

Research Paper

Longevity pathways converge on autophagy genes to regulate life span in *Caenorhabditis elegans*

Márton L. Tóth,^{1,†} Tímea Sigmond,^{1,†} Éva Borsos,¹ János Barna,¹ Péter Erdélyi,¹ Krisztina Takács-Vellai,¹ László Orosz,¹ Attila L. Kovács,² György Csikós,² Miklós Sass² and Tibor Vellai^{1,*}

¹Department of Genetics; ²Department of Anatomy, Cell and Developmental Biology; Eötvös Loránd University; Budapest, Hungary

[†]These authors contributed equally to this work.

Key words: autophagy genes, aging, life span, *C. elegans*, drosophila, insulin/IGF-1 signaling, TOR, mitochondrial respiration, caloric restriction

Aging is a multifactorial process with many mechanisms contributing to the decline. Mutations decreasing insulin/IGF-1 (insulin-like growth factor-1) or TOR (target of rapamycin) kinase-mediated signaling, mitochondrial activity and food intake each extend life span in divergent animal phyla. Understanding how these genetically distinct mechanisms interact to control longevity is a fundamental and fascinating problem in biology. Here we show that mutational inactivation of autophagy genes, which are involved in the degradation of aberrant, damaged cytoplasmic constituents accumulating in all aging cells, accelerates the rate at which the tissues age in the nematode *Caenorhabditis elegans*. According to our results *Drosophila* flies deficient in autophagy are also short-lived. We further demonstrate that reduced activity of autophagy genes suppresses life span extension in mutant nematodes with inherent dietary restriction, aberrant insulin/IGF-1 or TOR signaling, and lowered mitochondrial respiration. These findings suggest that the autophagy gene cascade functions downstream of and is inhibited by different longevity pathways in *C. elegans*, therefore, their effects converge on autophagy genes to slow down aging and lengthen life span. Thus, autophagy may act as a central regulatory mechanism of animal aging.

Introduction

The regulation of the aging process has been extensively studied in *Caenorhabditis elegans* with over 70 life-extension mutations known so far.¹⁻³ For example, mutant nematodes with reduced activity of the insulin/IGF-1 receptor homolog DAF-2 (Dauer larva formation abnormal-2) live two times longer than the wild type.^{1,4,5} The life span-lengthening effect of *daf-2* mutations requires the activity of the forkhead transcription factor DAF-16/FOXO, whose counterparts control longevity in *Drosophila* and mice, too.^{6,7} Lowering

mitochondrial respiration in *C. elegans* also doubles its natural life span.^{8,9} Life span extension in respiration-defective worms is increased much further by *daf-2* mutations.⁸ This additive interaction suggests that insulin/IGF-1 signaling and the mitochondrial respiratory system operate in parallel through a common mechanism that influences life span. Another signaling axis that affects nematode longevity is the nutrient-sensing TOR pathway.¹⁰⁻¹³ In worms, TOR deficiency does not increase further the extended life span of *daf-2* mutants,¹⁰ indicating that TOR interacts with the insulin/IGF-1 hormonal system to control life span.^{10,11} Indeed, *daf-15*, which encodes a Raptor-like (Regulatory associated protein of mammalian TOR) protein known to function with TOR in a complex,¹⁴ was shown to be regulated transcriptionally by DAF-16.¹¹ Furthermore, decreasing food intake by mutations that disrupt the function of the feeding organ, the pharynx, or by growing worms in liquid media with low concentration of bacteria (food) lengthens *C. elegans* life span by decreasing the rate at which the tissues age.^{15,16} Exposing *daf-2* mutants to dietary restriction further extends life span.¹⁶ Thus, insulin/IGF-1 signaling and dietary restriction may influence aging via different mechanisms, although there is some evidence that effects of dietary restriction on life span result from reduced TOR signaling¹⁶ and increased autophagy.¹⁷ Although aging appears to be a complex, multifactorial process, a general decrease in protein turnover and the intracellular accumulation of oxidative damaged macromolecules and organelles generated by reactive oxygen species produced during respiration¹⁸⁻²¹ are features common to all aged cells.²² This suggests that the aging process may be more specific than previously expected, and that reactive oxygen species might be the main determinant of animal life span.²³

Autophagy, a highly regulated cellular pathway used by eukaryotic cells to consume parts of their constituents during development or starvation, is the major route for bulk degradation of aberrant, damaged cytosolic components.²⁴⁻²⁶ During autophagic degradation, subcellular material is sequestered by a segregation membrane,^{26,27} delivered into lysosomes for subsequent breakdown, and the products of degradation are reused for cell functioning. Indeed, defects in autophagy have been associated with age-related cellular changes such as intracellular accumulation of damaged proteins and organelles.^{20-22,28-30} RNA interference- (RNAi) mediated downregulation

*Correspondence to: Tibor Vellai; Department of Genetics; Eötvös Loránd University; Pázmány Péter sétány 1/C; Budapest H-1117 Hungary; Tel.: +36.1.209.0555/8684; Fax: +36.1.209.0555/1841; Email: vellai@falco.elte.hu

Submitted: 09/28/07; Revised: 01/17/08; Accepted: 01/21/08

Previously published online as an *Autophagy* E-publication:
<http://www.landesbioscience.com/journals/autophagy/article/5618>

of certain autophagy genes has already implicated the autophagic process in the survival of *C. elegans*. Depleting BEC-1 (*C. elegans* Beclin1 ortholog-1), which is the worm counterpart of the human tumor suppressor Beclin1 (Bcl-2 interacting protein-1) and yeast Atg6 (Autophagy protein-6) proteins, suppressed the long-lived phenotype of *daf-2* mutant nematodes, and also slightly shortened life span in wild-type background.³¹ In a recent study, however, *bec-1* RNAi treatment had almost no effect on the life span of *daf-2* mutant and wild-type animals.³² Instead, knockdown of the *C. elegans* orthologs of yeast *Atg7* and *Atg12* autophagy genes partially inhibited life span extension in *daf-2* mutants, and significantly shortened mean, but not maximum, life span in wild-type worms.³² Moreover, *bec-1* and *atg-7* RNAi treatments reduced survival in mutant nematodes with inherent caloric restriction.³³ Despite its potential importance in aging regulation, it is unknown whether deregulating autophagy causes progeria (accelerated aging), or simply kills the animals for reasons unrelated to aging. Furthermore, no mutant analysis has been performed yet to assess the life span modulatory effect of autophagy. Using loss-of-function mutations in autophagy genes in a set of accurate epistasis (double mutant) experiments, this would be particularly important to uncover the molecular mechanisms by which autophagy affects the aging process. Finally, so far *C. elegans* is the only organism in which the influence of autophagy on life span has been demonstrated (note that while this paper was under review, a related work on the role of autophagy in *Drosophila* aging was published online; see ref. 34).^{31,32} Thus, it is unclear whether the autophagy-related regulation of life span is a nematode-specific feature or a common mechanism that operates in divergent animal species.

In this study we demonstrate that loss-of-function mutations in autophagy genes accelerate the rate at which the tissues age in *C. elegans*, and that inhibition of autophagy also shortens life span in *Drosophila* flies. Furthermore, we show that autophagy genes are required for life span extension in mutant worms with reduced food intake, TOR or insulin/IGF-1 signaling, and mitochondrial respiration. These results imply that the effects of mutations affecting distinct longevity pathways converge on common downstream processes that involve autophagy. Thus, autophagy might play a central role in the regulation of animal aging.

Results

Mutational inactivation of autophagy genes shortens *C. elegans* life span. To monitor the effects of loss-of-function mutations in autophagy genes on *C. elegans* life span (as a set of control experiments), we first examined single mutant nematodes deficient for BEC-1. This multifunctional protein is essential for development.³⁵ Thus, we rescued the lethality of *bec-1* loss-of-function mutants by an unstable (extrachromosomal) transgene array containing wild-type copies of *bec-1* [*bec-1(+)* denotes a translational fusion reporter construct labeled with green fluorescence protein (GFP): *Ex*[*pbec-1::BEC-1(+):GFP*]; see the Materials and Methods, for constructing the plasmid *pbec-1::BEC-1::GFP*, see refs. 35 and 36). Adults of *bec-1(-)*; *Ex*[*bec-1(+)*] genotype, especially those carrying *ok691* mutation, lived markedly shorter than the wild type (Fig. 1A and Table 1S). The short-lived phenotype of certain *bec-1(-)*; *Ex*[*bec-1(+)*] adults may result from incomplete rescue of *bec-1* in somatic cells. The majority of *bec-1* adults that were alive at day 15, i.e., at age that is close to the maximal life span of wild-type animals (Fig. 1A), showed

BEC-1::GFP expression in many if not all somatic tissues (data not shown).^{31,35,36} In these worms, the short-lived phenotype of *bec-1* mutants is likely to be completely rescued by *bec-1(+)* transgene. In contrast, the majority of 10-day-old *bec-1* adults with strong signs of tissue deterioration (i.e., at stage where they were expected to die within a few days) showed no or highly reduced transgene expression (data not shown). Note that at day 10 almost all wild-type worms were alive. Because the two independently isolated *bec-1* mutations, each backcrossed 6 times, behaved the same way, it is unlikely that a linked mutation caused the life span shortening effects. We next examined the life span of *unc-51* (*uncoordinated-51*) mutant animals. *unc-51* encodes a serine-threonine kinase³⁷ whose yeast counterpart Atg1 is a key regulator of autophagosome formation.³⁸ We found that mean, but not maximum, life spans were shorter in *unc-51* mutants than in wild-type animals (Fig. 1B and Table 1S). A similar life span-reducing effect was obtained by a loss-of-function mutation in *atg-18* (Fig. 1C and Table 1S), whose protein product is implicated in retrieving proteins from the preautophagosomal structure.³⁹

At present only a very limited number of *C. elegans* autophagy genes are available as mutant alleles. Thus, we used RNAi to knock down the function of two other autophagy genes: *lgg-1* (*LC3*, *GABARAP* and *GATE-16 family-1*)/*Atg8*,^{31,36,40} which encodes a ubiquitin-like protein whose lipidation is required for membrane dynamics during autophagy, and *T22H9.2/Atg9*,⁴¹ which encodes a transmembrane protein essential for autophagy. Silencing of these genes markedly reduced both mean and maximum life span (Fig. 1D and Table 1S). Together, these results show that autophagy genes influence the survival of *C. elegans*.

Autophagy genes have multiple roles in diverse cellular functions such as membrane remodeling, protein trafficking and endocytosis.^{24-26,29} It is therefore important to find indications of the autophagic process being affected in autophagy gene mutant nematodes which would implicate autophagy itself in aging. We used transmission electron microscopy to study the ultrastructural effects of *bec-1* and *unc-51* mutations, and found consistent and characteristic features reflecting compromised autophagy. Instead of distinct and well defined individual autophagosomes found in wild-type worms, the autophagic elements in *unc-51* and *bec-1* mutants tend to aggregate and accumulate abnormally large amount of myelinated membranous structures (Fig. 2A).

C. elegans longevity is regulated by signals from the nervous system and intestine.^{42,43} Consistent with their roles in life span control, three autophagy genes, *lgg-1*, *bec-1* and *atg-18*, for which reporters labeled with GFP are available, were expressed in many if not all adult somatic cells including neurons and intestinal cells throughout different larval stages and adulthood (Fig. 2B).

Impaired autophagy accelerates the anatomical and behavioral signs of aging. An important question is whether short-lived mutant nematodes deficient in autophagy age more rapidly than normal animals or become simply sick and die earlier for reasons unrelated to aging. To address this issue, we assayed the levels of lipofuscin granules in adults at different ages. Lipofuscin is a pigment that accumulates progressively, in particular during the postreproductive period, in aging tissues as a result of the oxidative degradation of cellular components.^{44,45} We found that *bec-1*, *unc-51* and *atg-18* mutants accumulated lipofuscin more rapidly during the course of life than wild-type animals (Fig. 3A). Although relative lipofuscin levels

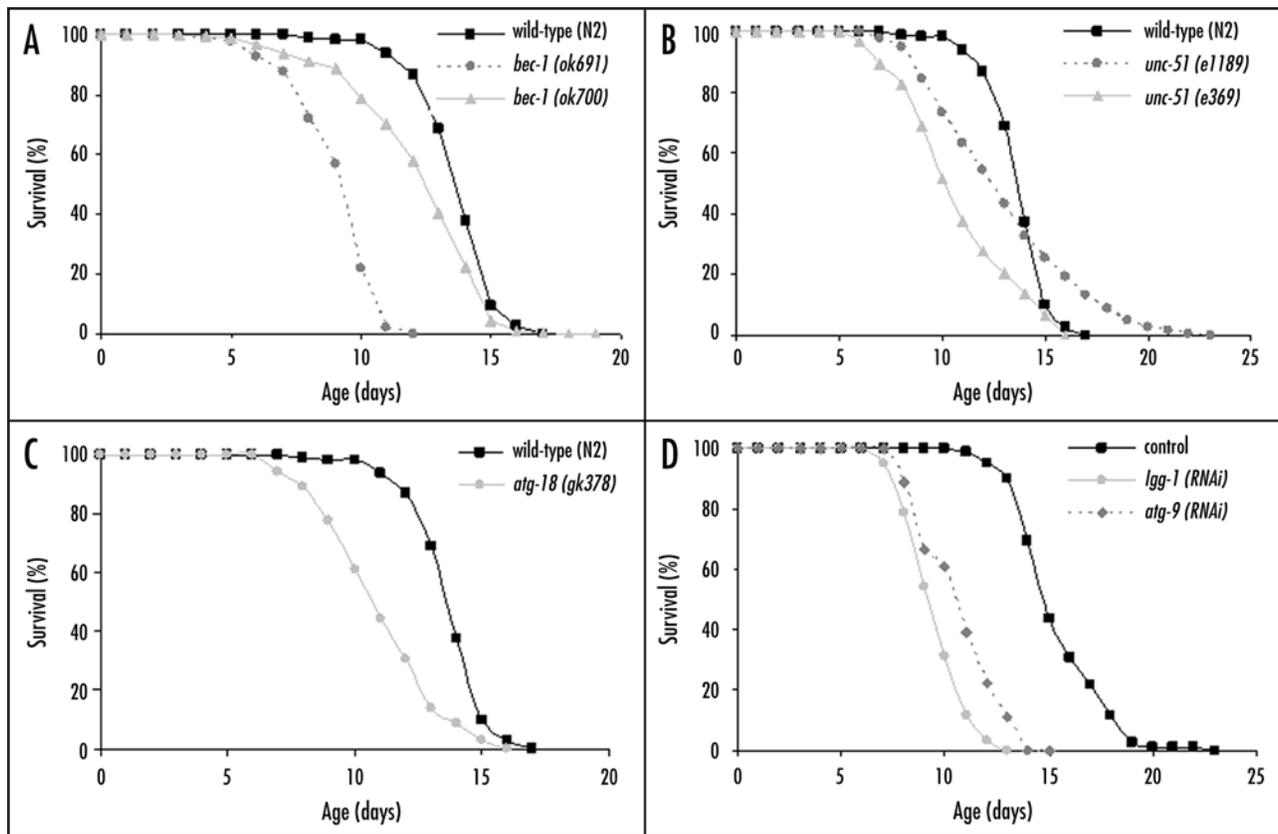


Figure 1. Mutational inactivation of autophagy genes shortens *C. elegans* life span. The percentage of living animals is plotted against animal age. Life span values and statistics are given in Supplementary Table 1. (A) Mutants deficient for BEC-1 live significantly shorter than the wild type. *bec-1(ok691)* and *bec-1(ok700)* denote the *bec-1(ok691); Ex[bec-1(+)]* and *bec-1(ok691); Ex[bec-1(+)]* genotypes, respectively. Randomly selected post-reproductive adults were used for scoring. Reduction in mean life span of these animals may result from incomplete rescue of *bec-1* in somatic cells. (B) Loss-of-function mutations in *unc-51* confer decreased mean life spans. (C) *atg-18(gk378)* mutants display a short-lived phenotype. (D) Depletion of LGG-1/Atg8 or T22H9.2/Atg9 shortens *C. elegans* life span. RNAi-sensitive *rrf-3(pk1426)* mutant animals were fed with bacteria (*E. coli* HT115) containing the empty vector (control) or vector expressing *lgg-1* or T22H9.2 double-stranded RNA.

at days of maximum life span were similar in autophagy mutant and wild-type animals, the latter reached these levels several days later.

A gradual, progressive decline in the cellular integrity of the muscle tissue is known to accompany *C. elegans* aging, causing severe defects in locomotion during the postreproductive period.⁴⁶ Hence we followed the locomotory behavior of individual autophagy gene mutant animals over time, and found that they became paralyzed at earlier times of onset than the wild type (Fig. 3B).

Using Nomarski (differential interference contrast) microscopy, we also visualized features of tissue deterioration as the animals grew older.⁴⁴ In autophagy mutant worms, necrotic cavities, nuclear cellularization and enlargement of germ cells, and accumulation of large vacuoles in the intestine became apparent earlier during adulthood than in wild-type animals (data not shown). Together, these results indicate that autophagy genes influence *C. elegans* life span by changing the rate at which the tissues age. Therefore, mutant nematodes with aberrant autophagy live shorter than normal as a result of progeria (accelerated aging).

Inhibiting autophagy shortens life span in *Drosophila*. Since autophagy is an evolutionarily conserved cellular pathway, we wondered whether autophagy genes influence life span in another animal phylum, the Arthropoda. In a screen for autophagy deficient *Drosophila* flies we have isolated a number of mutant strains that

are unable to form autophagic vacuoles in the larval fat body before pupariation and arrest development during metamorphosis (György Csikós and Miklós Sass, unpublished results). To monitor the effects of some of the corresponding *Drosophila* genes on life span, we generated fly strains expressing inverted repeat transgenes that induce RNAi interference of the following two genes: *Atg3*, which encodes the conjugative enzyme for Atg8;⁴⁷ and *SNF4 α* , which encodes the gamma subunit of the AMP-activated protein kinase.⁴⁸ The cellular energy-sensing AMP-activated protein kinase pathway is known to control life span in *C. elegans*⁴⁹ and to promote autophagy in human cell lines.⁴⁸ Silencing of these two genes from the first day of imaginal stage markedly reduced adult life span (Fig. 4 and Table 2S) and caused morphological and behavioral features of premature aging (data not shown). Consistent with a recently published work on the role of autophagy in *Drosophila* aging,³⁴ these results support the view that autophagy-related life span regulation might be a general mechanism in divergent animal phyla.

Mutational inactivation of autophagy genes partially suppresses life span extension in *daf-2* mutant nematodes. To explore the mechanisms by which autophagy genes influence life span in *C. elegans*, we performed a set of epistasis experiments. RNAi-mediated depletion of certain autophagy proteins has already been demonstrated to inhibit life span extension in *daf-2* mutant worms.^{31,32} This indicates

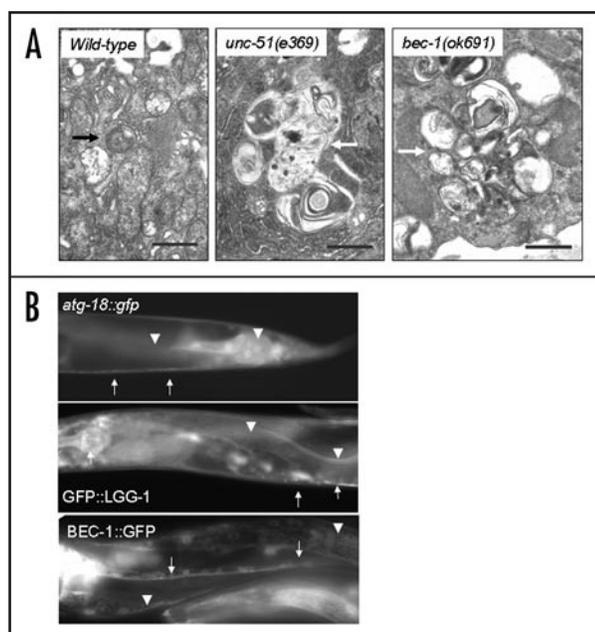


Figure 2. BEC-1 and UNC-51 mediate normal autophagosome formation. (A) Autophagosome formation is compromised in *bec-1* and *unc-51* mutant animals. In hypodermal cells of wild-type animals, autophagosomes are rather small and stand alone. In this specimen, the black arrow points to a small, single autophagosome. In the hypodermis of *unc-51* and *bec-1* mutants autophagic elements tend to be aggregated into big abnormal foci with excess amount of “myelinated” membrane whorls (white arrows). Such structures cannot be found in well-fed wild-type animals, and seems to indicate defective phagophore formation. Bars, 1 μ m. (B) Autophagy genes are expressed in many if not all somatic tissues including the intestine and neurons, which are known to emit signals regulating *C. elegans* life span. Arrows indicate neuronal structures, arrowheads point to intestinal cells.

that the effect of autophagy genes on life span is, at least in part, mediated through the insulin/IGF-1 signaling pathway. Consistent with these data, we found that *bec-1*, *atg-18* and *lgg-1* (Fig. 5 and Table 3S) were also required for the increased longevity of mutants with aberrant DAF-2 function. Strong loss-of-function mutations in *daf-2* inhibit reproductive growth, causing the animals to enter into a state of developmental diapause known as dauer larva that is triggered by starvation and crowding in the wild type. Thus, we maintained nematodes bearing the thermosensitive *daf-2(e1370)* mutation at 20°C until they reached the L4 larval stage to inhibit entry into dauer larva development, then transferred them at 25°C and scored their survival. Interestingly, mutations in *bec-1* and *atg-18* only partially suppressed the long-live phenotype of *daf-2(e1370)* mutant animals (Fig. 5 and Table 3S), although *ok691* and *gk378* are large deletions representing strong loss-of-function alleles of *bec-1* and *atg-18*, respectively (see ref. 35 and Wormbase: <http://www.wormbase.org>). *bec-1(ok691);daf-2(e1370)* and *atg-18(gk378);daf-2(e1370)* double mutant animals each lived longer than wild-type nematodes, and much longer than the corresponding single autophagy mutants. *lgg-1* and *T22H9.2* RNAi treatments also partly reduced the life span of *daf-2(e1370)* mutants (Table 3S). The incomplete inhibition of life-extension in *daf-2(e1370)* background by genetic null mutations in autophagy genes suggests that the autophagy gene cascade may act parallel with another system to mediate the effect of insulin/IGF-1 signaling on life span.

We also treated autophagy mutants with *daf-2* RNAi. Depletion of DAF-2 from hatching allows wild-type animals to develop as dauer larvae. In contrast, *daf-2* RNAi treatment from the L2 larval stage permits growth to adulthood, but significantly extends adult life span in wild-type background (Table 3S). However, we found no effect of *daf-2* RNAi on the survival of mutant animals defective for BEC-1 or ATG-18 (Table 3S). These results indicate that in epistasis analysis RNAi is often less effective than genetic mutations. Mutations in the insulin/IGF-1 signaling pathway alter the rate at which the tissues age.⁴⁵ Our results presented here further confirm that autophagy genes act downstream of and are regulated negatively by insulin/IGF-1 signaling to slow down the rate of aging.

Autophagy genes are required for life span extension in TOR-deficient nematodes. Inhibition of TOR signaling, which triggers autophagic degradation in eukaryotic cells,²⁴⁻²⁶ confers increased longevity in yeast, worms and flies.^{10-13,50,51} Moreover, genetic studies in *C. elegans* and on mammalian cell cultures indicate that TOR interacts with the insulin/IGF-1 signaling pathway to control life span,^{10,11} protein synthesis and cell growth.^{52,53} Thus, we asked whether the effects of TOR deficiency on life span are also mediated by concomitant upregulation of autophagy genes. If the answer is yes, inhibiting autophagy would eliminate the life-extending effect of TOR deficiency. Since loss-of-function mutations of the *C. elegans* *Tor* gene (*let-363/CeTor*) cause developmental arrest at the L3 larval stage,^{10,54} we assayed the life span of adult nematodes depleted for TOR from the L4 larval stage onwards. This procedure of *let-363/CeTor* RNAi treatment led to a significant increase in mean life span in an otherwise wild-type background, but did not change the life span of autophagy mutants (Fig. 6 and Table 3S). In other words, mutations in *bec-1*, *unc-51* and *atg-18* completely blocked life-extension in *let-363/CeTor(RNAi)* animals. Thus, we conclude that autophagy genes are required for increased longevity in worms with aberrant TOR signaling. This implies that the effect of TOR pathway on life span is mediated by autophagy genes.

Mutations in autophagy genes suppress the long-lived phenotype of mutant nematodes with inherent dietary restriction. Reduction in food intake increases life span and delays the onset of many age-related declines in function, as well as leads to downregulation of TOR signaling in organisms from yeast to mammals.^{15,19,24} The activity of the nutrient-sensing TOR signaling system is known to inhibit autophagy, whose major role is cellular response to starvation. Therefore, the TOR pathway and the autophagy gene cascade might constitute a common signaling axis. This prediction prompted us to investigate whether inactivation of autophagy genes interferes with the effect that dietary restriction has on *C. elegans* life span. We assess the effect of a loss-of-function mutation in *eat-2* (*eating-defective-2*) gene, *ad1116*, which lengthens life span by 50% via dietary restriction.¹⁵ According to our results, *ad1116* mutation did not affect life span in *bec-1* and *unc-51* mutant animals (Fig. 7A and Table 3S), consistent with previous observations.³³ These double mutants lived no longer than the corresponding single autophagy mutants. Complete suppression of the *Eat-2(ad1116)* long-lived phenotype by loss-of-function mutations in *bec-1* and *unc-51* suggests that these genes interact with and function downstream of the *eat-2*-mediated longevity pathway to influence aging.

We also measured relative autophagic activity in *eat-2(ad1116)* mutants, using an integrated GFP-tagged LGG-1 reporter³⁶ thought

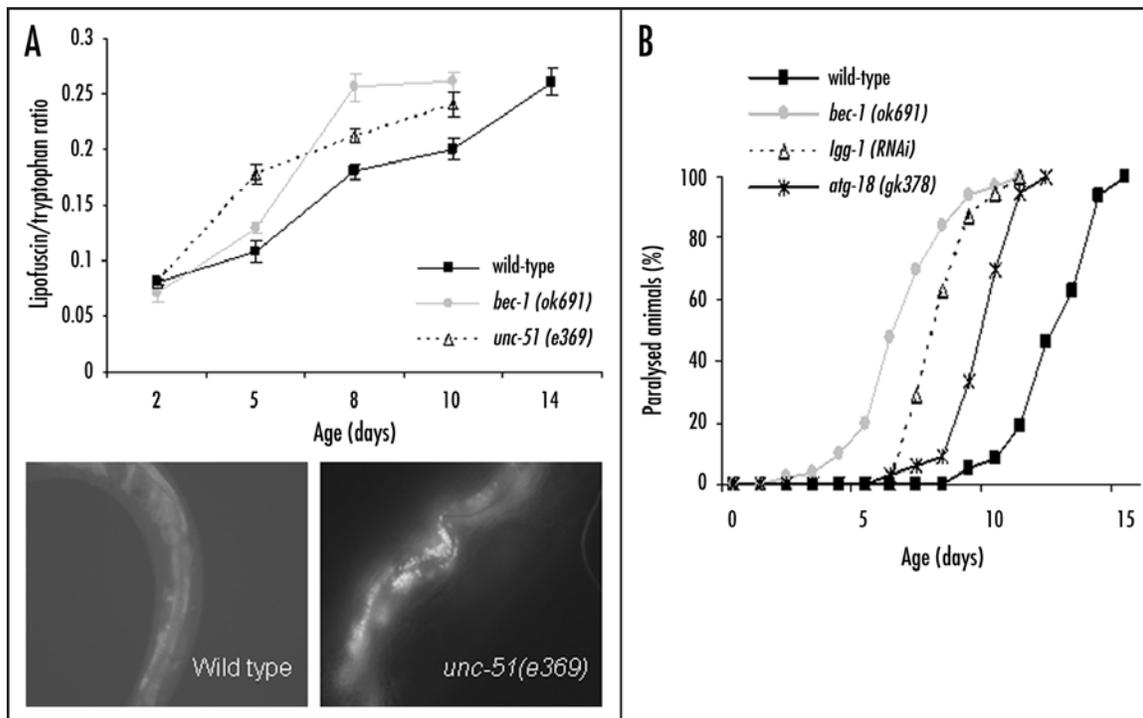


Figure 3. Mutant nematodes with aberrant autophagy show accelerated rate of tissue deterioration and decline in locomotion. (A) Autophagy mutant animals accumulate age pigments more rapidly than normal. The relative ratio of age pigment and tryptophan fluorescence was plotted against animal age. Quantification of age pigments was performed according to ref. Gerstbrein et al., 2005. Tryptophan fluorescence (relative protein level) was measured at 290 nm excitation and 330 nm emission, while age pigment fluorescence was detected at 340 nm excitation and 430 nm emission, using a spectrofluorimeter. Note that wild-type animals live longer than autophagy mutants, explaining their extended age-pigment curve. *bec-1(ok691)* refers to the *bec-1(ok691); Ex[bec-1(+)]* genotype. *p* values <0.001, using unpaired *t*-test. Data are mean \pm SEM. Fluorescence images show a 10-day-old wild-type animal (left) and a 10-day-old *unc-51* mutant (right). The mutant animal contains more age pigments than the wild type. (B) Age-associated locomotory defects occur with earlier times of onset in autophagy deficient mutants than in wild-type animals. The percentage of paralyzed animals (see the Material and Methods) is plotted against animal age. *bec-1(ok691)* denotes the *bec-1(ok691); Ex[bec-1(+)]* genotype. Control animals fed with bacteria containing the empty RNAi vector show wild-type rate (results not shown). For each genotype, *n* = 130. *p* value versus control <0.0001, using unpaired *t*-test.

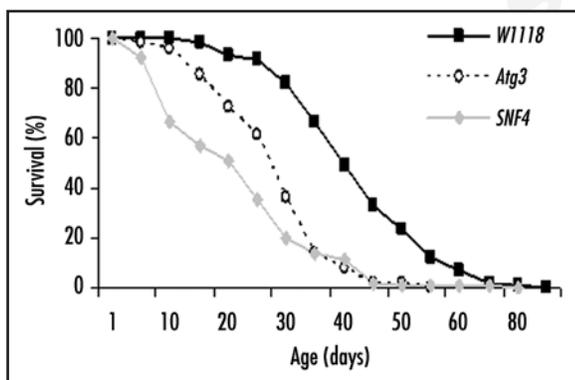


Figure 4. RNAi-mediated inactivation of autophagy shortens life span in *Drosophila*. Depletion of *Atg3* or *SNF4 γ* from the first day of imaginal stage shortens life span in *Drosophila*. *Atg3* is an autophagy-related protein, while *SNF4 γ* is also required for autophagic activity (see the text). W1118 strain was used as control. For RNAi-treated flies, *p* < 0.0001 (unpaired *t*-test). Life span values and statistics are given in Supplementary Table 2.

to mark (pre)autophagosomal structures in hypodermal cells.³¹ We stained animals transgenic for GFP::LGG-1 with LysoTracker Red, a dye known to be specific to lysosomes, and found that GFP-positive punctuate areas were co-localized with LysoTracker Red-labeled compartments (data not shown). We then monitored GFP::LGG-1 expression in wild-type and *eat-2(ad1116)* animals. GFP::LGG-1

was expressed mainly in diffuse cytoplasmic pattern in lateral seam cells in wild-type background (Fig. 7B). In contrast, there was a significant increase in the number of GFP-positive foci per lateral seam cells in *eat-2(ad1116)* mutants. These results further support that longevity in calorically restricted animals is coupled with enhanced autophagic activity.

Autophagy genes are essential for life span extension in mutant worms with decreased mitochondrial respiration. Lowered activity of the mitochondrial electron transport chain during development increases adult life span in *C. elegans*.^{8,9} Contrary, when mitochondrial activity is decreased only during adulthood nematodes have wild-type life span. Thus, the rate of respiration early in life establishes the rate of aging during adulthood. Recently, it was shown that mitochondrial membrane potential, oxygen consumption and ATP-synthetic capacity are influenced by TOR activity,⁵⁵ which, as we show in this study, regulates aging via inhibiting the activity of autophagy genes. Thus, we examined genetic interactions between mitochondrial respiratory components and autophagy genes in aging control. To extend *C. elegans* life span by impairing mitochondrial respiration, we knocked down *atp-3* (*ATP-synthase-3*), which encodes a component of the mitochondrial ATP-synthase, and *clk-1* (*clock abnormal-1*), whose product participates in ubiquinone production.^{8,9} We found that disruption of *atp-3* or *clk-1* function could not modulate life span in autophagy mutant background (Fig. 8 and Table 3S). Autophagy mutant nematodes treated with *atp-3* RNAi

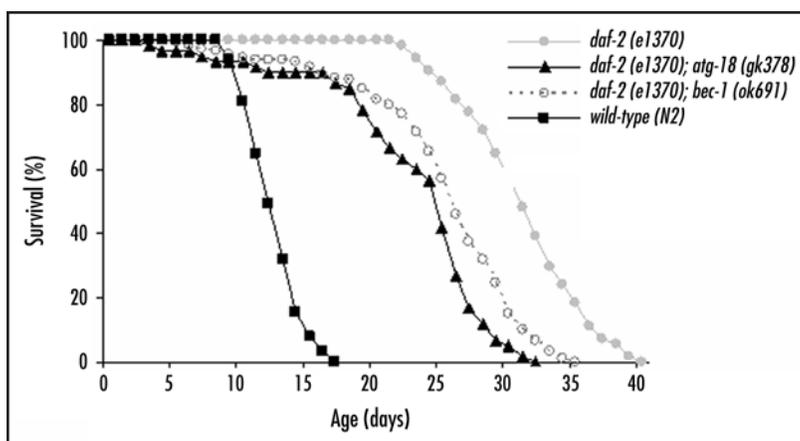


Figure 5. Loss-of-function mutations in autophagy genes partially suppress longevity in *daf-2(e1370)/Igf-1* mutant animals. Life span extension in *daf-2(e1370)* mutants is partially suppressed by mutations in *bec-1* and *atg-18*. *ok691* and *gk378* are strong loss-of-function mutations of *bec-1* and *atg-18*, respectively. These genetic interactions between *daf-2* and autophagy genes further support that the autophagy gene cascade functions downstream of and is inhibited by insulin/IGF-1 signaling to control life span. The percentage of living animals is plotted against animal age. Life span values and statistics for single and double mutant animals are given in Supplementary Table 3.

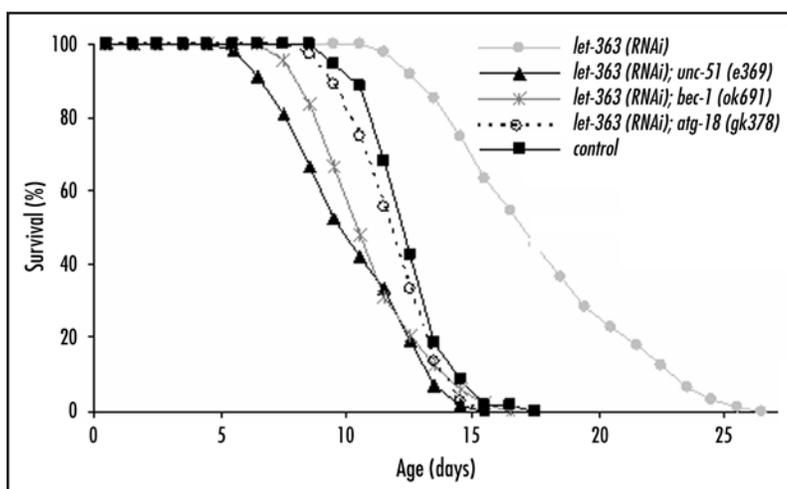


Figure 6. Autophagy genes are required for life-extension in TOR-deficient animals. Wild-type animals fed with bacteria expressing *let-363/Tor* double-stranded RNA live significantly longer than those fed with bacteria containing the empty RNAi vector (control). RNAi treatments were initiated from the L4 larval stage to avoid the animals to arrest development at the L3 larval stage (for details, see the text). For simplicity, life span curves of single autophagy deficient mutants treated with control RNAi (empty vector) are not shown. Data of single mutants are included into Table 3S. The percentage of living animals is plotted against animal age. Life span values and statistics for single and double mutant animals are given in Table 3S.

lived as long as when they were fed with control bacteria expressing the empty vector. Thus, the life-extending effects of *atp-3* and *clk-1* RNAi requires the activity of autophagy genes. These results suggest that mitochondrial dysfunction also shape nematode life span by interfering with the autophagy gene cascade.

Discussion

In this study we demonstrate that mutational inactivation of autophagy genes shortens life span in *C. elegans*, and that

Drosophila flies with compromised autophagy also die earlier than normal. We also present data suggesting that the rate at which tissue deterioration occurs is accelerated in autophagy deficient mutant nematodes, as compared with wild-type animals. Our major finding here is to show that autophagy genes are required for life-extension in various long-lived mutant strains, implying that the effects of mutations affecting distinct longevity pathways converge on common downstream processes that involve autophagy (Fig. 9). Increased longevity in nematodes with reduced mitochondrial activity, TOR signaling and inherent caloric restriction was completely suppressed by inhibiting autophagy genes (Figs. 5–9 and Table 3S); aberrant autophagy in animals defective for any of these longevity pathways conferred mean life spans that are comparable with that of the corresponding short-lived single autophagy mutants. Since the life-shortening effects of autophagy mutations on these long-lived mutants were more significant than their life-shortening effects on wild-type animals (Table 4S), we argue that autophagy genes specifically function in an anti-aging process instead of shortening life span because they are somehow toxic to the animal. Interestingly, inactivating autophagy by genetic null mutations only partially suppressed the long-lived phenotype of insulin/IGF-1 signaling deficient animals. This raises the possibility that another catabolic process may act in parallel to autophagy to mediate the effect of *daf-2* mutations on life span. Indeed, it has been recently demonstrated that a proteasomal system containing the Cullin-E3 ligase complex involved in the degradation of improperly folded and damaged proteins to maintain cellular homeostasis²⁴ is required for life-extension in *daf-2* mutants but not in other long-lived mutant animals (Fig. 4).⁵⁶ Alternatively, life span extension caused by aberrant DAF-2 activity is much greater than by mutations in other longevity pathways, raising the possibility that loss of autophagy is of sufficient magnitude to block weak extenders of life span but not more potent ones. Together, our results favor the view that autophagy might play a central role in animal aging.^{28,29}

How could autophagy genes mediate the effects of different longevity signals? Dietary restriction, decreased insulin/IGF-1 or TOR signaling, and lowered respiration are believed to increase life span, at least in part, by reducing the metabolic rate.¹⁶ Upregulation of autophagy in response to these longevity signals may supply the cells with energy under adverse conditions,^{24–26} maintaining critical levels of metabolism under which the cells would die. In addition, autophagy has been suggested to mediate the degradation of

oxidative damaged macromolecules and organelles, which are generated by reactive oxygen species produced during respiration.²² Thus, autophagic clearance and renewal of cytoplasmic materials is essential for the cells to survive. These cell protective functions of autophagy may be particularly important for long-lived cells such as those of the nervous system, which is known to act as a central regulatory tissue of the aging process.^{20,21,29}

Our data presented here suggest two important things. First, the effects of different longevity pathways may converge on autophagy

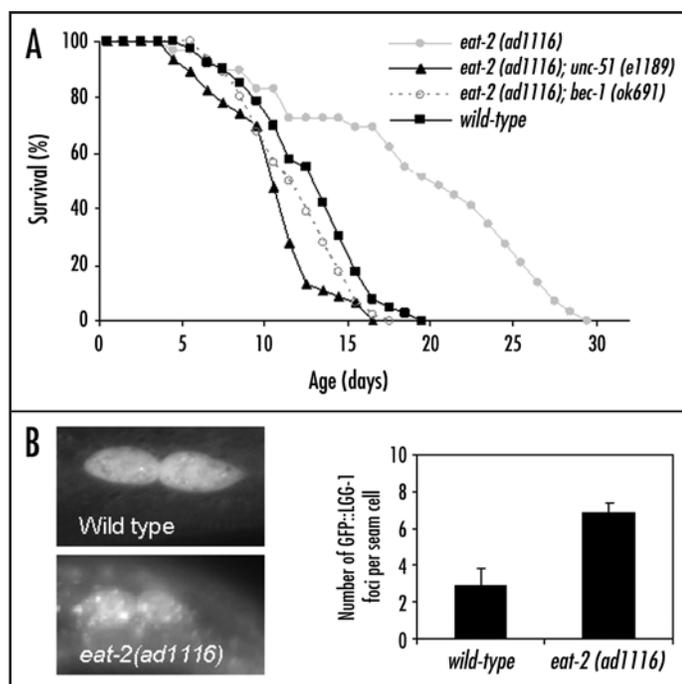


Figure 7. Mutations in autophagy genes suppress life span extension in *eat-2(ad1116)* mutant animals. (A) *eat-2(ad1116)* mutation, which confers dietary restriction by reducing the rate of pharyngeal pumping, extends life span in an otherwise wild-type background. Loss-of-function mutations in *unc-51* and *bec-1* block longevity in *eat-2(ad1116)* animals. These results suggest that in *C. elegans* autophagy genes play a pivotal role in longevity response to dietary restriction. The percentage of living animals is plotted against animal age. Life span values and statistics for single and double mutant animals are given in Table 3S. (B) Left show GFP-tagged LGG-1 accumulation in the lateral seam cells of wild-type and *eat-2(ad1116)* mutant animals. In *eat-2(ad1116)* animals, GFP::LGG-1 accumulates in punctuate areas that are supposed to indicate (pre)autophagosomal structures. Right shows the quantification of GFP::LGG-1 positive foci in individual seam cells of wild-type and *eat-2(ad1116)* animals.

genes to regulate aging. Thus, loss-of-function mutations decreasing insulin/IGF-1 and TOR signaling, mitochondrial respiration and food intake may lengthen life span by upregulating the autophagy gene cascade. Other aging-related factors identified so far, such as the NAD-dependent histone deacetylase Sir2,⁵⁷ proteins governing germ-line activity,⁵⁸ and molecular chaperons,⁵⁹ interact with (a) longevity pathway(s) and thus may also modulate autophagic activity to control the rate at which the tissues age. Second, our present results favor the assumption that the aging process is more specific than previously expected.²³ According to this view, the biology of reactive oxygen species produced by respiration might be the main determinant of animal life span. If autophagy operates downstream of different longevity pathways, they eventually may affect the rate of aging through regulating the efficiency of macromolecular, in particular protein, turnover. Indeed, recent experimental evidence suggested that inhibiting autophagy causes accumulation and subsequent aggregation of damaged intracellular proteins in various cell types, which eventually leads to massive cell loss and early death.^{20,21} Uncovering a central role of autophagy in life span control might also help to understand how metabolic diseases and overnutrition lead to premature aging and the risk of early death in humans.

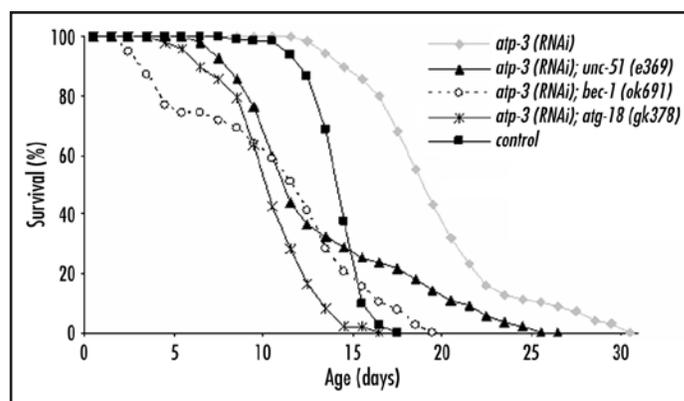


Figure 8. Suppression of the long-lived phenotype of *atp-3(RNAi)* animals by mutations in autophagy genes. Wild-type animals fed with bacteria expressing *atp-3* double-stranded RNA are long-lived. Life-extension in these animals is autophagy gene dependent. Wild-type animals fed with bacteria containing the empty RNAi vector were used as control. For simplicity, life span curves of single autophagy mutants are not shown. These data are included into Table 3S. The percentage of living animals is plotted against animal age. Life span values and statistics for single and double mutant animals are given in Table 3S.

To further support the role of autophagy in aging, one should enhance autophagic activity and test whether this treatment increases life span. However, both inhibition and overactivation of autophagy seem to induce cell loss, probably through interacting with the apoptotic and necrotic processes.^{29,30} Thus, fine-tuning of autophagy is critical for the eukaryotic cells to survive. This raises the need for genetically manipulated organisms with increased, but otherwise normally regulated levels of autophagy.

Materials and Methods

Strains and genetics. The following *C. elegans* strains were used in this study: wild-type N2 Bristol isolate, FR853 *bec-1(ok691)IV; swEx520[pbec-1::BEC-1::GFP + rol-6(su1006)]*, FR854 *bec-1(ok700)IV; swEx520[pbec-1::BEC-1::GFP + rol-6(su1006)]*, CB369 *unc-51(e369)V*, CB1189 *unc-51(e1189)V*, VC893 *atg-18(gk378)V*, BU071 *bulS1[GFP::plgg-1::LGG-1]*, BC13515 *sls13209[atg-18::gfp]*, NL2099 *rrf-3(pk1426)II, daf-2(e1370)III, CB4876 clk-1(2519)III*.

RNA interference. To generate *C. elegans* RNAi clones, 600–900 base pair-long cDNA fragments were amplified by RT-PCR, and cloned into pPD129.36. RNAi constructs were transformed into *Escherichia coli* HT115(DE3) used as food source. The following forward and reverse primers were used. For *lgg-1*: 5'-CAT GCC ATG GCA TGT GGG CTT ACA AGG AGG AGA AC-3' and 5'-CAT GCC ATG GCA TGT TCC CTT CTT TTC GAC CTC TCC-3'; for *daf-2*: 5'-TTG GAA GCT CTC GGA ACA ACC AC-3' and 5'-ATG AAC GAC GTT GAA GGA GAA GG-3'; for *T22H9.2/atg-9*: 5'-AGA ATG GCG GTT ATT TGT GC-3' and 5'-TGG TCA AGC TCG TTG AAG TG-3'; for *let-363/CeTor*: 5'-CAT GCC ATG GCA TGA ACA ATT GGC AAA TTT CGT G-3' and 5'-CAT GCC ATG GCA TGT GCA CGT AAC GAT GGA GAA C-3', and for *atp-3*: 5'-TAA TGG CGC AAC TCA TGA AA-3' and 5'-GCA AGG GCA TCC TTG TAT TT-3'. Experiments were performed at 25°C. For determining the life span of *lgg-1(RNAi)* and *T22H9.2(RNAi)* animals, RNAi-sensitive *rrf-3* mutant animals were treated with the

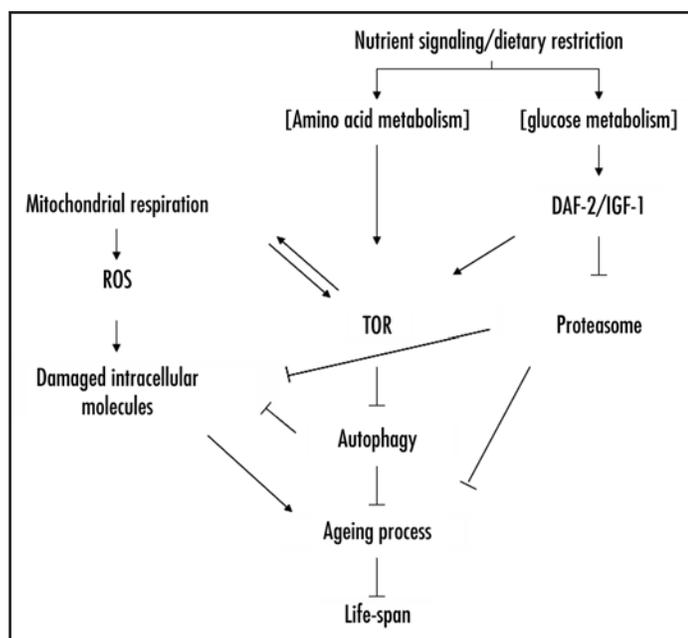


Figure 9. Working model for the regulation of *C. elegans* life span by autophagy. Longevity pathways, including the insulin/IGF-1-TOR signaling axis (nutrient signaling) and the mitochondrial respiratory chain components, converge on autophagy genes to extend nematode life span. Downstream of insulin/IGF-1 signaling, autophagy may act parallel to a proteasomal system to control aging. DAF-2, the worm insulin/IGF-1 receptor; TOR, the target of rapamycin kinase; ROS, reactive oxygen species. Arrows indicate activations, bars indicate negative regulatory interactions.

corresponding double-stranded RNA (*lgg-1* and *T22H9.2* RNAi treatments had only weak effects on life span in wild-type background). To create *Drosophila* RNAi constructs, 400–800 bp long cDNA fragments were cloned into the PCR cloning vector Topo-TA (Invitrogen), and then subcloned into pWizMod in inverted repeat arrangement. Transgenic flies were crossed with line hsGal4, and then synchronised animals were maintained at 24°C, and scored for survival. W1118 strain was used as wild-type.

Life span analysis. Nematode life span assays were carried out at 25°C. *daf-2* mutant animals were maintained at 20°C until the L4 larval stage, then transferred at 25°C and scored for mean life span. For synchronization, 20–30 gravid well-fed adults (P) were transferred to a new agar plate containing nematode growth medium (NGM) seeded with *E. coli* OP50 to lay eggs for 4–5 hours, and then removed. F1 young (not gravid) adults were transferred to NGM plates supplemented with 300 mg/ml FUDR (5-fluoro-2'-deoxyuridine) for 1 day ($t = 0$). Sterile F1 adults were then transferred to the final assay plates and scored. Animals were considered dead when they stopped pharyngeal pumping and responding to touching. SPSS 14 software was used to calculate mean life span and perform statistical analysis. *p* values for comparing Kaplan-Meier survival curves between two groups were determined using log-rank (Mantel-Cox) tests.

Electron microscopy. For fixation and embedding of transmission electron microscopic samples, the nematodes were treated individually. They were cut open under a dissecting microscope in a drop of fixative composed of 0.2% glutaraldehyde and 3.2% formaldehyde in 0.15 M cacodylate buffer. After an overnight fixation at 4°C, the fixative was changed to washing buffer (0.1 M

cacodylate buffer) and the samples were embedded in agar, post-fixed with 0.5% cacodylate-buffered OsO₄, stained with 2% uranyl acetate, dehydrated in ethanol and propylene oxide and embedded in Durcupan (Fluka). Thereafter the samples were cut along the longitudinal body axis with Reichert-Jung Ultracut-E type ultramicrotome, stained with lead citrate and examined in JEM100CX II electron microscope.⁶⁰

Age pigment measurement. Relative lipofuscin levels were measured by fluorescence spectroscopy (using Spex Fluoromax spectrofluorimeter, Edison, NJ, USA) as described,⁴⁵ with slight modification. Briefly, for one measurement 30 hand-picked animals of a given age were collected and washed with water to remove residual bacterial contamination. Nematodes were then sonicated (Branson Sonifier 250, output level 2, duty cycle 50%, Branson Ultrasonics Corp. Danbury, CT, USA) and incubated on ice three times for 30–30 seconds in 1 ml UP water. From this point, samples were kept on ice. Floating particles were removed by centrifugation (at 3000 rpm, for 0.5 min) and supernatants were measured. Tryptophan (used as control) fluorescence was measured at 290/330 nm (excitation/emission wavelength), while age pigment was detected at 340/430 nm.

Behavioral assay. Age-synchronized nematodes were distributed (5–10 nematode per plate) and scored every day for spontaneous movement during adulthood. We distinguished two classes of behavioral phenotypes: in one (wild-type) class, animals move constantly in a sinusoidal pattern, while in the other class animals either do not move or move hard, leaving non-sinusoidal tracks in the bacterial lawn through which they migrate. Animals that belong to the second class were considered as uncoordinated (paralyzed). All animals began adulthood in the first class. *unc-51* mutants were excluded from this study.

Acknowledgements

Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). We thank A. Fire for plasmid vectors. We are grateful for two anonymous reviewers for their valuable comments on the manuscript. We are also grateful to Sára Simon and Tünde Péntzes for excellent technical help, and to all members of our group for helpful comments on the manuscript. This work was supported by grants from Ministry of Health (167/2006), National Office for Research and Technology (NKFP 1A/007/2004) and the Hungarian Scientific Research Foundation (OTKA K68372) to T.V., and the Hungarian Scientific Research Foundation (OTKA T047241) to A.L.K. T.V. is a grantee of the János Bolyai scholarship.

Note Added in Revision

While this paper was under review, a related work on the role of autophagy in *Drosophila* aging was published online.³⁴

Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/TothAUTO4-3-Sup.pdf

References

1. Kenyon C. The plasticity of aging: insights from long-lived mutants. *Cell* 2005; 120:449–60.
2. Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. *Nature* 2000; 408:255–62.
3. Kenyon C. Environmental factors and gene activities that influence life span. In: Riddle DL, Meyer BJ, Priess JR, editors. *C. elegans* II. Cold Spring harbor Lab Press, Woodbury, NY. 1997; 791–814.

4. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R. A *C. elegans* mutant that lives twice as long as wild type. *Nature* 1993; 366:461-4.
5. Dorman JB, Albinder B, Shroyer T, Kenyon C. The *age-1* and *daf-2* genes function in a common pathway to control the life span of *Caenorhabditis elegans*. *Genetics* 1995; 141:1399-406.
6. Tatar M, Kopelman A, Epstein D, Tu MP, Yin CM, Garofalo MS. A mutant *Drosophila* insulin receptor homolog that extends life span and impairs neuroendocrine function. *Science* 2001; 292:107-10.
7. Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloën A, Even PC, Cervera P, Le Bouc Y. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* 2003; 421:182-7.
8. Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, Kamath RS, Ahringer J, Kenyon C. Rates of behavior and aging specified by mitochondrial function during development. *Science* 2002; 298:2398-401.
9. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat Genet* 2003; 33:40-8.
10. Vellai T, Takács-Vellai K, Zhang Y, Kovacs AL, Orosz L, Müller F. Influence of TOR kinase on lifespan in *C. elegans*. *Nature* 2003; 426:620.
11. Jia K, Chen D, Riddle DL. The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development* 2004; 131:3897-906.
12. Pan KZ, Palter JE, Rogers AN, Olsen A, Chen D, Lithgow GJ, Kapahi P. Inhibition of mRNA translation extends lifespan in *Caenorhabditis elegans*. *Aging Cell* 2007; 6:111-9.
13. Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, Kenyon C. Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*. *Aging Cell* 2007; 6:95-110.
14. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, Tokunaga C, Avruch J, Yonezawa K. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* 2002; 110:177-89.
15. Lakowski B, Hekimi S. The genetics of caloric restriction in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 1998; 95:13091-6.
16. Walker G, Houthoofd K, Vanfleteren JR, Gems D. Dietary restriction in *C. elegans*: from rate-of-living effects to nutrient sensing pathways. *Mech Aging Dev* 2005; 126:929-37.
17. Mörck C, Pilon M. *C. elegans* feeding defective mutants have shorter body lengths and increased autophagy. *BMC Dev Biol* 2006; 6:39.
18. Harman D. Free radicals in aging. *Mol Cell Biochem* 1988; 84:155-61.
19. Koubova J, Guarente L. How does caloric restriction work? *Genes Dev* 2003; 17:313-21.
20. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* 2006; 441:885-9.
21. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* 2006; 441:880-4.
22. Cuervo AM, Bergamini E, Brunk UT, Droge W, Ffrench M, Terman A. Autophagy and aging: the importance of maintaining "clean" cells. *Autophagy* 2005; 1:131-40.
23. Hekimi S, Guarente L. Genetics and the specificity of the aging process. *Science* 2003; 299:1351-4.
24. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000; 290:1717-21.
25. Klionsky DJ. The molecular machinery of autophagy: unanswered questions. *J Cell Sci* 2005; 118:7-18.
26. Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human. *Autophagy* 2007; 3:181-206.
27. Kovács AL, Pálfi Z, Réz G, Vellai T, Kovács J. Sequestration revisited. Integrating traditional electron microscopy, de novo assembly and new results. *Autophagy* 2007; 3:655-62.
28. Donati A. The involvement of macroautophagy in aging and anti-aging interventions. *Mol Aspects Med* 2006; 27:455-70.
29. Takács-Vellai K, Bayci A, Vellai T. Autophagy in neuronal cell loss: a road to death. *BioEssays* 2006; 28:1126-31.
30. Vellai T, Tóth ML, Kovács AL. Janus-faced autophagy. A dual role for cellular self-eating in neurodegeneration? *Autophagy* 2007; 3:461-3.
31. Meléndez A, Tálloczy Z, Seaman M, Eskelinen EL, Hall DH, Levine B. Autophagy genes are essential for dauer development and life span extension in *C. elegans*. *Science* 2003; 301:1387-91.
32. Hars ES, Qi H, Ryazanov AG, Jin S, Cai L, Hu C, Liu LF. Autophagy regulates aging in *C. elegans*. *Autophagy* 2007; 3:93-5.
33. Jia K, Levine B. Autophagy is required for dietary restriction-mediated life span extension in *C. elegans*. *Autophagy* 2007; 3: 597-9.
34. Simonsen A, Cumming RC, Brech A, Isakson P, Schubert DR, Finley KD. Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult *Drosophila*. *Autophagy* 2008; 4: in press.
35. Takács-Vellai K, Vellai T, Puoti A, Passannante M, Wicky C, Streit A, Kovács AL, Müller F. Inactivation of the autophagy gene *bec-1* triggers apoptotic cell death in *C. elegans*. *Curr Biol* 2005; 15:1513-7.
36. Tóth ML, Simon P, Kovács AL, Vellai T. Influence of autophagy genes on ion-channel-dependent neuronal degeneration in *Caenorhabditis elegans*. *J Cell Sci* 2007; 120:1134-41.
37. Ogura K, Wicky C, Magnenat L, Tobler H, Mori I, Müller F, Ohshima Y. *Caenorhabditis elegans unc-51* gene required for axonal elongation encodes a novel serine/threonine kinase. *Genes Dev* 1994; 8:2389-400.
38. Matsuura A, Tsukada M, Wada Y, Ohsumi Y. Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. *Gene* 1997; 192:245-50.
39. Krick R, Tolstrup J, Appelles A, Henke S, Thumm M. The relevance of the phosphatidylinositolphosphat-binding motif FRRGT of Atg18 and Atg21 for the Cvt pathway and autophagy. *FEBS Lett* 2006; 580:4632-8.
40. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, Ohsumi Y. A ubiquitin-like system mediates protein lipidation. *Nature* 2000; 408:488-92.
41. Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, Klionsky DJ. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. *J Cell Biol* 2000; 148:465-80.
42. Wolkow CA, Kimura KD, Lee MS, Ruvkun G. Regulation of *C. elegans* life span by insulin-like signaling in the nervous system. *Science* 2000; 290:147-50.
43. Apfeld J, Kenyon C. Cell non-autonomy of *C. elegans daf-2* function in the regulation of diapause and life span. *Cell* 1998; 95:199-210.
44. Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, Kenyon C. Genetic analysis of tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation. *Genetics* 2002; 161:1101-12.
45. Gerstbrein B, Stamatias G, Kollias N, Driscoll M. In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in *Caenorhabditis elegans*. *Aging Cell* 2005; 4:127-37.
46. Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, Paupard MC, Hall DH, Driscoll M. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature* 2002; 419:808-14.
47. Schlumpberger M, Schaeffeler E, Straub M, Bredschneider M, Wolf DH, Thumm M. AUT1, a gene essential for autophagocytosis in the yeast *Saccharomyces cerevisiae*. *J Bacteriol* 1997; 179:1068-76.
48. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol* 2007; 9:218-24.
49. Apfeld J, O'Connor G, McDonagh T, DiStefano PS, Curtis R. The AMP-activated protein kinase AAK-2 links energy levels and insulin-like signals to lifespan in *C. elegans*. *Genes Dev* 2004; 18:3004-9.
50. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of life span in *Drosophila* by modulation of genes in the TOR signaling pathway. *Curr Biol* 2004; 14:885-90.
51. Kaeberlein M, Powers RW, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* 2005; 310:1193-6.
52. Inoki K, Li Y, Zhu T, Wu J, Guan K-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signaling. *Nat Cell Biol* 2002; 4:648-57.
53. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. *Curr Opin Cell Biol* 2005; 17:596-603.
54. Roggo L, Bernard V, Kovacs AL, Rose AM, Savoy F, Zetka M, Wymann MP, Müller F. Membrane transport in *Caenorhabditis elegans*: an essential role for VPS34 at the nuclear membrane. *EMBO J* 2002; 21:1673-83.
55. Schieke SM, Phillips D, McCoy JP, Aponte AM, Shen RF, et al. (2006) The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 281: 27643-52.
56. Ghazi A, Henis-Korenblit S, Kenyon C. Regulation of *Caenorhabditis elegans* life span by a proteasomal E3 ligase complex. *Proc Natl Acad Sci USA* 2007; 104:5947-52.
57. Imai S-I, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 2000; 403:795-800.
58. Hsin H, Kenyon C. Signals from the reproductive system regulate the life span of *C. elegans*. *Nature* 1999; 399:362-6.
59. Solti C, Sreedhar AS, Csermely P. Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. *Aging Cell* 2003; 2:39-45.
60. Kovács AL, Vellai T, Müller F. Autophagy in *Caenorhabditis elegans*. In: *Autophagy*, ed. Daniel J. Klionsky, Landes Bioscience, Georgetown, Texas, USA, pp. 219-225, 2004.