Τα αποτελέσματα υποδεικνύουν ότι επιμέρους παράγοντες με θεμελιώδη ρόλο στο μηχανισμό της αυτοφαγίας είναι απαραίτητοι για το αυξημένο προσδόκιμο ζωής που σχετίζεται με αναστολή της πρωτεϊνοσύνθεσης. Δοκιμές γήρανσης, με τη χρήση μεταλλαγμάτων και αποσιώπηση, μέσω RNA παρεμβολής, βασικών παραγόντων του μεταφραστικού μηχανισμού, υποδεικνύουν ότι η αυτοφαγία είναι απαραίτητη για το αυξημένο όριο ζωής που παρατηρείται υπό αναστολή της πρωτεϊνοσύνθεσης. Σίγηση (RNAi) ριβοσωμικών γονιδίων επάγει την αυτοφαγία ενώ ο αριθμός των αυτοφαγοσωμάτων αυξάνεται σε στελέχη που φέρουν μεταλλαγές σε βασικούς παράγοντες του μεταφραστικού μηχανισμού. Επιπρόσθετα, αποσιώπηση μέσω RNAi της ριβοσωμικής πρωτεΐνης RPS-24, που χαρακτηρίζεται από ένα μοτίβο αλληλεπίδρασης με πρωτεΐνες της αυτοφαγίας, οδηγεί σε μείωση των συσσωματωμάτων που προκαλούνται από επαναλήψεις γλουταμίνης σε ένα διαγονιδιακό μοντέλο *C. elegans* της νόσου Huntington. Συνοψίζοντας, τα αποτελέσματα υποδεικνύουν τη συμμετοχή της αυτοφαγίας στη ρύθμιση της μακροζωίας υπό συνθήκες μειωμένης πρωτεϊνοσύνθεσης. Επιπρόσθετα πειράματα απαιτούνται για την περαιτέρω διαλεύκανση της σύνθετης συσχέτισης μεταξύ αυτοφαγίας και πρωτεϊνοσύνθεσης και των μηχανισμών ρύθμισής της.

Ευχαριστίες

Το να έχεις γύρω σου ανθρώπους που σε υποστηρίζουν και σε βοηθούν, αποτελεί έναν πολύ σημαντικό παράγοντα στην πορεία σου προς την επίτευξη οποιουδήποτε, ομολογουμένως, στόχου. Είτε αυτοί οι άνθρωποι είναι οικογένεια, καθηγητές, συνεργάτες, φίλοι καλοί και φίλοι καρδιακοί, για την κάθε συμβουλή και καλή κουβέντα που σου χάρισαν σίγουρα αξίζουν ένα ευχαριστώ.

Για το λόγο αυτό θέλω πρωτίστως να ευχαριστήσω τον καθηγητή μου, κ. Νεκτάριο Ταβερναράκη, που με δέχτηκε στο εργαστήριό του και μου έδωσε τη δυνατότητα να εργαστώ πάνω στον τομέα Νευρογενετικής και Γήρανσης μέσω της εκπόνησης της μεταπτυχιακής μου εργασίας. Νιώθω ευτυχής για όλες τις ευκαιρίες που μου προσέφερε και τη βοήθεια που μου παρείχε όλο αυτό το διάστημα.

Θέλω ολόψυχα να ευχαριστήσω την επιβλέπουσά μου στο εργαστήριο, κ. Μαίρη Μαρκάκη, που με εκπαίδευσε και με στήριξε καθ' όλη τη διάρκεια της πορείας μου. Την ευχαριστώ για τις κουβέντες της –πάντοτε καθησυχαστικές και ενθαρρυντικές- , τις συμβουλές της και το γενικότερο ενδιαφέρον της για τη δουλειά μας και τον καθένα μας ξεχωριστά. κ. Μαίρη ένα μεγάλο ευχαριστώ που μου μεταδώσατε τις γνώσεις σας και ήσασταν εκεί για μένα όποτε σας χρειάστηκα!

Θέλω επίσης να ευχαριστήσω από καρδιάς όλους τους συναδέλφους και φίλους που απέκτησα μέσα σ' αυτό το εργαστήριο. Την Αγγέλα, τον Κώστα, την Andrea, την Ιωάννα, τον Ηλία, τον Κώστα, το Μάνο, την Ειρήνη, την Πέρσα, τη Μάρθα, τη Διονυσία, τη Μαργαρίτα και το Θάνο. Ευχαριστώ που με κάνατε να αισθανθώ τόσο ευπρόσδεκτη στο εργαστήριο, μοιραστήκατε τις γνώσεις σας, με συμβουλεύσατε σε θέματα που με απασχολούσαν και δημιουργήσατε ένα όμορφο κλίμα συνεργασίας.

Και φυσικά, ευχαριστώ πολύ την Αγγελικούλα, την Ιωάννα, τη Χριστίνα και το Νίκο για τη βοήθεια, την υποστήριξη και τις αμέτρητες ευχάριστες στιγμές που θα έχω να θυμάμαι. Σας ευχαριστώ εκτός από τις συμβουλές σας σε θέματα εργαστηριακά, για το γέλιο, τη δύναμη και τη καλή διάθεση που μου χαρίσατε μέσα από σχετικές και άσχετες συζητήσεις, αστεία, αγκαλιές και πειράγματα.

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Contents

Introduction

A. Introduction to *Caenorhabditis elegans* biology

Caenorhabditis elegans was established as a multicellular eukaryotic model organism for the study of developmental biology and neurobiology by Sydney Brenner in 1963. It is a transparent, free-living nematode reaching 1 mm at length during adulthood. It mostly exists as a self-fertilizing hermaphrodite, although males arise naturally at a frequency of 1‰.

Hermaphrodites initially produce haploid sperm which is stored in the spermatheca at the L4 stage, and subsequently during early adulthood they start to produce oocytes. Therefore, in cases where there are no males for cross-fertilization, the hermaphrodites can self-fertilize producing an entire population (~300) of progeny (Wormbook).

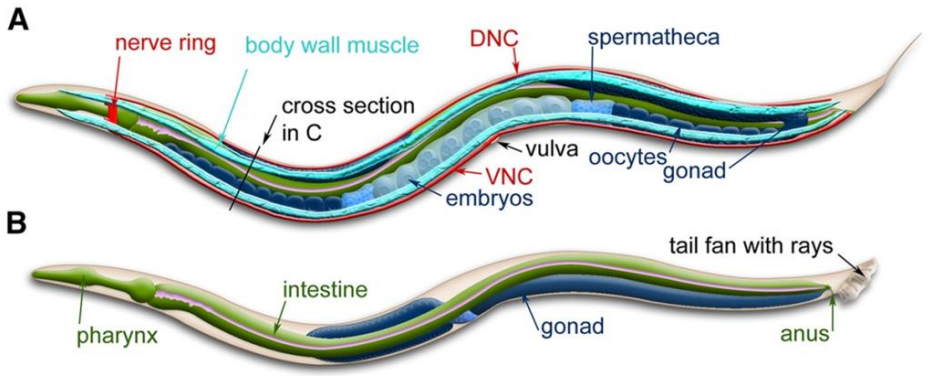


Figure 1: *C. elegans* anatomy. Major anatomical features of a hermaphrodite (A) and a male (B). Image adapted from WormAtlas.

C. elegans has a short life cycle (Figure 2). After fertilization embryogenesis lasts approximately 16 hours at 20°C. Embryos are retained within the hermaphrodite until the 24-cell stage when they are laid. After hatching the animals gradually develop through four larval stages, L1-L4. The L1 stage lasts for ~16 hours while the rest larval stages have a ~ 12 hour-long duration. After the L4 molt, adult hermaphrodites enter the reproductive period where they begin to produce progeny for ~6 days. Animals can live several more weeks (~3-4) before they die. In case they are starved, overcrowded or experience elevated temperatures, L2 larvae enter an alternative developmental pathway where they transcend into an L3 larval stage called the dauer larva (WormBook). Dauer larvae are more resistant and manage to survive under environmental stresses until conditions become favorable again, at which point they exit this stage and continue their development as slightly different L4 larvae (Golden and Riddle, 1984).

In the laboratory, *C. elegans* can be easily maintained through culturing in solid or liquid medium using *Escherichia coli* bacteria as a food source. Nematodes can live at different temperatures, 16°C, 20°C and 25°C, and can also be frozen at -80°C for long-term maintenance.

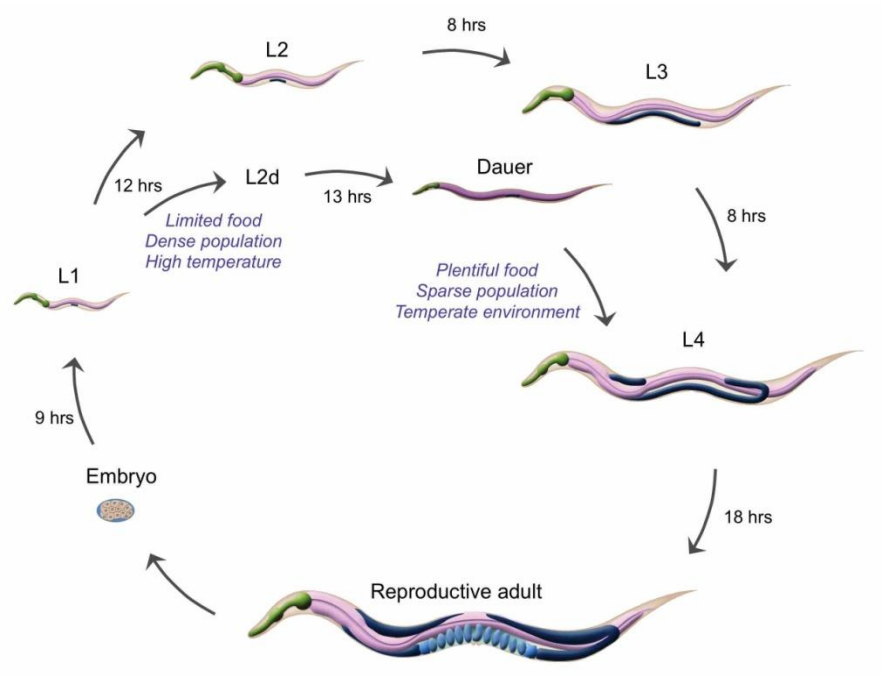


Figure 2: *C. elegans* life cycle with dauer branch. Image adapted from WormAtlas.

The entire genome of *C. elegans* is 100 Mb; individual genes are organized in five sets of autosomal chromosomes named I, II, III, IV, V and the X chromosome, and they are arranged in a conventional eukaryotic fashion with 5' untranslated regions, open reading frames (ORFs) containing exons and introns, and 3' untranslated regions. *C. elegans* is, in fact, the first multicellular organism whose genome was sequenced (WormBook).

C. elegans has been proved a powerful system to study genetics as well as general eukaryotic biology. Key features of the model include its transparency, large brood size, ease of cultivation in the lab, low maintenance expense and its detailed characterization (genomic sequence, development, anatomy etc.). In addition, its rapid life cycle (2.5 days at 25°C) and short lifespan renders it an ideal tool for ageing studies. Most importantly, the conservation of molecular and cellular processes that take place in *C. elegans* and other more complex organisms across evolution makes it an excellent model system of eukaryotic biology, even at the level of human physiology and pathogenesis.

B. Autophagy

Cells undergo constant morphological and functional changes during differentiation, development and ageing. This need of continuous renovation requires the existence of recycling and quality control systems which in eukaryotes involve autophagy and the ubiquitin-proteasome system (Mizushima and Komatsu, 2011).

Autophagy is a mechanism of vacuolar or lysosomal degradation that eliminates damaged or excessive cellular structures and misfolded proteins. In contrast to the proteasome-ubiquitin system, autophagy is an adaptive response for the degradation of longer-lived constituents and damaged or unwanted organelles (Anding and Baehrecke, 2017).

The term “autophagy” usually refers to the process of macroautophagy which is characterized by the formation of a double membrane compartment, called the autophagosome, which engulfs cytoplasmic material and ultimately fuses with the vacuole or lysosome for degradation (Figure 3). Due to the variety of autophagic cargoes, including hydrophilic molecules and large structures that need to cross membrane barriers, there are two other subtypes of autophagy, microautophagy and chaperone-mediated autophagy (CMA). Microautophagy refers to the direct uptake of cytoplasmic components by the vacuole or lysosome through the invagination of their limiting membrane. Chaperone mediated-autophagy takes place in mammalian cells, where in an *ATG* (autophagy-related genes) independent manner, soluble proteins bearing a KFERQ-like motif are transferred to the lysosome for degradation by cytosolic chaperones (Mizushima, 2018).

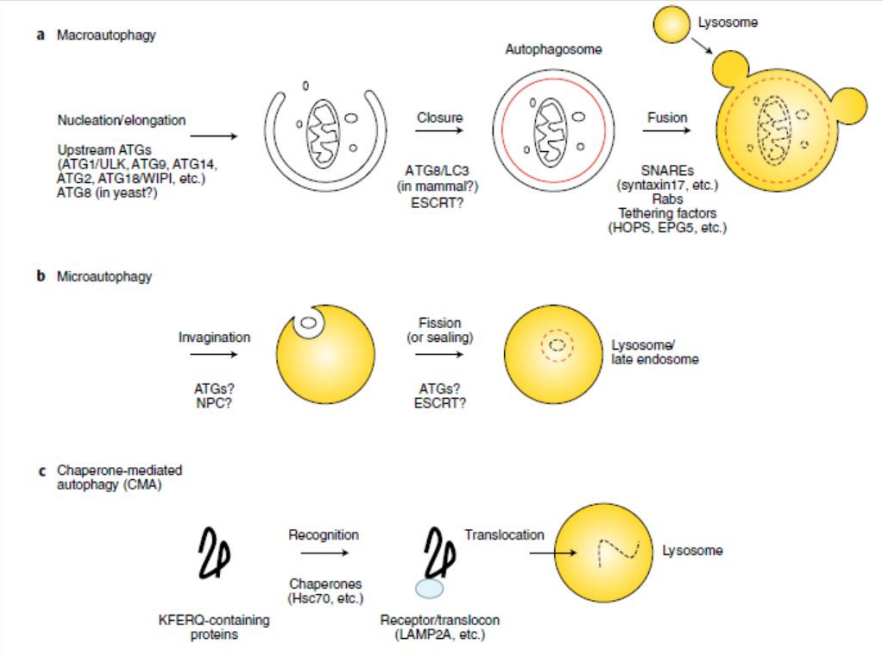


Figure 3: The three types of autophagy: macroautophagy, microautophagy and chaperone mediated autophagy (CMA). Image adapted from Mizushima, 2018.

ATGs (Autophagy-related genes) are highly conserved among eukaryotes (Table 1). Research conducted over the last two decades has led to the identification of 42 *ATGs*; sixteen genes have been characterized as 'core' autophagy genes based on their involvement on both selective and non-selective degradation pathways, in contrast to a more exclusive role of the rest (Nakatogawa *et al.*, 2009). This core autophagic machinery is considered to function in six complexes: Atg1/ULK1 kinase complex, Atg9 vesicles, Atg14 containing PI3K complex, Atg2/Atg18 complex, Atg8 and Atg12 ubiquitin –like conjugation systems. To a large extent the molecular functions of ATG proteins remain elusive. Atg1/ULK1 kinase complex participates in autophagosome formation (Zachari and Ganley, 2017) while the rest of the core machinery is suspected to act in membrane elongation and fusion. Atg9 vesicles act as a partial source for autophagosome formation (Suzuki and Ohsumi, 2007; Yang and Klionsky, 2010; Mizushima, Yoshimori and Ohsumi, 2011).

In more detail, a complex containing Atg6, or its mammalian homolog Beclin 1, recruits the PI3 kinase Vps34, necessary for autophagosome nucleation. After phagophore formation and nucleation, multimerization of the phagophore is achieved through the action of the ubiquitin –like conjugation systems. The E1-like Atg7 and E2-like Atg10 covalently link Atg12 with Atg5, which together bind to Atg16 to form the pre-autophagosomal structure. In parallel, after Atg8 is cleaved by Atg4, Atg7 and E2-like Atg3 perform its conjugation with phosphatidylethanolamine (PE). This lipidated form of Atg8 associates with newly formed autophagosomal membranes (Ichimura *et al.*, 2000). The generated vesicle subsequently fuses with the lysosome and its cargo is degraded by proteases, nucleases, lipases and glycosidases. As a final step, permeases of the lysosomal membrane release the degradation products back to the cytoplasm (Mizushima, Yoshimori and Ohsumi, 2011).

Table 1: Autophagy genes in yeast, *C. elegans* and mammals.

Yeast *ATG* gene	*C. elegans* homolog	Mammalian homolog
ATG1	*unc-51*	ULK1
ATG2	*atg-2*	ATG2A, ATG2B
ATG3	*atg-3*	ATG3
ATG4	*atg-4.1*	ATG4A, ATG4B, ATG4C, ATG4D
	atg-4.2	
ATG5	*atg-5*	ATG5
ATG7	*atg-7*	ATG7
ATG8	*lgg-1*	GABARAP, GABARAPL1, GABARAPL2
	lgg-2	LC3
ATG9	*atg-9*	ATG9
ATG10	*atg-10*	ATG10
ATG12	*lgg-3*	ATG12
ATG13	*epg-1*	ATG13
ATG14	*epg-8*	ATG14
ATG16	*atg-16.1*	ATG16L1, ATG16L2
	atg-16.2	
ATG17		RB1CC1
ATG18	*atg-18*	WIPI1, WIPI2, WIPI3, WIPI4
	epg-6	
VPS30/ATG6	*bec-1*	BECN1
VPS34	*vps-34*	PI3KC3
	epg-3	VMP1
	epg-4	EI24
	epg-5	EPG5
	epg-9	ATG101
	epg-2	
	epg-7	

Initially, macroautophagy (hereafter referred to as autophagy) was considered a bulk process, a non-selective degradation pathway in which random portions of the cytoplasm are degraded and recycled as a response to nutritional starvation. Indeed, the two major functions of autophagy entail the generation of degradation products and the clearance of defective organelles and macromolecules, ensuring not only new building blocks but also quality control necessary for cellular and organismal growth and survival. However, it has been established that these functions can be highly selective and target specific proteins and organelles (Reggiori *et al.*, 2012). Moreover, autophagy can take place at basal levels independently of any environmental stimuli, having a housekeeping role throughout life.

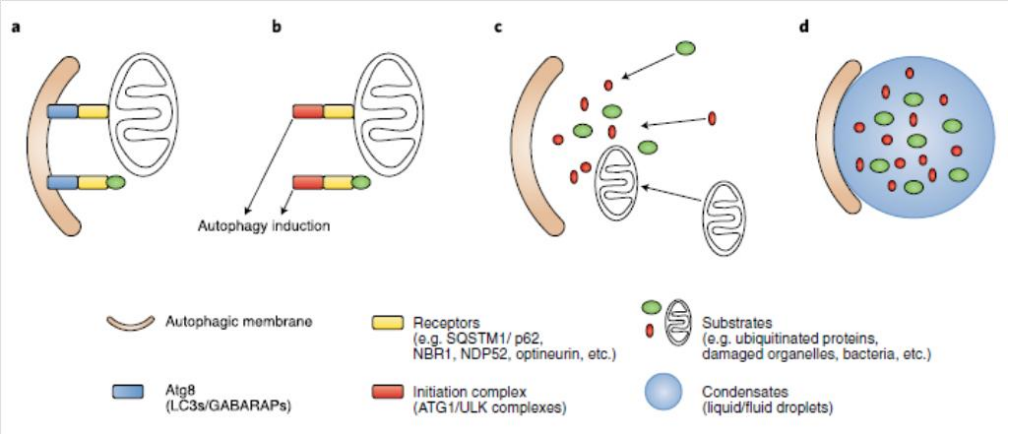


Figure 4: Mechanisms of selective macroautophagy. Image adapted from Mizushima, 2018.

The best characterized proteins of the autophagic machinery are members of the Atg8 family (Shpilka *et al.*, 2011; Mizushima *et al.*, 1998). In mammals two subfamilies of Atg8 proteins exist, LC3 (microtubule-associated proteins 1 light chain 3) and GABARAP (γ-aminobutyric acid receptor-associated protein). LC3 can be used as a marker to follow the entire autophagic pathway. Yeast has only one homolog while *Drosophila melanogaster* and *Caenorhabditis elegans* have two (Table 1). There are three distinct, yet not mutually exclusive ways, through which autophagy can be established as a selective process (Figure 4). LC3- interacting region (LIR) motifs, or Atg8-interacting motifs (AIMs), or GABARAP-interacting motifs (GIMs) are crucial for the recruitment of cargo to the inner surface of the phagophore and the recruitment of effector proteins to the outer autophagosomal membrane where they mediate autophagosomal maturation and transport to the lysosome (Birgisdottir, Lamark and Johansen, 2013). LIR motifs have been detected on either selective substrates or autophagy receptors suggesting selective engulfment through interaction with Atg8 on the autophagosomal membrane (Fracchiolla, Sawa-Makarska and Martens, 2017). Some specific substrates have also been found to recruit the Atg1/ULK1 kinase complex to induce autophagosome formation. Additionally, certain substrates seem to be enriched at

the site of autophagosome formation through an unknown Atg8-independent mechanism. Finally, it is hypothesized that some substrates may possess properties which allow them to act as a surface for preferential membrane elongation, facilitating autophagosome formation at the site of their presence (Mizushima, 2018).

Throughout the process of ageing molecular damage accumulates and protein homeostasis declines (López-Otín *et al.*, 2013). Loss of function mutations in many autophagy genes are linked with decreased lifespan in *D. melanogaster*, *S. cerevisiae* and *C. elegans* (Tóth *et al.*, 2007). These findings, combined with reduced levels of autophagy in ageing and disease, suggest that ageing is associated with insufficient autophagy. Additionally, increased autophagy has been found to contribute to longevity (Meléndez *et al.*, 2003). Several studies provide evidence which place autophagy as a downstream mechanism that mediates lifespan extension, in response to a variety of upstream regulators (Rubinsztein, Mariño and Kroemer, 2011; Madeo *et al.*, 2015).

Mechanistic Target of Rapamycin (TOR) is a highly conserved serine/threonine protein kinase that acts as an amino acid and nutrient sensor. It is a major regulator of cell growth, division and proliferation. Based on the key role of the autophagic process in cellular homeostasis, it is no wonder that autophagy and mTOR are tightly connected. In mammals, upon nutrient rich conditions mTORC1 suppresses the ULK1 complex, therefore autophagy, and promotes cell growth and metabolic activity. Upon starvation, mTORC1 dissociates from the ULK1 complex enabling the initiation of autophagosome formation and promoting cell maintenance and survival. Inactivation of mTOR even in nutrient-rich conditions upregulates autophagy, indicating that the mTOR pathway negatively regulates starvation-induced autophagy (Mizushima, 2010). Additional studies in model organisms including *S. cerevisiae* and *C. elegans* have demonstrated that mTORC1 inhibits autophagy (Noda and Ohsumi, 1998; Tóth *et al.*, 2007; Hansen *et al.*, 2008).

The mTORC1 - autophagy axis has been extensively studied in ageing regulation. Hansen *et al.* (2008) demonstrated that both mTOR inhibition and dietary restriction cause an autophagic phenotype in *C. elegans*. Inactivation of core autophagy genes abolishes the otherwise seen lifespan extension under these conditions.

Also as a degradation pathway, autophagy naturally targets protein aggregates. Intracellular aggregate-prone proteins associated with various diseases, of neurodegenerative nature or not, constitute crucial substrates of the autophagic process (Labbadia and Morimoto, 2015). Models of Parkinson's disease, Alzheimer's disease and Huntington's disease have been extensively used to verify the protective role of autophagy (Ravikumar *et al.*, 2010; Menzies *et al.*, 2017).

C. Protein synthesis

C. elegans shares many characteristics with higher eukaryotes and therefore has been proven a very useful model organism for the study of protein synthesis and its regulation in the context of various cellular responses (Rhoads, Dinkova and Korneeva, 2006). Many homologs of known initiation, elongation and release factors as well as components of the signal transduction pathways implicated in protein synthesis control have been characterized in *C. elegans*. Moreover, most of the key components of the core translational machinery have been identified, including rRNAs (Albertson, 1984; Files & Hirsh, 1981), tRNAs (Khosla & Honda, 1989; Lee et al., 1990), ribosomal proteins (Jones & Candido, 1993; Zorio, Cheng, Blumenthal, & Spieth, 1994; Gönczy et al., 2000) and aminoacyl tRNA synthetases (Amaar & Baillie, 1993; Gönczy et al., 2000).

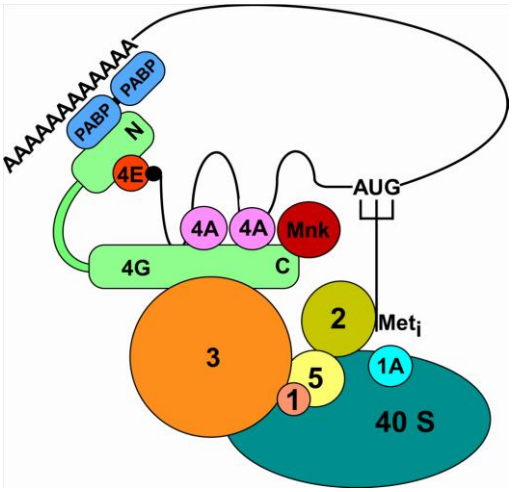


Figure 5: Model of the eukaryotic translation initiation complex. Image adapted from WormBook.

eIF4E (eukaryotic initiation factor 4E) is a 25 kDa cap-binding protein; it is one of the eIF4E proteins required for the recruitment of mRNA on the 40S initiation complex during translation initiation (Rhoads, Dinkova and Korneeva, 2006). Due to its role in the recruitment of the rest of the machinery it constitutes a major regulator of protein synthesis. In *C. elegans* five isoforms of eIF4E are encoded by genes *ife-1* through *ife-5* (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). Elimination of a specific isoform of eIF4E in somatic tissues, IFE-2, either through RNAi or a deletion, is associated with reduced global protein synthesis, increased oxidative stress resistance and extended lifespan (Syntichaki, Troulinaki and Tavernarakis, 2007).

Control of protein synthesis is fundamental for several aspects of cellular and organismal homeostasis such as development, differentiation, growth and apoptosis (Merrick, 2010). Protein synthesis is also one of the downstream targets of mechanistic TOR. The mTOR signaling pathway positively regulates protein synthesis by modulating the activities of key

regulators of the process including the ribosomal protein p70 S6 kinase (S6K) and the eIF4E-binding protein 4E-BP1. Upon nutrient limitation, mTOR inhibits protein synthesis initiation in part through the downregulation of S6K activity and 4E-BP1 dephosphorylation (Gingras, Raught and Sonenberg, 2001). 4E-BP1 normally suppresses initiation of protein synthesis by binding to eIF4E; under normal growth conditions phosphorylation of 4E-BP1 by mTOR negates its inhibitory role and renders eIF4E accessible to eIF4G for translation initiation (Ma & Blenis, 2009; Magnuson, Ekim, & Fingar, 2012).

TOR (LET-363) deficiency in *C. elegans*, either by mutation or RNA interference (RNAi), causes developmental arrest and a distinct phenotype of intestinal atrophy that can be attributed to inhibition of mRNA translation (Long *et al.*, 2002). Moreover, in *C. elegans* a 4E-BP1 homolog has been recently identified. Despite the restricted homology in sequence, IFET-1 shows functional conservation through binding to several eIF4E homologs *in vitro* (Li *et al.*, 2009).

Long et al. (2002) identified the worm ortholog of p70S6K, *rsks-1*. Despite the fact that direct phosphorylation targets of mTOR have not been found in *C. elegans*, studies in mammals and yeast have shown that mTORC1 directly phosphorylates S6 kinase (Ma & Blenis, 2009; Magnuson, Ekim, & Fingar, 2012). Mutation of *rsks-1* results in many phenotypes seen upon LET-363 depletion concerning lifespan, translation regulation and germline proliferation (Long *et al.*, 2002).

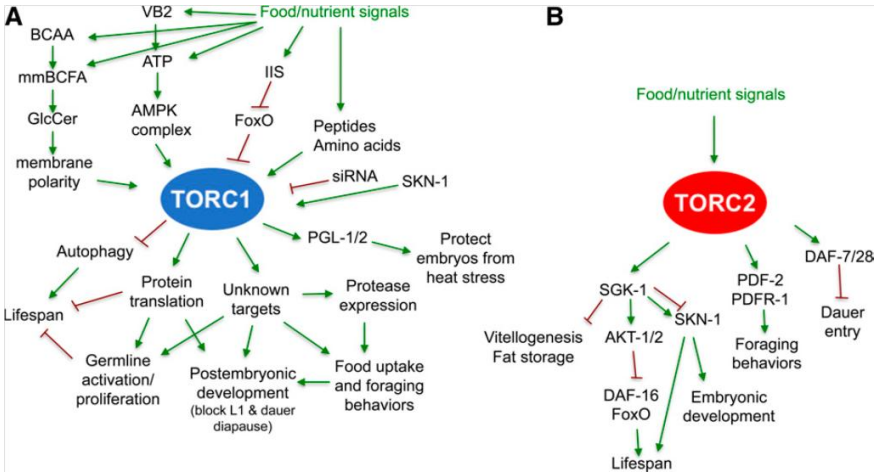


Figure 6: Upstream regulators and downstream targets of mTOR in *C. elegans*. Image adapted from Blackwell, Sewell, Wu, & Han, 2019.

A number of studies have been conducted in *C. elegans* in order to investigate the effect of protein synthesis inhibition on ageing. Interestingly, apart from IFE-2 depletion, reducing the levels of any of a large number of ribosomal proteins during adulthood enhances longevity

(Hansen *et al.*, 2007). Lifespan is also extended upon depletion of the worm homolog of the ribosomal protein S6 kinase, RSKS-1, and various translation initiation factors (Hansen et al., 2007; Pan et al., 2007; Tohyama, Yamaguchi and Yamashita, 2008). Moreover, treatments that inhibiting translation result in increased tolerance to various stresses (Hansen et al., 2007). In agreement with these findings, genetic mutations that attenuate protein synthesis in flies, mice and yeast also lead to increased lifespan (Kapahi *et al.*, 2004; Kaeberlein, 2012).

Based on the longevity effect observed upon loss of translation-related proteins, Hipkiss (2007) proposed a model on how decreasing protein synthesis can lead to lifespan extension. He suggested that within a context of general protein synthesis inhibition there is decreased synthesis of both normal and erroneous polypeptides. The generation of less misfolded or damaged proteins combined with the remaining action of various chaperones could alleviate the ageing phenotype and result in stress resistance and lifespan extension (Hipkiss, 2007). An excess in energy resources could also have a beneficial effect, however, the nature of this interconnection between translation and longevity is far more complex.

It is generally accepted that in a variety of organisms the rate of total protein synthesis declines with age (Labbadia and Morimoto, 2014; Ward, 2000). However, the molecular mechanisms that mediate these changes in protein synthesis during ageing are unknown. Even more importantly, it still remains elusive whether these changes in protein turnover are an effect of the ageing process or act as a causative mechanism (Tavernarakis, 2007).

D. Ribosomes

Ribosomes constitute a critical component of the translational machinery. They are large molecular complexes that carry out cellular protein synthesis. Their importance is reflected on the high amount of energy that cells dedicate for ribosome biogenesis and assembly: up to half of all protein and RNA synthesis is invested for the production of thousands of ribosomes every minute (Warner, 1999).

Disruptions to ribosome biogenesis are associated with acute loss of proteostasis in *Saccharomyces cerevisiae*. Perturbations in the synthesis of ribosomal proteins or rRNAs lead to proteostasis collapse due to the accumulation of orphan ribosomal proteins; newly-synthesized ribosomal proteins form insoluble aggregates and cause proteotoxicity (Tye *et al.*, 2019).

Under the same concept of homeostatic importance and energetic balance, ribosomes have been linked with lifespan regulation (Macinnes, 2016). Longevity studies using *S. cerevisiae* and *C. elegans* demonstrate that reduction in ribosomal proteins and inhibition of ribosome biogenesis genes extend lifespan (Hansen *et al.*, 2007; Steffen *et al.*, 2008; Macinnes, 2016). In parallel, reduction of such ribosomal factors has been implicated in increase of cellular autophagy (Artero-Castro *et al.*, 2015; Heijnen *et al.*, 2014). Loss of ribosomal proteins is shown to induce autophagy, in part through an increase in S6 kinase phosphorylation

(Heijnen *et al.*, 2014). Deficiency of ribosomal proteins in cancer cells causes a stress response through which cells survive by employing the autophagy as a protective mechanism (Artero-Castro *et al.*, 2015). Moreover, autophagy-dependent degradation of ribosomal RNA has been proven critical in maintaining nucleotide homeostasis during development in *C. elegans* (Liu *et al.*, 2018; Hillwig *et al.*, 2011).

The mTOR signaling pathway acts as common regulator of autophagy and protein synthesis, even specifically to ribosome biogenesis, in the coordination of lifespan extension (Macinnes, 2016). While acknowledging the complexity of this interplay, high mTOR signaling is associated with ribosome biogenesis, increased protein synthesis, cell growth and metabolic activity while inhibition of the mTOR pathway is associated with autophagy induction and reduced protein synthesis, both of which have been separately linked with enhanced longevity (Blackwell *et al.*, 2019)(Figure 7).

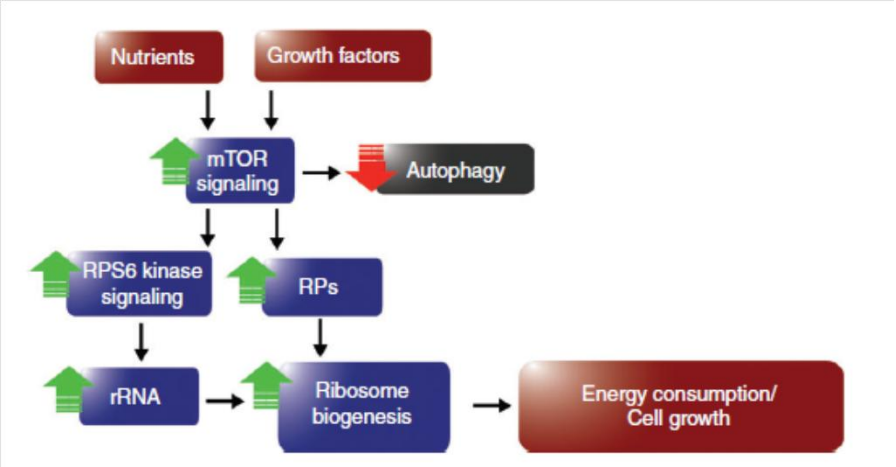


Figure 7: Basic pathways implicated in cell growth through the biogenesis and assembly of ribosomes in response to nutrients and growth factors. Image adapted from Macinnes, 2016.

Based on their biological significance, both protein translation and ribosome biogenesis are expected to be tightly controlled. Ribosomes are highly stable complexes with a half-life up to several days that comprise almost half of the cell's protein content (Warner, 1999). However, quality control mechanisms responsible for their degradation have been poorly understood. In yeast, excess ribosomal proteins are rapidly degraded by the ubiquitin-proteasome pathway; ribosomal proteins that fail to assemble into ribosomes, and would otherwise form insoluble aggregates, are rapidly ubiquitinated and degraded by the proteasome (Sung *et al.*, 2016). Interestingly, autophagy may also act as a degradation response mechanism to promote survival in the face of ribosomal stress or generally under conditions where the cell would benefit by selective and non-selective ribosomal degradation. In a more simplistic view, modulation of every aspect of ribosomal turnover could be considered as a large part of protein synthesis regulation. It is crucial to ensure that both of these closely related pathways are tightly controlled under conditions of stress or

changing metabolic needs to ensure translational fidelity and protein homeostasis (Frankel, Lubas and Lund, 2017; Lafontaine, 2010)

E. Ribophagy: selective degradation of ribosomes

Ribophagy, as a form of selective autophagy, was first discovered and studied in *Saccharomyces cerevisiae*. Kraft *et al.* (2008) were the first to use the term in order to describe the selective degradation of ribosomes under conditions of nitrogen starvation.

Kraft *et al.* (2008) showed that under nutrient rich conditions, the signal of a GFP-tagged ribosomal protein of the 60S ribosomal subunit was distributed throughout the cytoplasm. However, upon nitrogen starvation the protein accumulated in the vacuole. This relocalization was abolished in mutants of core autophagy proteins, including Atg7, indicating that autophagy was required. Compared to cytoplasmic control proteins, turnover kinetics of ribosomal proteins were significantly increased suggesting selective and rapid degradation of ribosomes under nitrogen starvation rather than non-selective uptake of cytoplasm (Kraft *et al.*, 2008).

Initial screening of a variety of starvation sensitive mutants with defective accumulation of ribosomal proteins in the vacuole, as well as many additional experimental findings, have implicated Ubp3-Bre5 de-ubiquitination complex as the principal component of the ribophagy mechanism in yeast. In yeast cells lacking Ubp3 (ubiquitin-specific processing protease 3) or Bre5 (brefeldin A sensitivity 5), general autophagy and trafficking pathways were not affected. However, ubp3Δ and bre5Δ cells died upon prolonged nitrogen starvation or upon treatment with the mTOR inhibitor rapamycin. Moreover, ubp3Δ cells were significantly enriched in ribosomal subunits and ribosome-associated proteins (Kraft *et al.*, 2008).

Based on their findings, Kraft *et al.* (2008) suggested a protective role of ubiquitination in the regulated degradation of (60S) mature ribosomes and proposed two different mechanisms for ribophagy in *Saccharomyces cerevisiae*. According to the first mechanism, de-ubiquitination by itself acts as a signal for the engulfment of the ribosomal components, as well as further maturation of the autophagosome and subsequent degradation. The second mechanism suggests that initial ubiquitination is the one acting as an ‘engulf me’ signal, while subsequent de-ubiquitination is what actually triggers autophagosome maturation and fusion with the vacuole (C. Kraft et al., 2008; Beau, Esclatine, & Codogno, 2008).

In agreement with the latter mechanism, Rsp5 and Ltn1 are E3 ubiquitin ligases in *Saccharomyces cerevisiae* that enable ubiquitination of ribosomal proteins of the 60S subunit. Beside their role in ribophagy, Rsp5 plays a major role in ubiquitin-dependent trafficking in the endocytic system, while Ltn1 has an active role in mRNA surveillance and ribosome-associated quality control. A temperature sensitive mutant of Rps5 when combined with a mutant for Ubp3 presented increased synthetic sickness upon rapamycin treatment. Additionally, the same double mutant depicted delayed cleaved-GFP accumulation in the vacuole, from the GFP-tagged ribosomal reporter, during nitrogen

starvation suggesting a potential cooperation between the two proteins (Kraft and Peter, 2008). Concerning Ltn-1, both Ubp3 and Ltn1 were found to target a protein of the large ribosomal subunit, but at the same time LTN1 deletion rescued the defects in vacuolar accumulation of ribosomal proteins in ubp3Δ yeast cells. Moreover, its levels were significantly reduced under nitrogen starvation. These findings pointed to an antagonistic relation between the two proteins which was further explained by the following model: Ltn-1 adds an ubiquitin mark or proteins of the 60S subunit which, under nutrient-rich conditions, is subsequently removed by Ubp3. Upon nitrogen starvation Lnt1 levels rapidly decay, negating the protective role of ubiquitin, so the remaining process of de-ubiquitination leads to rapid degradation of ribosomal proteins (Ossareh-Nazari *et al.*, 2014). Finding the unknown enzymes that mediate selective ribosomal degradation confirmed the respective mechanism introduced by Kraft *et al.* (2008) (Figure 8).

It was also suggested that ubiquitin could exert its protective role by inhibiting a potential interaction between the selected substrate and a receptor of the autophagosomal membrane (Ossareh-Nazari *et al.*, 2014) . Such a ribophagy receptor has not been discovered in yeast but NUFIP1 (nuclear FMRI interacting protein 1) has been attributed this role in mammalian cells. NUFIP1 has been found to co-localize with ribosomes and autophagosomes. Upon mTORC1 inhibition with rapamycin treatment, NUFIP1 re-localizes from the nucleus to the lysosomes in an autophagy dependent manner. Also, it is characterized by three LIR motifs and mutation in one of them leads to defects in ribosomal degradation (Wyant et al., 2018; Denton & Kumar, 2018; Jin & Klionsky, 2018)

As mentioned, ubiquitination of ribosomal substrates by these ligases acts as a signal for their engulfment by the elongating autophagosomal structure. Cdc48 (cell division cycle 48), a significant protein in proteasomal escort pathway, and its ubiquitin binding adaptor Ufd3, were new partners found to form a complex with Ubp3 and Bre5. Ubp3 and Cdc48 interact with distinct regions of Bre5; Ufd3 independently interacts with Cdc48 and Ubp3, but not Bre5. All components were vital for selective and efficient ribosomal degradation leading the authors to suggest that the Ubp3-Bre5/Cdc48-Ufd3 complex acts as a molecular scaffold for

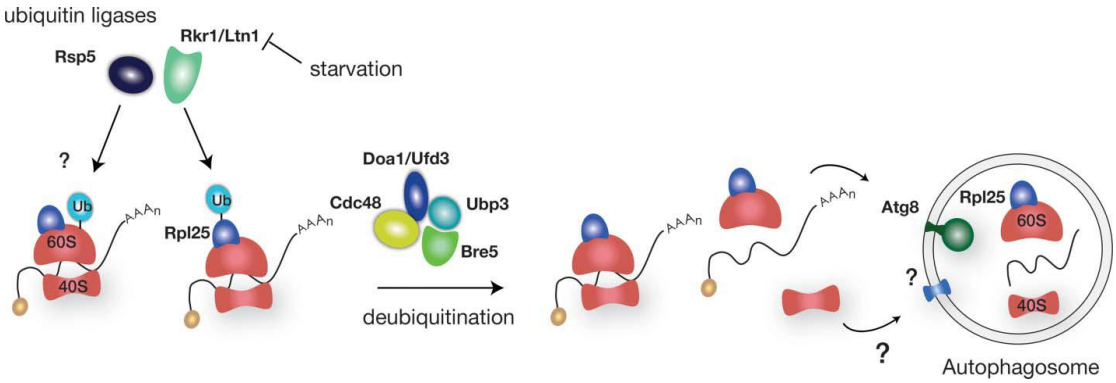


Figure 8: Mechanisms of ribophagy in yeast. Image adapted from Frankel, Lubas, & Lund, 2017

ubiquitinated ribosomal proteins of the 60S subunit; ubiquitin is removed to induce maturation of the autophagosome and fusion with the vacuole (Ossareh-Nazari *et al.*, 2010).

The mechanisms of ribophagy in mammalian cells still remain unknown. Ribosomes have been detected inside mammalian autophagosomes by electron microscopy (Eskelinen *et al.*, 2011). Moreover, degeneration of murine Purkinje cells involves, among other responses, disassembly of polyribosomes into monoribosomes and ribophagy; cytoplasmic portions containing monoribosomes appear to be sequestered in autophagic vacuoles (Baltanás *et al.*, 2011). USP10 was characterized as a novel ubiquitin-specific protease and a mammalian homolog of Ubp3, along with its modulator G3BP1, a mammalian homolog of Bre5 (Soncini, Berdo and Draetta, 2001). These findings combined with the characterization of NUFIP1 as a receptor for the selective degradation of ribosomes upon induction of starvation (Wyant *et al.*, 2018), support that ribophagy is an evolutionary conserved pathway, also occurring in mammals.

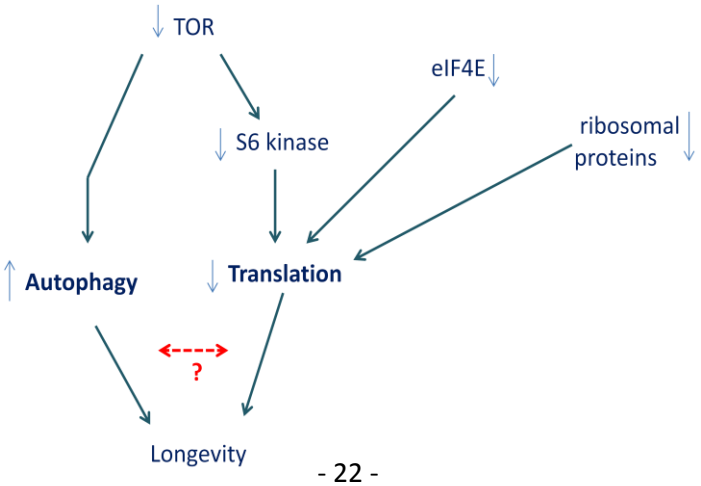
F. Scientific aim

Based on its cytoprotective role, autophagy has been implicated in lifespan regulation. Induction of autophagy, to a certain extent, has been associated with lifespan extension (Meléndez *et al.*, 2003). On the other hand, translation inhibition has also been shown to enhance longevity (Hansen et al., 2007; Pan et al., 2007; Syntichaki, Troulinaki and Tavernarakis, 2007). More specifically, reduction in ribosomal proteins and genetic inhibition of genes involved in ribosomal biogenesis and assembly, result in lifespan extension.

Then the question arises:

Is there a link between autophagy and protein synthesis in the regulation of lifespan and healthspan?

Our scientific aim was to investigate whether autophagy is one of the implicated mechanisms that mediate lifespan extension under conditions of reduced protein synthesis. And if so, whether autophagy could act by selectively targeting ribosomes in a process of ribophagy.



Materials & Methods

NEMATODE STRAINS:

Nematodes were cultured using the described techniques at 20°C, unless otherwise noted. All *C. elegans* strains used in this study are:

N2 Bristol strain, wild type

KX15: *ife-2(ok306) X*

RB1206: *rsks-1(ok1255) III*

RB1311: *edc-3(ok1427) I*

DA2123: *adIs2123*[p_{lgg-1} GFP::LGG-1, *rol-6(df)*]

AM141: *rmIs133*[p_{unc-54} Q40 ::YFP]

HZ589: *bpIs151*[p_{sqst-1} SQST-1::GFP, *unc-76(+)*]

ife-2(ok306); adIs2123[p_{lgg-1} GFP::LGG-1, *rol-6(df)*]

edc-3(ok1427); adIs2123[p_{lgg-1} GFP::LGG-1, *rol-6(df)*]

lgg-2(tm5755) IV

PRIMERS:

The following sets of primers were used in order to specifically amplify the desired sequences:

ife-2:
5'- CAAGTGGGCTCAATCCACC-3' & 5'- CTCACCAGTACTTGAGACACATCAC-3'

edc-3:
5'- TTTGACCATGGCGAATGTTA-3' & 5'- TAGAGGGATCGCTGGAGAAA-3'

rpl-6:
5'- TTCCGGTCATCAGCAGAAAC-3' & 5'- GCTCTGGGTGCTTCTTGATG-3'

rps-15:
5'- CCGCAAGTTCATGTACAGAGG-3' & 5'- AATCGGGAAGAGTGGGTAGC-3'

rps-24:
5'- ATTCGCTCCATTTTCGTTGT-3' & 5'- AGACAACCGCATGGAAATGA-3'

rpl-6(RT-PCR):
5'-ACTTCTCCTTGTCACTGGACC-3' & 5'-CCAAGGTATCCGAAGAGGAACT-3'

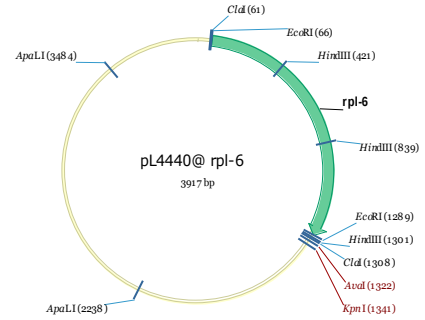
rps-15(RT-PCR):
5'- ACTTTTGGACATGTCCCGTGAAC-3' & 5'- CTGGTTGAAAACCTTTCCGTTG-3'

rps-24(RT-PCR):
5'- CTCTACAGAAAGCAGATGGTTGT-3' & 5'- GTGGCAAGTCCCATGCGAA-3'

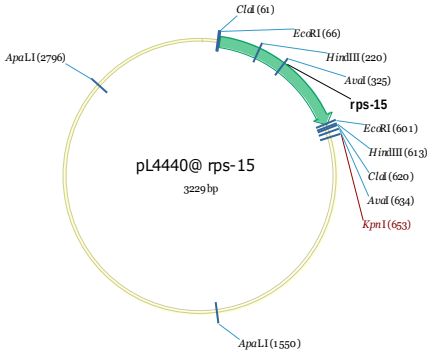
act-1(RT-PCR):
5'- AGGCCCAATCCAAGAGAGGTATC-3' & 5'- TGGCTGGGGTGTTGAAGGTC-3'

PLASMIDS:

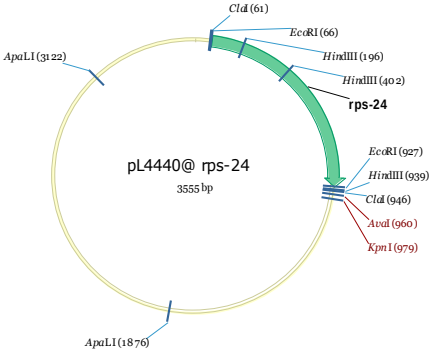
- *rpl-6* – pL4440



- *rpl-15* – pL4440



- *rps-24* – pL4440



The constructs *rpl-6* – pL4440, *rps-15* – pL4440 and *rps-24* – pL4440 were used to knockdown the expression of the respective ribosomal genes through RNAi. In each case, the desired sequence was amplified by PCR using genomic DNA from wild type nematodes and a specific set of primers. Sequences were initially cloned in PCRII-TOPO (Invitrogen). With the use of specific restriction enzymes, inserts were cut and ligated to the pL4440 vector.

A. *Caenorhabditis elegans* techniques

1. Growth media preparation

C. elegans is maintained in petri dishes containing an agar-based medium called Nematode Growth Medium (NGM). This medium is subsequently seeded with a bacterial culture which constitutes the food source of the worms, as described below. There are several sizes of petri plates available: 35 mm diameter plates are mostly used for mating or when using expensive drugs, 60 mm diameter plates are used for general maintenance while 100 mm diameter plates are used when larger amounts of worms are needed. After its preparation the NGM medium is aseptically poured into petri plates with the use of a pump; 4 ml, 8 ml and 16 ml of medium is usually poured into the 35, 60 and 100 mm diameter plates, respectively. If drugs are used, they can be added to the NGM medium prior to pouring (WormBook).

The growth medium of nematodes is slightly altered in the case of RNA interference (RNAi) experiments.

Reagents & equipment:

- NaCl
- Agar
- Bactopeptone
- Streptomycin
- KPO_4 buffer 1 M, pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, H_2O to 1 litre)
- $MgSO_4$ 1M
- $CaCl_2$ 1M
- Cholesterol (5 mg/ml, dissolved in ethanol)
- Nystatin (10 mg/ml, dissolved in ethanol)
- ddH_2O
- Petri plates
- Pump

Method:

1. For a 1 litre preparation mix 3 g NaCl, 17 g agar, 0.2 g streptomycin and 2.5 g bactopeptone in a flask containing approximately 700 ml of water. If preparing RNAi medium do not add streptomycin. Cover the mouth of flask with aluminum foil and autoclave for 1 hour.
2. Let the flask cool at room temperature until it reaches 55°C.
3. Add 1 ml $CaCl_2$, 1 ml $MgSO_4$, 25 ml KPO_4 solution, 1 ml cholesterol and 1 ml nystatin. Supplement with autoclaved ddH_2O till 1 litre and swirl to mix well. Add 0.5 ml ampicillin (100 mg/ml) if preparing RNAi medium.
4. Using sterile procedures dispense the NGM medium into petri plates using a pump.
5. Leave the plates at room temperature for 2 days before using to allow detection of contaminants, and to allow excess moisture to evaporate. Plates stored in an air-tight container at room temperature will be usable for several weeks.

2. Bacterial food preparation

In the laboratory an *Escherichia coli* strain is used as a food source for *C. elegans*. *E. coli* OP50 is a uracil auxotroph; a culture of *E. coli* OP50 can be initially acquired from the CGC (*Caenorhabditis* Genetics Center) and further maintained by streaking on LB agar plates. A single colony can be used for inoculation of LB broth, and cultures are ready to use for seeding after incubation for approximately 4-5 hours at 37°C. When seeding NGM plates a limited lawn is usually preferred, as it better enables the observation and mating of the worms. Therefore, using a pipette, 200 µl of culture is seeded on medium NGM plates while a single drop is enough for the small petri plates (WormBook).

Another *Escherichia coli* strain, HT115, is used as a food source when performing RNAi (RNA interference). This strain is tetracycline resistant.

3. Culturing *C. elegans*

C. elegans stocks can be maintained at 20°C, 16°C and 25°C. *C. elegans* nematodes grow 1.3 times faster at 20°C compared to 16°C and 2.1 times faster at 25°C compared to 16°C.

Due to its transparent nature *C. elegans* can be visualized with the use of a stereoscope. Transferring of worms can be achieved either by chunking, which involves the dissection of a small piece of agar by a sterilized scalpel or spatula and its transfer to a new plate along with the containing worms, either by using a worm picker, which allows the user to control the number of the transferred worms. Transferring frequency mostly depends on the genotype of the strain and the culturing temperature (WormBook).

4. Generating males

C. elegans has two sexes, hermaphrodite and male. Both males and hermaphrodites posses five sets of autosomal chromosomes; hermaphrodites are also characterized by the presence of two X chromosomes, while males have only one.
Males can normally occur by rare non-disjunction of the X chromosome (1‰ incidence) during meiosis. In the lab, generation of males can be induced by heat stress.

Method:

1. Place 4-5 hermaphrodite L4 animals in each of 4 seeded NGM plates.
2. Incubate at 37°C for 50 min.
3. Maintain at 20°C and examine the descendants for the presence of males.

5. Crossing strains

Males and hermaphrodites carrying individual genetic elements can be crossed for the purpose of generating double homozygous strains. In such case, animals are placed in 35 mm diameter NGM plates with a small OP50 lawn in order to facilitate their encounter.

Method:

Transfer a single L4 hermaphrodite in the presence of 5-7 males. Occurrence of males at a high percentage between the progeny (~50%) is a positive indicator of a successful cross.
As a next step, select individual hermaphrodite F1 progeny at the L4 stage and place them in new NGM plates. Allow them to reproduce and as soon as the F2 generation hatches, select individual L4 animals for subsequent transfer.
The process can be repeated until homozygous animals are generated.
At each step where selection is required, animals can be screened based on their possible phenotype (due to a mutation or a marker) or if that is not an option, by single worm PCR using specific primers for the desired gene.

6. Decontaminating / Synchronizing strains

From time to time, *C. elegans* stocks can be contaminated by bacteria or fungi. Contamination, apart from making observation and transferring of the worms more difficult, can actually trigger physiological responses of the organism that may affect biological processes studied under experimental conditions. Therefore, it is essential to decontaminate any strain used in an experiment.

Mold can be removed by chunking and serially transferring, allowing the worms to crawl away from the contaminant. Also mold as well as bacterial contaminants are easily removed by treating with a hypochlorite solution, which kills the contaminant and all worms beside their eggs. This can be done using an entire plate that is contaminated. Since only eggs of *C. elegans* survive this process this method can also be used in order to synchronize a population of worms (WormBook).

Reagents:

Bleaching solution

- 1 ml 5N NaOH
- 2 ml 5% solution of sodium hypochlorite NaOCl (household bleach)
- 7 ml ddH_2O

Method:

1. Wash contaminated plates containing many gravid adults with sterile H_2O (or M9 buffer). Plates should be washed thoroughly along their surface with a pipette so that worms stuck in the bacterial lawn can also be retrieved.
2. Collect the liquid in an eppendorf tube.

3. Centrifuge all the prepared samples for 30 seconds at 3000 rpm.
4. Dispose of the supernatant (*E. coli* OP50) and resuspend the pellet in ddH_2O. Repeat steps 3 and 4 two more times.
5. Instead of water resuspend the worms in bleaching solution and ddH_2O (3:1 ratio).
6. Shake well or vortex the eppendorfs every 2 minutes till the solution is clear of intact worms and only eggs can be seen.
7. Centrifuge for 1 minute at full speed.
8. Dispose of the supernatant and resuspend the pellet in ddH_2O. Repeat steps 7 and 8 two more times.
9. Use a pipette to transfer the eggs to a seeded NGM plate.

The next day the eggs will have hatched and the larvae will have crawled into the *E. coli* OP50 lawn. Transfer the hatched larvae to a new NGM plate when needed.

7. Freezing and recovery of stocks

Long-term storage of *C. elegans* strains can be achieved by freezing them at -80°C or in liquid nitrogen (-196°C). For successful freezing and maintenance of the strain, it is vital to perform the procedure in freshly starved plates where the majority of the worms are young larvae of the L1 developmental stage. This is based on their ability to enter dauer stage and survive the freezing as opposed to the rest developmental stages where worms usually do not recover (WormBook).

Reagents & equipment:

M9 buffer (1lt):
- 3gr KH_2PO_4
- 6gr Na_2HPO_4
- 5g NaCl
- 1 ml $MgSO_4$ 1M (added after autoclave)

Freezing buffer (1lt):
- 5,85g NaCl
- 50 ml KPO4 1M, pH 6.0
- 200ml glycerol
- 3 ml $MgSO_4$ 1M (added after autoclave)

Cryotube vials

Method:

Freezing

Initially, depending on the growth rate of each strain, 5-10 L4 animals are placed on each one of 4 NGM plates. As soon as the second generation of worms hatches, the OP50 lawn is freshly consumed and the plate is filled with L1 larvae. At this point:

1. Use cold M9 buffer to wash the nematodes from the plates and subsequently place them in a 15ml falcon residing on ice.
2. Add equal volume of cold freezing buffer.
3. Mix the buffers thoroughly and put 2ml of the solution in each of 10 labeled cryotube vials residing on ice.
4. Transfer the cryotube vials to -80°C.

Thawing

1. Remove the cryotube vial from the freezer and allow it to gradually thaw at room temperature.
2. Centrifuge at 3000 rpm for 2 min and discard most of the supernatant.
3. Mix and pour the content on NGM plate(s) seeded with OP50. You should soon see worms moving.
4. After 2-3 days transfer the worms that recovered to new NGM plates.

8. RNA interference *in C. elegans*

RNA interference (RNAi) is a form of sequence-specific gene silencing induced by double-stranded RNA molecules (dsRNAs) that are processed into small hairpin RNAs (shRNAs) (Fire, 2007). In *C. elegans* RNAi is systemic and heritable, therefore constitutes a useful genetic tool (Grishok, Tabara and Mello, 2000; Winston, Molodowitch and Hunter, 2002). It has been established that worms fed with bacteria engineered to produce double strand RNA present a robust RNAi response (Kamath *et al.*, 2001; Lisa Timmons and Andrew Fire, 1998).

In order to knockdown the expression levels of the desired gene, a part or the whole corresponding genomic sequence has to be cloned on the pL4440 vector. The genomic sequence is placed between two T7 promoters juxtaposed to each. The pL4440 vector is used to transform a specific *E. coli* strain, HT115, containing the T7 polymerase gene under the control of the *lac* operon. HT115 strain lacks RNase III. Upon treatment with IPTG (isopropyl-β-D-thiogalactopyranoside) induced expression of T7 polymerase leads to the simultaneous transcription of the cloned sequence by both promoters and the generation of complementary RNA molecules that eventually form dsRNA.

Reagents &equipment:

- RNAi plates
- LB liquid medium with ampicillin 100 μg/ml
- Tetracycline 10 μg/ml
- IPTG 2 mM

Method:

1. Use a single colony of HT115 cells expressing the desired double-stranded RNA molecule to inoculate LB liquid medium with ampicillin and tetracycline. Incubate at 37°C overnight (14-16 hours).

2. Use the overnight culture to inoculate LB-ampicillin liquid medium (7:100 overnight to overday culture). Incubate the overday culture at 37°C for a few hours (~3 hours).
3. Add IPTG at a final concentration 0.2 mM, mix well and seed the RNAi plates.
4. Place the animals on the bacterial lawn the next day.

9. Monitoring survival using *C. elegans*

Lifespan has been established as an effective measure of the ageing process. *C. elegans* is extensively used as a model organism for studying ageing (Olsen, Vantipalli and Lithgow, 2006). Therefore, lifespan assays can be used in order to investigate the effect of various mutations or treatments and elucidate aspects of this complex biological process (Amrit *et al.*, 2014)

Method:

Synchronized nematodes of the selected developmental stage are placed on NGM or RNAi plates seeded with OP50 or HT115 bacteria, respectively. In the case of RNAi lifespan assays HT115 cells are transformed with the desired RNAi constructs and as a control, the empty pL4440 vector. Animals are transferred in new plates every other day in order to prevent contamination by progeny, but even after egg-laying ceases transferring is necessary to avoid contamination by worms that undergo 'bagging'. Alive, dead and censored animals are counted every other day. Lack of movement after a repeated gentle touch with the pick is indicative of death. Animals that are lost or they die to another cause, unrelated to ageing, like drying up, undergo 'bagging' or exhibit 'protruding vulva', are considered censored.

In this study, *E. coli* HT115 bacteria expressing the double-stranded RNAs against core autophagy genes (*lgg-1*, *unc-51*, *atg-7*, *bec-1*) or components of the translational machinery (*rsks-1*, *rpl-6*, *rps-15*, *rps-24)* were used as a food source for nematodes. Synchronized animals at the L4 developmental stage were placed on RNAi plates seeded with HT115 bacterial lawn. Approximately 200 worms were the initial population for each treatment.

B. Molecular biology techniques

1. Bacterial growth media

Luria-Bertani medium (LB)

- 10 g bactotryptone
- 5 g yeast extract
- 10 g NaCl
- 15 g agar (for solid culture plates)
- ddH_2O

Method:

Suspend the solid reagents in ddH_2O to a total volume of 1 litre. Divide the medium in smaller flasks of the desired size and autoclave for 1 hour. For preparation of LB plates pour the medium aseptically in 100 mm diameter petri plates after autoclaving.

Ampicillin can be added to LB medium at a final concentration of 100 µg/ml prior to pouring. Tetracycline can be added to ready LB or LB-ampicillin plates at a final concentration of 10 µg/ml.

2. Genomic DNA extraction

Extraction of DNA can be used for cloning a specific sequence or screening for mutations. Genomic DNA of lower quality can be extracted from a single nematode.

Reagents & equipment:

Worm Lysis Buffer:

- KCl 50 mM
- Tris 10 mM, pH 8.5
- $MgCl_2$ 2.5 mM
- NP-40 0.45%
- Tween-20 0.45%
- Proteinase K 10 mg/ml at final concentration 200 µg/ml (add before use)

PCR tubes

Method:

1. Place a single worm in a 200 µl PCR tube containing 10 µl of lysis buffer.
2. Incubate for 30 min at -80°C.
3. Incubate at 65 °C for 1 hour.
4. Inactivate the proteinase K by heating at 95°C for 15 minutes.
5. Perform the PCR reaction using the same tube or store at -20°C.

High quality genomic DNA can be obtained with the use of a kit. We used the Genomic DNA purification kit (Macherey-Nagel, NucleoSpin Tissue).

3. Polymerase chain reaction (PCR)

Polymerase chain reaction is a method used for exponential amplification of a single copy of a DNA segment. Multiple copies of the selected segment are generated *in vitro* trough repeated thermal cycles and the use of a heat-resistant DNA polymerase. Apart from the enzyme and the DNA template, a set of specific primers is required as well as the enzyme buffer and dNTPs. The process takes place is three basic steps. At first the DNA template is denatured by heating the sample at 92-95°C. The temperature is then dropped at 55-65°C to achieve annealing of the primers at the single-stranded DNA. Finally, the temperature is increased at 72°C where the DNA polymerase synthesizes the included DNA sequence.

Reagents:

	Initial concentration	Final concentration
Enzyme buffer	10x	1x
dNTPs	2 mM	0.2 mM
Forward primer	100 pmol/μl	25 pmol/μl
Reverse primer	100 pmol/μl	25 pmol/μl
DNA template		
Enzyme	10 U/μl	~0.2 U/μl
ddH_2O		

Method:

1. Initialization: 92-95°C for 3-5 min
2. Denaturation: 92-95°C for 30 sec
3. Annealing: 55-65°C for 45 sec (based on melting temperature Tm of the primers)
4. Elongation: 68-72°C ~1 min/1 kb
5. Repetition of steps 2-4 for 35 cycles
6. Final elongation: 68-72°C for 10 mim
7. Hold: 4°C

PCR purification and gel extraction were performed with the use of PCR Purification Kit (Qiagen-QIAquick) and Gel Extraction Kit (Qiagen), respectively.

4. Restriction endonuclease reaction

Restriction enzymes and buffers were provided by New England Biolabs (NEB) and Minotech and were used according to each manufacturer.

5. Ligation / dephosphorylation reaction

Ligation enzymes and buffers were provided by Minotech. Dephosphorylation of DNA was performed using alkaline phosphatase (CIP) provided by New England Biolabs (NEB).

6. Transformation of competent cells

Reagents:

- LB medium
- LB plates

Method:

1. Retrieve the competent cells and the ligation reaction from -80°C and -20°C, respectively.
2. Shortly incubate on ice.
3. In 100 µl of competent cells add 3 µl of the ligation reaction and mix.
4. Incubate for 30 min on ice (4°C).
5. Perform heat shock at 42°C for 60-90 sec and place the tube back on ice.
6. Add 900 µl of LB and incubate at 37°C for 1 hour.
7. Under sterile conditions spread ~100 µl of the cell suspension on an LB plate (containing the required antibiotics).
8. Centrifuge the rest of the suspension for 5 min at 3000 rpm.
9. Discard most of the supernatant, mix and spread the remaining quantity on a new LB plate.
10. Incubate the plates overnight (16-18 hours) at 37°C.

7. Plasmid DNA extraction and purification

Minipreparation of plasmid DNA is a rapid, small-scale isolation of plasmid DNA from bacteria. It is based on the alkaline lysis method and normally offers a yield of 50 to 100 µg plasmid DNA, depending on the cell strain.

Reagents & equipment:

P1 and P3 solutions were provided by the MACHEREY-NAGEL kit

P1 solution (on ice):

- Glucose 50 mM
- Tris-HCl 25 mM, pH 8.0
- EDTA 10 mM
- RNase A at a final dilution 1‰

P3 solution (on ice):

- potassium acetate 3 mM
- glacial acetic acid

P2 buffer (room temperature):

- NaOH 0.2 N
- SDS 1%
- ddH_2O

Absolute ethanol and ethanol 70% v/v

Eppendorf tubes

Method:

1. Inoculate 2-3 ml LB medium containing the necessary antibiotics with a bacterial clone and incubate at 37°C for 14-16 hours.
2. Aliquot 1.5 ml of the grown bacterial cultures in eppendorf tubes.
3. Harvest the bacteria by centrifugation for 1 min at full speed.
4. Remove the supernatant and resuspend the bacterial pellet in 100 µl of P1 solution.
5. Add 100 µl of P2 buffer and mix gently by inverting the tube.
6. Incubate for 3 minutes at room temperature.
7. Add 100 µl of P3 solution, mix and incubate on ice for 5-10 min.
8. Centrifuge the samples at 4°C for1 10 min at 10000 rpm.
9. Transfer the supernatant in new eppendorf tube and add 2.5x volume of ethanol.
10. Centrifuge at 4°C for 20 min, full speed.
11. Discard the supernatant and wash the pellet with 100 µl ethanol 70% v/v.
12. Centrifuge for 1 min at 10000 rpm.

13. Discard the supernatant and air dry the pellet.
14. Resuspend in 30 µl of ddH_2O.

8. RNA isolation

Reagents & equipment:

- M9 buffer
- Trizol
- Chloroform ($CHCl_3$)
- Isopropanol
- Ethanol 70% v/v
- RNase-free water

Method:

1. Pick 10 worms into 20 µl M9 in an RNase-free eppendorf tube.
2. Pellet worms by spinning at 14000 rpm.
3. In the hood add 250 µl Trizol.
4. Vortex by hand for about 30 sec and then vortex for 4 min.
5. Place at -80°C for 10 min (samples can be stored temporarily at this stage).
6. Heat at 65°C for 5 min.
7. In the hood add 50 µl of chloroform.
8. Vortex for 15 sec and place at room temperature for 3 min.
9. Centrifuge at 12000 rpm for 15 min at 4°C.
10. Transfer the clear layer (~125 µl) into a new RNase-free eppendorf tube.
11. Repeat steps 6-9.

12. Add 125 µl 2-propanol and invert to mix.
13. Place at room temperature for several minutes (~3min).
14. Spin down at 12000 rpm for 10 min at 4°C.
15. Keep the pellet.
16. Add 500 µl ethanol 70% v/v (use RNase-free ddH_20).
17. Spin down at 14000 rpm for 5 min at 4°C.
18. Remove the supernatant and air dry the pellet.
19. Dissolve the pellet in 20 µl of RNase-free ddH_20.
20. Quantify the samples by measuring the ratio OD_{260}/OD_{280} (2.0 for pure RNA).
21. Store at -20°C temporarily or at -80°C for long-term usage.

9. cDNA synthesis and Real-Time PCR

RNA can be used as a template for cDNA synthesis by a reverse transcriptase. Further on, cDNA can be used for quantification of gene expression by performing real-time PCR. A fluorescent dye which intercalates with the double-stranded DNA is used for the detection of PCR products in real time.

cDNA synthesis

20 µl reaction set-up:

- 4 µl 5x iScript Reaction Mix (Bio-Rad)
- 1 µl iScript Reverse Transcriptase
- x µl RNA template (100 fg to 1 µg RNA)
- (15-x) µl Nuclease-free H_2O

Reaction protocol:

1. 5 min at 25°C
2. 30 min at 42°C
3. 5 min at 85°C (gradually elevate 42°C --> 85°C)
4. 4°C

Real-Time PCR

Real-time PCR reaction was performed with the use of Bio-Rad CFX96. Samples were placed on 96 well plates in duplicates. *act-1* was used as a reference gene.

20 µl reaction set-up:

- 10 µl SSoFast EvaGreen Supermix (Bio-Rad)
- 1 µl (10 pmol/µl forward primer
- 1 µl (10 pmol/µl) reverse primer
- 6 µl RNase/DNase H_2O
- 2 µl cDNA template (diluted 1:20)

Reaction protocol:

1. 30 min at 98°C
2. 2 sec at 98°C
3. 15 min at 56°C
4. Steps 2-3 for 39 cycles
5. Melt curve 65°C --> 95°C, increment 0.1°C/sec

C. Microscopy techniques

Reagents & equipment:

- M9 buffer
- NaN_3 solution (sodium azide) 20 mM
- Slides and over slips 18 x 18 mm

Method:

1. Mix equal volume of NaN_3 and M9 buffer and place a single drop on the slide.
2. Pick the desired number of worms and place them inside the drop.
3. Cover the drop with a cover slip and seal it with some nail polish.

The microscope that was used was Zeiss Axio Imager Z2. Analysis of the pictures was performed by ImageJ 1.48 V.

D. Statistical analysis

All statistical analyses were performed in GraphPad Prism 6.01 (GraphPad Software, USA).

Results

A. Autophagy is required for longevity mediated by protein synthesis inhibition

In order to address whether autophagy is involved in lifespan extension under conditions of reduced protein synthesis, we performed survival assays, where we monitored the lifespan of protein synthesis mutant strains subjected to RNAi (RNA interference) against selected autophagy genes. RNAi has been successfully used to clarify the role of specific autophagy genes in many biological processes (Meléndez *et al.*, 2003).

In this case, we used RNAi constructs against core autophagy genes, namely *bec-1*, *lgg-1*, *atg-7* and *unc-51*. BEC-1 is the worm homolog of mammalian and yeast BECN1 and Atg6, respectively. It is a part of the PI3 kinase complex that plays a role in localizing autophagic proteins to pre-autophagosomal structures. BEC-1 is also required for the regulation of endocytic retrograde transport (Ruck *et al.*, 2011) and has a role in normal growth, movement and vulval morphogenesis (Meléndez *et al.*, 2003; Jia, Hart and Levine, 2007). LGG-1 is the homolog of mammalian LC3 and Atg8 in yeast, and is a key player of the autophagic process (Meléndez *et al.*, 2003). *atg-7* encodes an E1 ubiquitin-activating like enzyme involved in the regulation of autophagosome assembly and is homologous to the *ATG7* in *Saccharomyces cerevisiae* and ATG7 in mammals. Lastly, UNC-51 is the homolog of ULK1 (unc-51 like autophagy activating kinase) in vertebrates and Atg1 in yeast. It exhibits protein kinase activity and is needed in early steps of autophagosomal biogenesis (Meléndez *et al.*, 2003).

We monitored the survival of two mutant strains, RB1206 and RB1311. RB1206 is a mutant for RSKS-1 which is the worm homolog of the ribosomal protein S6 kinase. Genetic inhibition of RSKS-1 is associated with reduced protein synthesis and prolonged lifespan (Hansen *et al.*, 2007). RB1311 is a mutant for EDC-3, the homolog for Edc3 enhancer of mRNA decapping in *Saccharomyces cerevisiae*. Depletion of EDC-3 is also linked with protein synthesis inhibition and extended lifespan (Rieckher *et al.*, 2018).

1. Components of the autophagic machinery are required for lifespan extension in *rsks-1(ok1255)* mutants

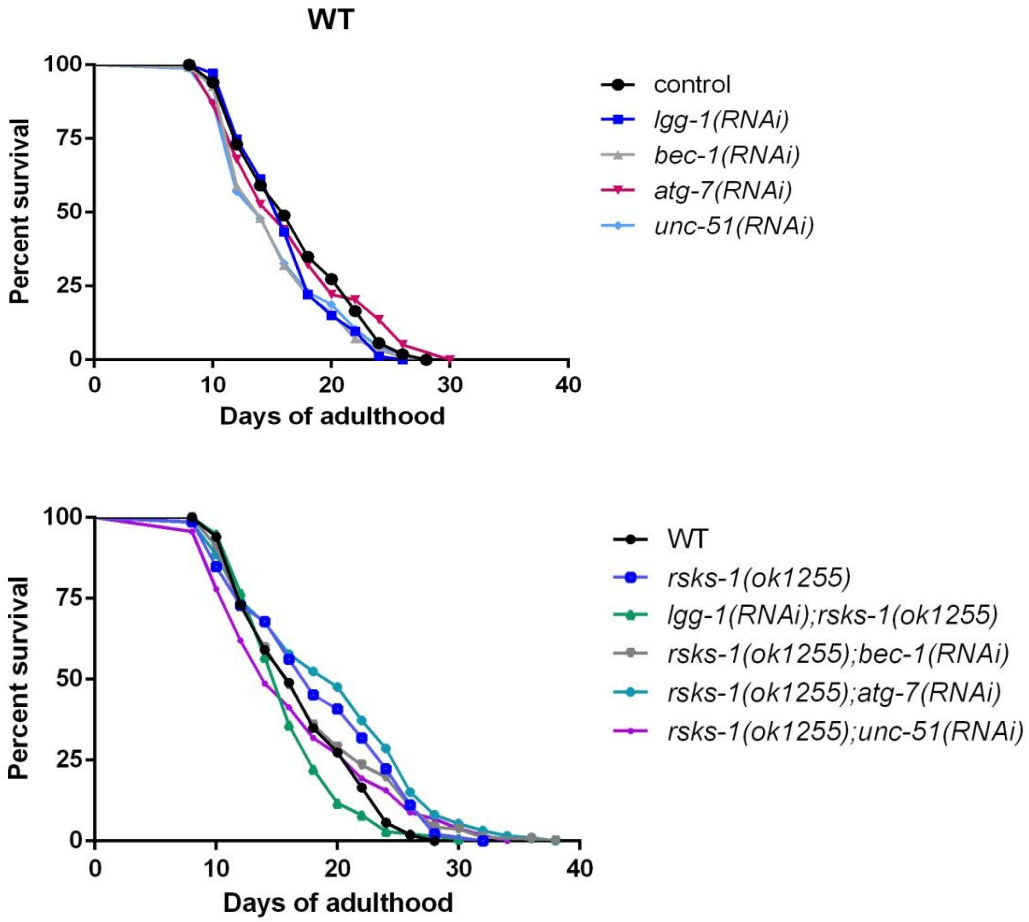


Figure 1: Effects of autophagy deficiency on lifespan. Survival curves of wt and *rsks-1(ok1255)* mutant nematodes upon RNAi-mediated knockdown of core autophagy genes.

RNAi-mediated knockdown of *atg-7* did not cause any alterations in lifespan of wild-type (wt) animals. Animals subjected to RNAi against *lgg-1, unc-51 or bec-1* exhibited a slight lifespan shortening (Figure 1, Table 1).

In contrast, suppression of autophagy shortened the lifespan of long-lived *rsks-1(ok1255)* mutants. More specifically, knockdown of either *lgg-1* or *unc-51* abrogated the lifespan extension observed under conditions of reduced protein synthesis (Figure 1, Table 2).

Table 1: Survival parameters of wt animals upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	16	24	
lgg-1(RNAi)	16	22	*
bec-1(RNAi)	14	22	**
atg-7(RNAi)	16	26	ns
unc-51(RNAi)	14	22	*

Table 2: Survival parameters of *rsks-1(ok1255)* mutants upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	16	24	
rsks-1(ok1255)	18	26	**
lgg-1(RNAi);rsks-1(ok1255)	16	20	****
rsks-1(ok1255);bec-1(RNAi)	16	26	ns
rsks-1(ok1255);atg-7(RNAi)	20	28	ns
rsks-1(ok1255);unc-51(RNAi)	14	26	**

We repeated the survival assay in order to verify our findings. In agreement with previous results, when animals were fed with bacteria expressing the empty vector, *rsks-1(ok1255)* mutants lived significantly longer compared to the wild type strain. This extension was abolished when animals were treated with *lgg-1* or *unc-51* RNAi (Figure 2, Tables 3 & 4) suggesting that autophagy is required for longevity mediated by a mutation in the *rsks-1* gene.

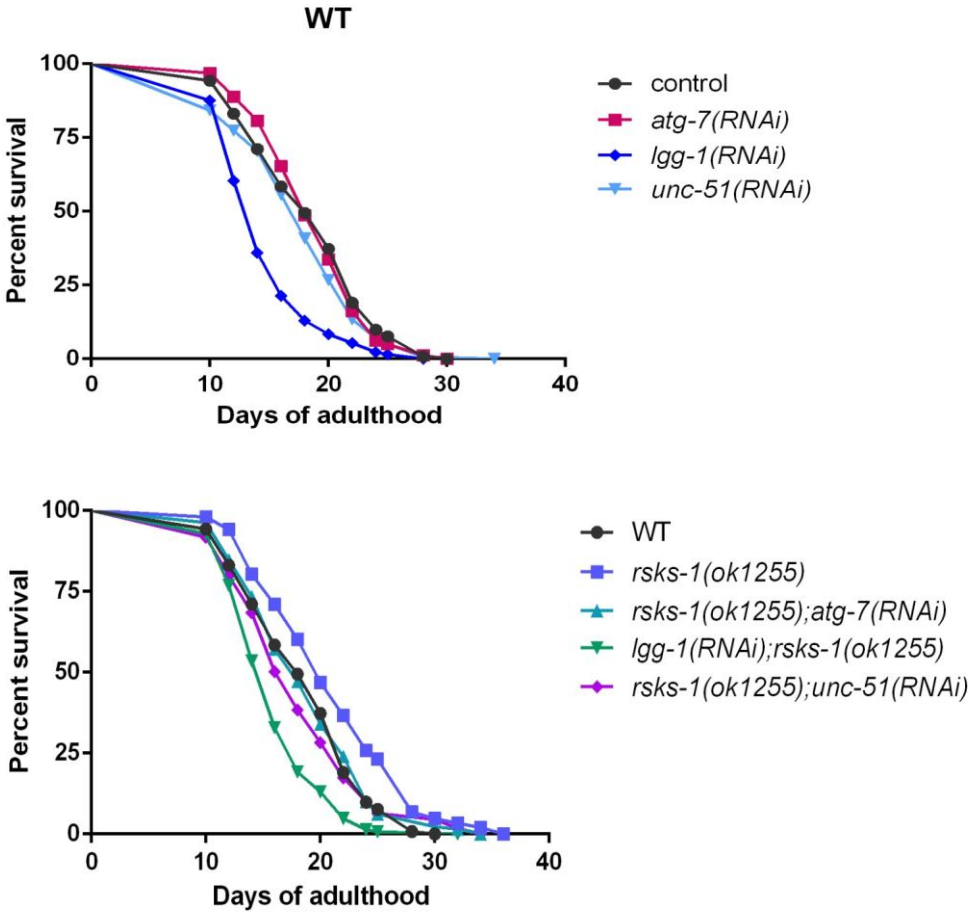


Figure 2: Autophagy deficiency shortens the lifespan of *rsks-1* mutants. Survival curves of wt and *rsks-1(ok1255)* mutant nematodes upon RNAi-mediated knockdown of core autophagy genes.

Table 3: Survival parameters of wt animals upon different RNAi treatments.

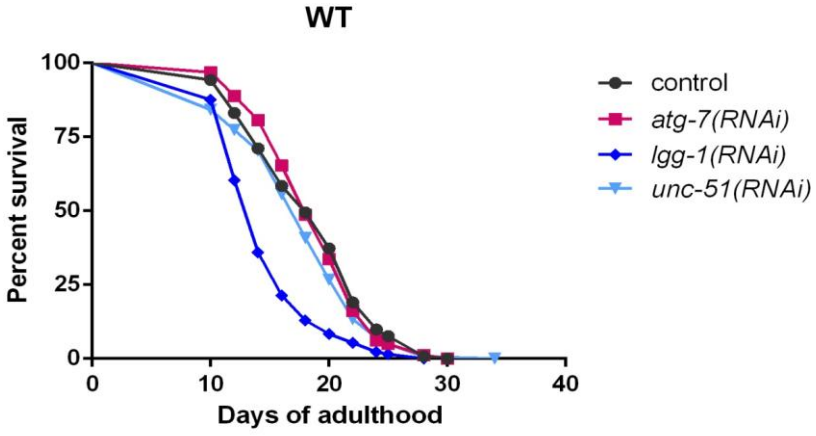
Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
lgg-1(RNAi)	14	28	****
atg-7(RNAi)	18	30	ns
unc-51(RNAi)	18	34	ns

Table 4: Survival parameters of *rsks-1(ok1255)* mutants upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
rsks-1(ok1255)	20	36	***
lgg-1(RNAi);rsks-1(ok1255)	16	32	****
rsks-1(ok1255);atg-7(RNAi)	18	34	**
rsks-1(ok1255);unc-51(RNAi)	18	34	****

2. Components of the autophagic machinery are required for lifespan extension in *edc-3(ok1427)* mutants

The *edc-3(ok1427)* mutant was used as an alternative strain characterized by prolonged lifespan and reduced protein synthesis. The results we obtained resembled those observed in *rsks-1(1255)* mutants; wild type animals treated with RNAi against *atg-7* or *unc-51* did not display significant changes compared to control. Only *lgg-1(RNAi)* exhibited lifespan shortening (Figure 3, Table 5). Additionally, the enhanced longevity of *edc-3(ok1427)* mutants was abolished when animals were treated with *atg-7* RNAi or *unc-51* RNAi (Figure 3, Table 6), suggesting that autophagy was once again required for lifespan extension mediated by a mutation in the EDC-3 encoding gene.



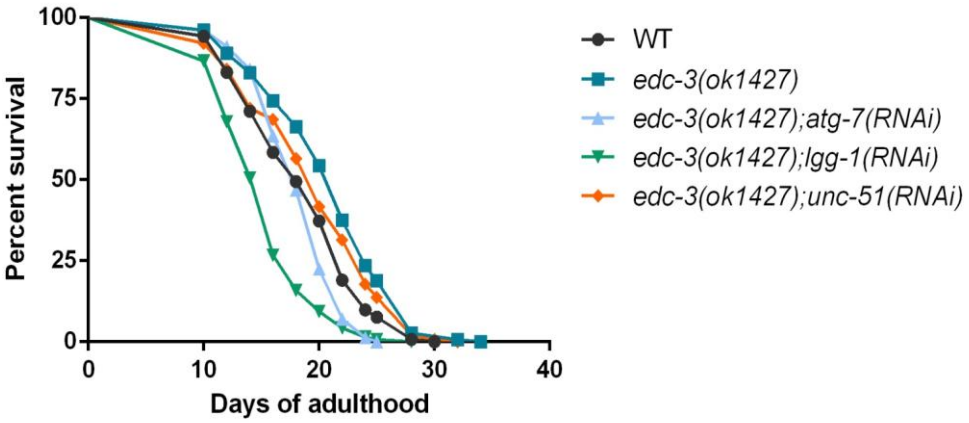


Figure 3: Autophagy deficiency shortens the lifespan of *edc-3* mutants. Survival curves of wt and *edc-3(ok1427)* mutant nematodes upon RNAi-mediated knockdown of core autophagy genes.

Table 5: Survival parameters of wt animals upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
lgg-1(RNAi)	14	28	****
atg-7(RNAi)	18	30	ns
unc-51(RNAi)	18	34	ns

Table 6: Survival parameters of *edc-3(ok1427)* mutants upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
edc-3(ok1427)	22	34	***
edc-3(ok1427);lgg-1(RNAi)	16	28	****
edc-3(ok1427);atg-7(RNAi)	18	25	****
edc-3(ok1427);unc-51(RNAi)	20	32	*

3. Efficiency of RNAi-mediated knockdown of ribosomal genes

At this point, it should be mentioned that in order to evaluate the efficiency of all three RNAi constructs that were used to knockdown the expression of ribosomal genes, we performed real-time PCR using specific primers as reported in the materials and methods section.

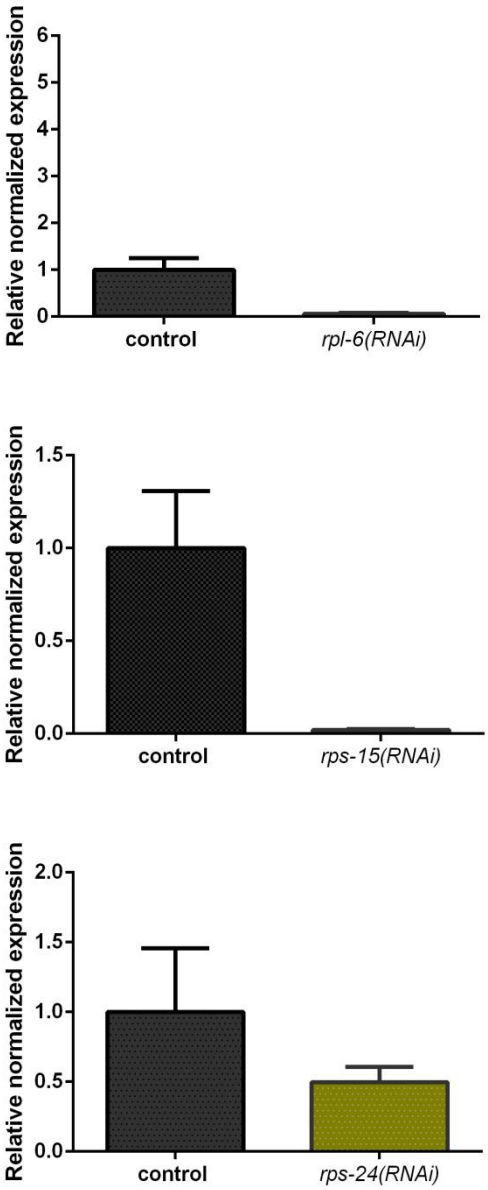


Figure 4: RNAi constructs efficiently supress the expression of ribosomal genes. Relative normalized expression of *rpl-6*, *rps-15* and *rps-24* in animals treated with the respective construct**.**

All target genes exhibited a reduction in relative normalized expression, suggesting that feeding nematodes with bacteria that express these double-stranded RNAs can effectively and selectively knockdown the expression of the respective ribosomal gene.

4. Autophagy is required for lifespan extension mediated by *rps-24* knockdown

We also monitored the lifespan of an autophagy mutant strain upon reduction of protein synthesis, in an effort to perform the reverse experiment.

C. elegans genome contains two homologs of *ATG8*, *lgg-1* and *lgg-2*, which have been found to act synergistically in *C. elegans* longevity and dauer formation. *lgg-1(tm3489)* mutants exhibit embryonic lethality but *lgg-2(tm5755)* mutants are viable in a homozygous state (Alberti *et al.*, 2010).

We used synchronous wild type nematodes and *lgg-2(tm5755)* mutants of the L4 developmental stage. Treatment involved RNAi constructs against main components of the translational machinery: *rsks-1*, *rpl-6*, *rps-15* and *rps-24. lgg-1* RNAi was used to underline the synergistic relationship between the two LC3 homologs. As previously mentioned, RSKS-1, is the worm homolog of the ribosomal protein S6 kinase in mammals. *rpl-6* encodes a protein which is a structural constituent of the large (60S) ribosomal subunit. In contrast, *rps-15* and *rps-24* encode protein components of the small (40S) ribosomal subunit (Hansen *et al.*, 2007). In addition, RPS-24 is one of two ribosomal proteins in *C. elegans*, along with RPL-5, that possess a LIR (LC3 interacting region) motif (Jacomin *et al.*, 2016).

It is known that mutations or environmental cues that reduce ribosome manufacturing and assembly in eukaryotes lead to lifespan extension (Macinnes, 2016). Therefore, knockdown of ribosomal genes was selected as means of lifespan extension and protein synthesis inhibition, based on the core role of the ribosome in the translational process. More specifically, *rpl-6* and *rps-15* ribosomal genes were selected based on data that support both protein synthesis inhibition and a robust increase in lifespan (Hansen *et al.*, 2007). Moreover, recent studies have shown that deficiency or genetic loss of ribosomal proteins may lead to induction of autophagy (Heijnen *et al.*, 2014; Artero-Castro *et al.*, 2015). Since the central aim of this project was to elucidate the relationship between autophagy and protein synthesis in lifespan regulation, we selected a ribosomal protein with an LC3 interacting motif to further enhance this link and examine the possibility of selective ribosomal degradation through autophagy (Birgisdottir, Lamark and Johansen, 2013).

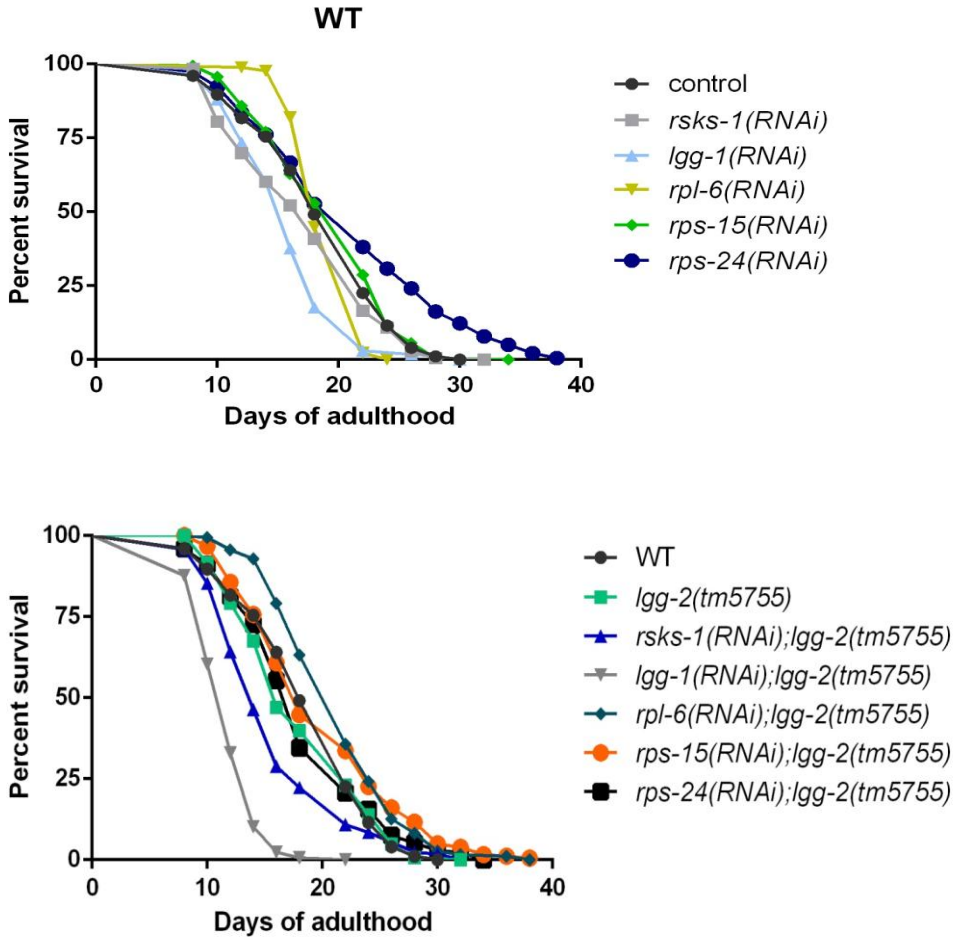


Figure 5: Effects of protein synthesis attenuation and LGG-1/LGG-2 inhibition on lifespan. Survival curves of wt and *lgg-2(tm5755)* mutant nematodes upon RNAi-mediated knockdown of genes involved in protein synthesis.

RNAi-mediated knockdown of *rsks-1* extended the lifespan of wild type animals. Also, *rps-24* RNAi had the same effect. Surprisingly, both *rpl-6* RNAi and *rps-15* RNAi, despite their reported lifespan prolonging effect (Hansen *et al.*, 2007) did not seem to significantly extend lifespan. *lgg-1(RNAi)* animals exhibited lifespan shortening as in previous cases.

In agreement with the literature, *lgg-2(tm5755)* mutants did not exhibit alterations in lifespan compared to wild type animals (Alberti *et al.*, 2010). However, in an *lgg-2(tm5755)* background, the longevity mediated by *rsks-1(RNAi)* and *rps-24(RNAi)* was abolished. Mutation in the LGG-2 encoding gene diminished the extension of lifespan that these nematodes exhibited in an otherwise wild type background, supporting once again the role of autophagy in longevity mediated by protein synthesis inhibition.

Table 7: Survival parameters of wt animals upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
rsks-1(RNAi)	18	32	*
lgg-1RNAi)	16	30	****
rpl-6(RNAi)	18	24	ns
rps-15(RNAi)	22	34	ns
rps-24(RNAi)	22	38	**

Table 8: Survival parameters of *lgg-2(tm5755)* animals upon different RNAi treatments.

Genotype	Median Survival (days)	Maximum Survival (days)	Statistical Significance
WT	18	30	
lgg-2(tm5755)	16	32	ns
rsks-1(RNAi);lgg-2(tm5755)	14	32	**
lgg-1(RNAi);lgg-2(tm5755)	12	22	****
rpl-6(RNAi);lgg-2(tm5755)	22	38	****
rps-15(RNAi);lgg-2(tm5755)	18	38	**
rps-24(RNAi);lgg-2(tm5755)	18	34	ns

B. Autophagy is induced upon inhibition of ribosomal genes

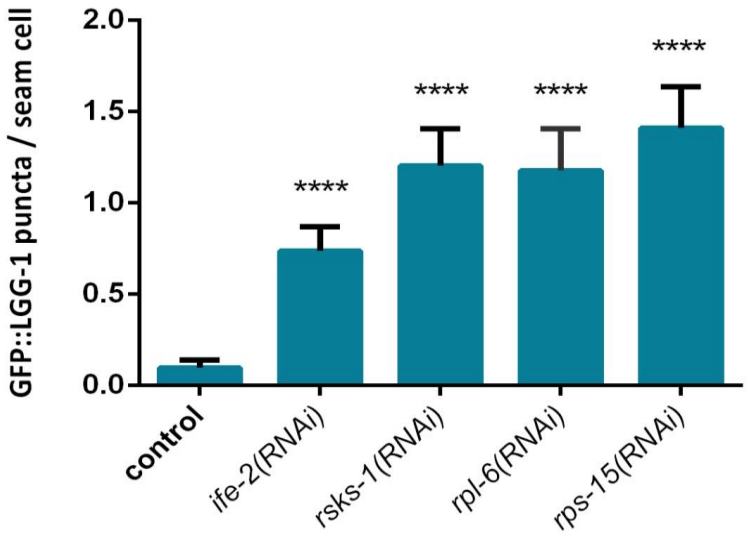
With the combined use of various mutants and RNAi treatments, we have already established that autophagy is involved in lifespan regulation under conditions that reduce protein synthesis. In order to address the question of how the autophagic process may be altered under these conditions and ultimately has this end effect in longevity we performed a series of RNAi experiments. In these experiments we used specific transgenic strains which allowed us to monitor autophagy while interfering with gene expression of key players in protein synthesis.

For this purpose, we used the former RNAi constructs against the ribosomal protein S6 kinase homolog, RSKS-1, as well as the eukaryotic initiation factor 4E (eIF4E) worm homolog, IFE-2. Loss of the specific eIF4E isoform in somatic cells, IFE-2, reduces global protein synthesis and extends lifespan in *C. elegans* (Syntichaki, Troulinaki and Tavernarakis, 2007). In addition we used RNAi constructs against the ribosomal genes *rpl-6* and *rps-15* (Hansen *et al.*, 2007).

1. Autophagosome formation is increased under conditions that inhibit protein synthesis

Having shown that autophagy is required for lifespan extension under conditions of reduced protein synthesis, we sought to assess autophagy levels in animals upon protein synthesis inhibition. To this end, we monitored autophagy by fluorescent image analysis of transgenic nematodes expressing the GFP::LGG-1 reporter (Palmisano and Meléndez, 2016). Atg8/LC3, based on its presence at the autophagosomal membrane even during autophagosome maturation, has been extensively used as a means of monitoring autophagy (Mizushima *et al.*, 2004). In *C. elegans*, GFP::LGG-1 under normal conditions is expressed in a diffused pattern while under conditions that induce autophagy, its overall expression levels increase and punctate structures are formed (Meléndez *et al.*, 2003; Kang, You and Avery, 2007; Lapierre *et al.*, 2011).

E. coli HT115 bacteria expressing the double-stranded RNAs against the mentioned genes were used as a food source for nematodes, as described in materials and methods. Transgenic animals expressing LGG-1 fused with GFP (DA2123: *adls2123*[p_{lgg-1} GFP::LGG-1, *rol-6(df)*]) were placed on RNAi plates seeded with HT115 bacterial lawn either as eggs (after bleaching) or at the L4 stage. Both developmental stages were selected as initiation points of the experiment in order to test for embryonic lethality or possible developmental defects upon RNAi treatment against ribosomal genes. When animals were treated from the egg stage, autophagy was monitored at early L4 stage, approximately 2 days post hatching. When animals were treated from the L4 stage autophagy was monitored in early L4 animals of the first generation. In all cases, autophagy was monitored by the formation of GFP::LGG-1 puncta at hypodermal seam cells of early L4 animals (Palmisano and Meléndez, 2016).



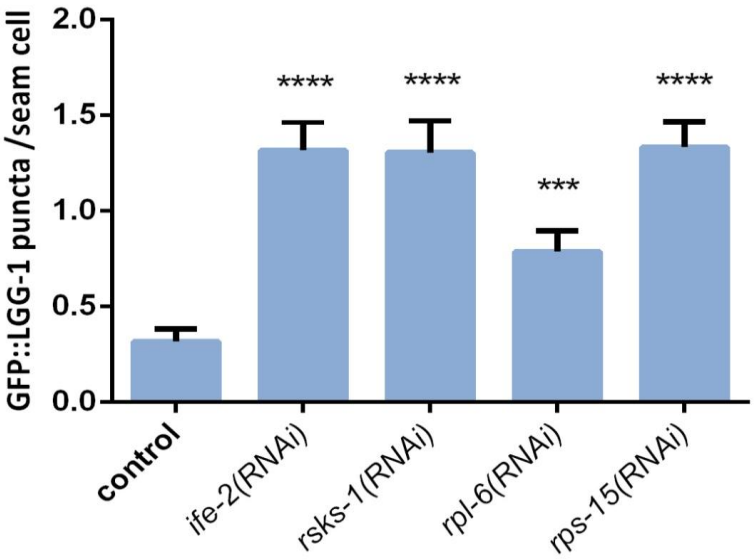


Figure 6: Autophagosomes accumulate under conditions of reduced protein synthesis. Number of GFP::LGG-1 puncta at seam cells of early L4 animals treated with *rsks-1*, *ife-2*, *rpl-6* and *rps-15* RNAi from the egg stage (above) and the L4 stage(below).

We observed a statistically significant increase in the formation of GFP::LGG-1 puncta (Figure 6) upon all treatments that interfered with protein synthesis. This was also the case for first generation animals, suggesting that autophagy is increased upon conditions that reduce protein synthesis.

2. Autophagy is induced upon RNAi-mediated knockdown of *rpl-6* and *rps-15*

An increase in the number of autophagosomes does not necessarily mean that autophagy is induced. The same end effect can be reached either due to autophagy induction or because autophagy is inefficient or blocked (Klionsky *et al.*, 2016).

In order to clarify that, we also monitored autophagy by fluorescent image analysis of animals expressing the SQST-1::GFP reporter. SQST-1 (sequestosome 1) exhibits sequence similarity to mammalian SQST1 and is also degraded by autophagy. Thus, blocking autophagy increases the levels of SQST-1 and leads to accumulation of SQST-1 aggregates (Tian *et al.*, 2010).

E. coli HT115 bacteria expressing the double-stranded RNAs against the mentioned genes were used as a food source for nematodes, as described in materials and methods. Transgenic nematodes expressing SQST-1 fused with GFP (HZ589: *bpls151*[p_{sqst-1} SQST-1::GFP, *unc-76(+)*]) were placed on RNAi plates seeded with HT115 bacterial lawn either as

eggs (after bleaching) or at the L4 stage. When animals were treated from the egg stage SQST-1::GFP puncta were monitored at the first day of adulthood, approximately 3 days post hatching. When animals were treated from the L4 stage, these puncta were monitored in D1 animals of the first generation. In all cases, autophagic flux was monitored by the formation of SQST-1::GFP puncta in the anterior region of the worms, around the area of the head, at the first day of adulthood (Nakamura *et al.*, 2016).

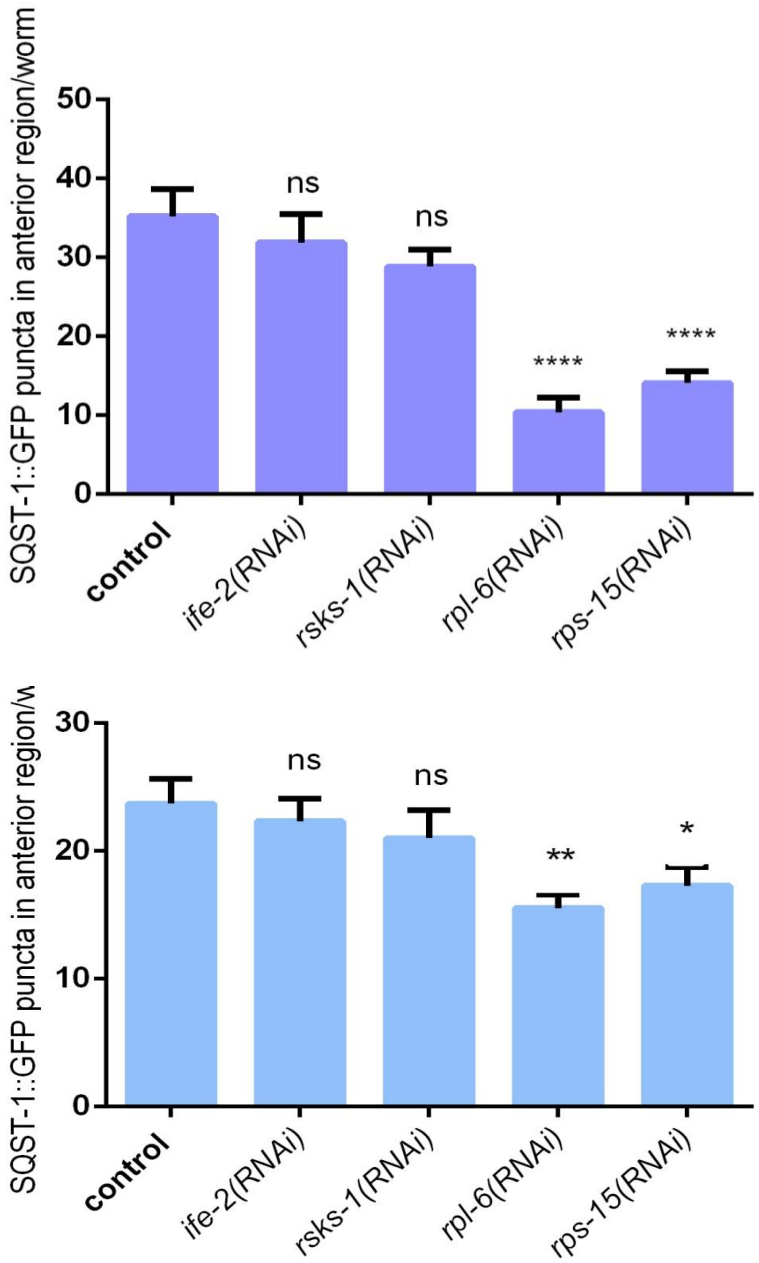


Figure 7: SQST-1 degradation upon different cases of protein synthesis inhibition. Quantification of SQST-1::GFP puncta in the pharyngeal region of D1 animals treated with RNAi against *rsks-1*, *ife-2*, *rpl-6* and *rps-15* from the egg stage (above) and the L4 stage(below).

RNAi-mediated knockdown of *rsks-1* and *ife-2* did not result in any alterations concerning the formation of SQST-1::GFP puncta in the pharyngeal region of the worms (Figure 7) although it enhanced the formation of GFP::LGG-1 puncta (Figure 6). Based on these results, we postulate that the increase in the number of autophagosomes could be due to blockage of the autophagic flux.

On the contrary, RNAi-mediated knockdown of *rpl-6* and *rps-15* significantly reduced the number of SQST-1::GFP puncta in the pharyngeal region of the worms when treated from both egg and L4 stage (Figure 7). Therefore, the increased number of autophagosomes seen previously by the GFP::LGG-1 reporter can be attributed to induction of autophagy. Combined, these results suggest that autophagy is induced upon depletion of the selected ribosomal proteins.

C. Autophagosome formation is enhanced in protein synthesis mutants

As an additional measure of determining how the autophagic process may be altered upon protein synthesis inhibition we performed genetic crosses between animals that carry mutations in key components of the translational machinery, and therefore are characterized by reduced protein synthesis, and animals that express an autophagy reporter. These crosses would allow us to monitor autophagy under these conditions.

To this end, we used the transgenic strain DA2123: *adIs2123*[p_{lgg-1} GFP::LGG-1, *rol-6(df)*] and crossed it with two mutant strains with reduced global protein synthesis: *ife-2(ok306)* and *edc-3(1427)*. As we have previously mentioned, loss of IFE-2 in *C. elegans* results in reduced global protein synthesis and prolonged lifespan (Syntichaki, Troulinaki and Tavernarakis, 2007). Depletion of EDC-3 has the same features, based on its role in controlling eIF4E availability for translation initiation (Rieckher *et al.*, 2018).

1. Loss of IFE-2 increases autophagosome accumulation

Generation of males (KX15) and genetic crosses between the two strains were performed as described in the section of materials and methods. Animals homozygous for the mutation while expressing the GFP::LGG-1 reporter were cultured on OP50 seeded NGM plates. We quantified autophagy by monitoring the formation of GFP::LGG-1 puncta in seam cells of early L4 animals (Palmisano and Meléndez, 2016).

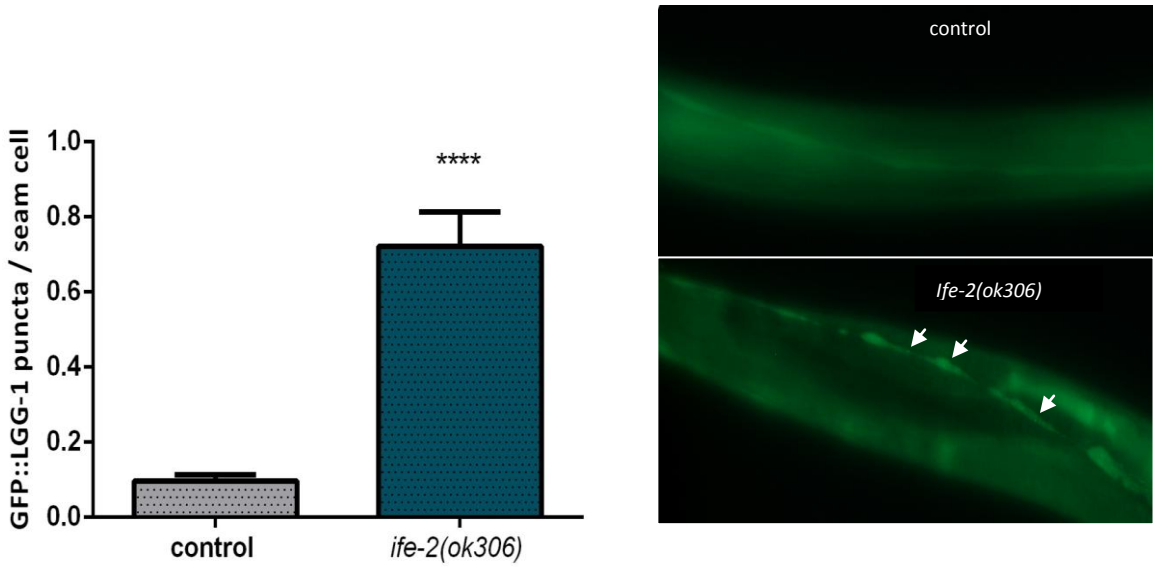


Figure 8: Autophagosomes accumulate in *ife-2* mutants. Quantification of GFP::LGG-1 puncta in seam cells of early L4 wt animals and *ife-2(ok306)* mutants.

Under conditions of reduced protein synthesis, simulated by a mutation in the *ife-2* gene, the GFP::LGG-1 signal was increased. Crossed animals exhibited a significant increase in the formation of GFP::LGG-1 puncta (Figure 8).

2. Autophagosome abundance is increased upon EDC-3 depletion

Following the same procedure homozygous animals for the *edc-3(ok1427)* mutation expressing the GFP::LGG-1 reporter were cultured on OP50 seeded NGM plates. Once again autophagy was quantified by monitoring the formation of GFP::LGG-1 puncta in seam cells of early L4 animals (Palmisano and Meléndez, 2016).

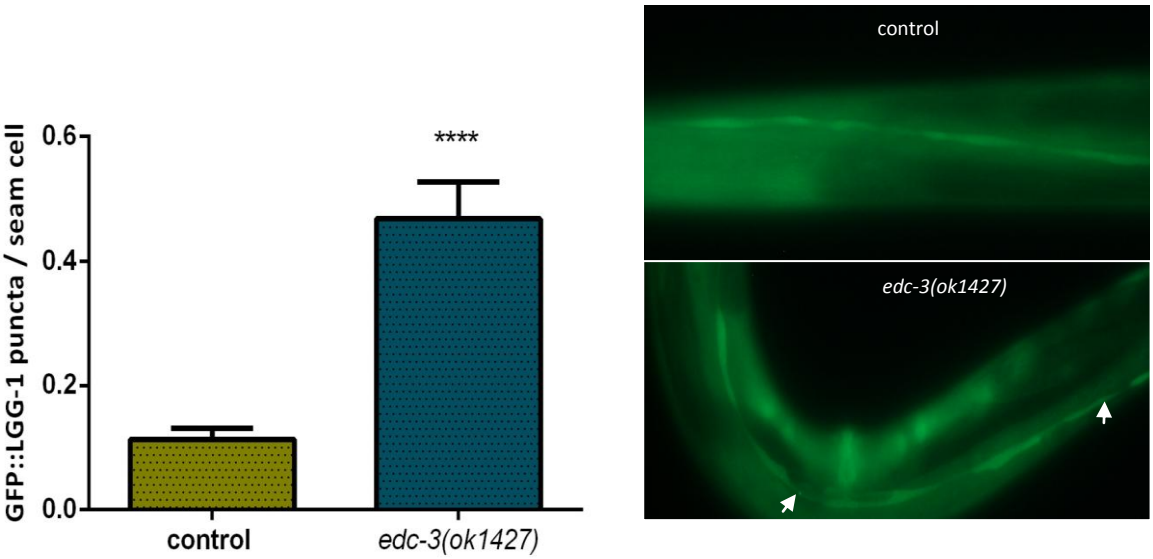


Figure 9: Autophagosomes accumulate in *edc-3* mutants. Quantification of GFP::LGG-1 puncta in seam cells of early L4 wt animals and *edc-3(ok1427)* mutants.

Under conditions of reduced protein synthesis, simulated by a mutation in the *edc-3* gene, animals exhibited a significant increase in the number of GFP::LGG-1 puncta suggesting an increase in the number of autophagosomes (Figure 9).

D. Inhibition of ribosomal genes differentially affects Huntington's Disease pathology in a *C. elegans* model

C. elegans has been established as a model for studying several human diseases. In this study, we used a nematode model of Huntington's Disease (HD) in order to investigate the effects of protein synthesis inhibition in terms of healthspan apart from lifespan.

The nematode does not have a huntingtin homolog. However, several transgenic *C. elegans* strains that express an N-terminal human huntingtin fragment with different numbers of glutamine repeats have been generated (Li and Le, 2013). These strains have mostly been used to model HD and identify genes that ameliorate its phenotype by reducing the number of polyglutamine (polyQ) aggregates.

It has been found that autophagy genes exert a protective role against polyQ toxicity in *C. elegans* models of Huntington's disease by suppressing the accumulation of polyQ aggregates (Jia, Hart and Levine, 2007). In this study, we wanted to investigate the phenotype of the disease under conditions of reduced proteins synthesis.

To this end, we used the transgenic strain AM141: *rmIs133*[p_{unc-54} Q40 ::YFP] which expresses an N-terminal human huntingtin fragment with 40 polyglutamine repeats fused with YFP. After hatching, these animals exhibit a Q40::YFP distribution to the body wall muscle and as they age Q40::YFP aggregates are rapidly formed.

Synchronized animals were placed as eggs after bleaching on RNAi plates seeded with HT115 bacteria expressing double stranded-RNA against *rpl-6*, *rps-15* or *rps-24*. At the first and third day of adulthood we measured the formation of Q40::YFP foci at the body wall muscle cells by fluorescent image analysis. As in all previous RNAi experiments empty pL4440 vector was used as a control.

As expected, Day 3 animals treated with the empty pL4440 vector displayed more Q40::YFP foci compared to Day 1 animals. This was also the case upon *rpl-6(RNAi)* and *rps-15(RNAi)* treatment: as nematodes aged, Q40::YFP foci in the body wall muscle cells increased. However, *rps-24(RNAi)* animals at the first and third day of adulthood did not display any statistically significant difference concerning the number of Q40::YFP foci.

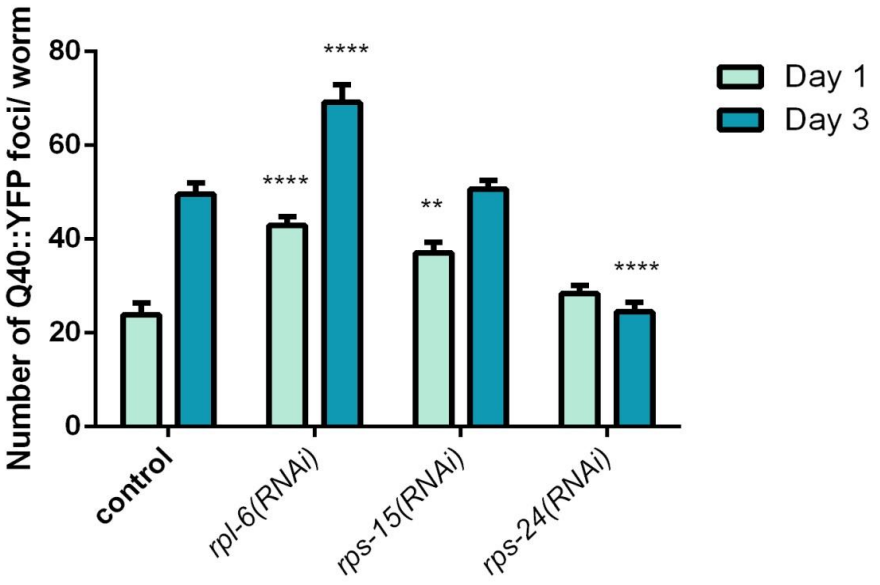


Figure 10: PolyQ aggregates upon knockdown of ribosomal genes. Quantification of Q40::YFP foci at D1 and D3 animals subjected to RNAi against *rpl-6, rps-15* and *rps-24* from the egg stage.

RNAi-mediated knockdown of *rpl-6* led to an increase of the formed Q40::YFP foci at both measurement points of Day 1 and Day 3. Treatment with *rps-15* RNAi resulted in increased formation of Q40::YFP aggregates of D1 animals while Q40::YFP foci of Day 3 worms were not significantly altered compared to control. On the contrary, *rps-24* RNAi significantly reduced the number of Q40::YFP foci of Day 3 animals. At Day 1 animals the formation of Q40::YFP aggregates remained at the same levels as in the case of the control (Figure 10). This suggests a potential protective role of the genetic inhibition of the LIR-bearing ribosomal protein RPS-24 in the aggregation phenotype of Huntington's disease.

Discussion

Autophagy is a major degradation mechanism, with a critical role in cellular recycling and quality control. Due to its implication in the clearance of excessive or defective macromolecules and organelles as well as the degradation of misfolded and aggregated proteins, autophagy induction, to a certain extent, is considered beneficial for cellular homeostasis. Moreover, considering how these alterations correlate with cellular senescence, organismal ageing and pathogenesis, autophagy acts as a protective mechanism and has beneficial effects against ageing and disease (Meléndez et al., 2003; Madeo et al., 2015; Menzies et al., 2017)

Regulation of both protein synthesis and degradation is equally crucial for homeostasis. Protein synthesis is highly energy consuming, utilizing an estimated 50% of the total cellular energy. This is invested to promote reproductive growth and metabolic activity. Despite its importance for cellular physiology and energetic balance, information concerning the interplay between protein synthesis and ageing is scarce. Ageing is accompanied by marked alterations in both general and specific protein synthesis, and accumulating findings suggest that these changes do not simply arise as a corollary of ageing but play a causative role in the ageing process (Tavernarakis, 2007). However, multiple studies have demonstrated that translation inhibition extends lifespan in a variety of model organisms (Hansen et al., 2007; Pan et al., 2007; Tohyama, Yamaguchi and Yamashita, 2008).

Ribosomal biogenesis and assembly are highly energy consuming. Ribosomes are stable and complex molecular machines and a core component of the translational machinery. Inhibition of ribosomal proteins and ribosome biogenesis genes enhances longevity (Hansen *et al.*, 2007; Steffen *et al.*, 2008; Macinnes, 2016).

The aforementioned biological processes should be tightly regulated by sensors of energy and nutrient levels. mTORC1 acts as a common modulator of autophagy, protein synthesis, and ribosomal biogenesis, in lifespan regulation (Macinnes, 2016). Inhibition of mTOR signaling induces autophagy and simultaneously blocks protein translation, mainly through the function of S6 kinase.

To address whether autophagy is involved in lifespan extension under conditions of reduced protein synthesis, we performed survival assays, where we assessed the lifespan of protein synthesis mutants upon RNAi-mediated knockdown of autophagy genes. Our results indicate that certain autophagy components are required for extended longevity of *rsks-1(ok1255)* and *edc-3(ok1427)* mutants. Specifically, RNAi against *unc-51* and *lgg-1* abolish the lifespan extension in *rsks-1(ok1255)* animals, while RNAi-mediated knockdown of *atg-7* and *unc-51* yield similar effects in *edc-3(ok1427)* mutants. Similarly, mutation in the *lgg-2* gene abrogates lifespan extension conferred by RNAi treatments that inhibit protein synthesis. In summary, our findings suggest that components of the autophagic machinery are essential for longevity associated with reduced protein synthesis.

Additionally, we utilized specific transgenic strains to monitor autophagy when interfering with protein synthesis. By counting autophagic puncta and SQST-1/p62 degradation, we report that autophagy is induced upon genetic inhibition of ribosomal proteins. Moreover, the number of autophagosomes is increased in animals with reduced protein synthesis, notably *ife-2(ok306)* and *edc-3(ok1427)*.

Interestingly, inhibition of protein synthesis specifically through RNAi-mediated knockdown of *rps-24*, ameliorates the polyglutamine aggregation phenotype in a nematode model of Huntington's disease. This indicates that suppression of the LIR-bearing ribosomal protein RPS-24 may protect against disease, eventually enhancing healthspan, apart from lifespan. The effects of genetic inhibition of RPS-24 and the rest ribosomal proteins on the aggregation phenotype of HD, among other proteotoxic disorders modeled by *C. elegans,* such as Alzheimer's disease and Parkinson's disease, is worth considering in the future.

We suggest that autophagy is involved in the extended longevity associated with reduced protein synthesis. We speculate that ribosomes which are inactive or excessive due to translation inhibition could constitute a novel target of autophagy. We anticipate that our future experiments will delineate the intricate interplay between autophagy, ribosomal turnover and protein synthesis. In addition behavioral assays in nematode models of human diseases such as Alzheimer's and Parkinson's will unravel the possibility of targeting ribosome-specific autophagy to tackle severe human pathologies.

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