Roles of autophagy genes in the regulation of neurodegeneration, cell size and aging in *Caenorhabditis elegans*

PhD Thesis

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Introduction and aims

Autophagy is one of the major catabolic processes of eukaryotic cells. Autophagy involves the formation of membrane structures termed autophagic vacuoles to sequester cytoplasmic cargo for breakdown by acidic lysosomal hydrolases. In mammals, autophagy plays a critical role in embryogenesis, maturation of red blood cells and the function of immune system. Impaired autophagy results in tumor development, neurodegenerative disease, cardiovascular disease and overwhelming infection by intracellular parasites. Molecular characterisation of autophagy is far from complete and its role has not been examined from many physiological and pathological aspects. Necrotic cell death stimulated by excitotoxicity, regulation of cell size or aging are such aspects. I used the small, transparent and short-lived roundworm *Caenorhabditis elegans* to study the role of autophagy in these pathophysiological processes.

Role of autophagy genes is neurodegeneration

Necrotic cell death is a common feature in numerous human neurodegenerative disorders. In the nematode *Caenorhabditis elegans*, gain-of-function mutations in genes that encode specific ion channel subunits such as the degenerins DEG-1 and MEC-4, and the acetylcholine receptor subunit DEG-3 lead to excitotoxic-like degeneration of a subset of neurons. Recent results indicate a role for starvation in excitotoxic-like neurodegenerative models in *C. elegans*. Under conditions of limited food supply, activity of aspartyl proteases drops, insulin/IGF-1 signaling is inhibited and autophagy is stimulated at the cellular level. Neuronal cell death caused by ion channel hyperactivity in *C. elegans* is preceded by intense degradation of cytoplasmic contents, membrane infolding, and formation of vacuoles and electron-dense whorls. These results strongly suggest that autophagy has a role in excitotoxic like neurodegeneration. Thus, I examined how mutational or RNA interference-mediated inactivation of certain autophagy genes affect ion channel-dependent, or 6-hydroxydopamine induced neuronal degeneration in *C. elegans*.

Role of autophagy genes in cell size regulation

In multicellular organisms, cell growth is intimately linked to nutrient availability and growth factor signaling including insulin/IGF-1, TGF-β and TOR, as well as requires a well-controlled balance in macromolecule and organelle turnover (i.e., between synthesis
and degradation). Despite its developmental and medical significance, little is known about cellular pathways that mediate the growth of cells. A large body of evidence demonstrates a role for autophagy - a mechanism of eukaryotic cells to digest their own constituents during development or starvation - in cell size determination. Increasing autophagic activity by prolonged starvation, rapamycin treatment inhibiting TOR (target of rapamycin) signaling, or genetic intervention, causes cellular atrophy in worms, flies and mammalian cell cultures. Therefore in my experiment I investigated how inactivation of certain autophagy genes influences cell size regulation.

**Role of autophagy genes in the regulation of the aging process**

Aging is a multifactorial process with many mechanisms contributing to the decline. During aging damaged macromolecules and organelles are persistently accumulated in the cell. Autophagy is one of the cellular pathways used by eukaryotic cells to degrade aberrant, damaged cytosolic components and the only one capable of degrading membrane covered organelles. Defects in autophagy have been associated with age-related diseases. RNA interference- (RNAi) mediated downregulation of certain autophagy genes has already been implicated autophagy in the survival of *C. elegans*. Therefore I investigated how mutational inactivation of autophagy genes influences lifespan and the aging process in this organism.

Regulation of the aging process has been extensively studied in *Caenorhabditis elegans* with almost 300 life-extension mutations known so far. Mutant nematodes with inherent dietary restriction, aberrant insulin/IGF-1 or TOR signaling and lowered mitochondrial respiration display extended lifespan. Interaction of these genes has been poorly studied. Therefore I investigated the inactivation between autophagy genes and various longevity pathways.
Materials and Methods

Cell death analysis

The number of vacuolated cells in synchronized, well fed degenerin mutant animals was counted at specific developmental stages by Nomarski microscopy, at 20°C. Treatment of nematodes with 6-OHDA: nematodes were exposed in liquid suspension to 50 mM 6-OHDA in 10 mM ascorbic acid solution for 1 hour, and then transferred to plates lacking the toxin. pdat-1::GFP expression was analyzed the following day at 25°C. The percentage of nematodes with intact head dopaminergic neurons was determined. Control animals were treated with 10 mM ascorbic acid.

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 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) 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 to perform statistical analysis. p values for comparing Kaplan-Meyer survival curves between two groups were determined using log-rank (Mantel-Cox) tests.

Age pigment measurement

Relative lipofuscin levels were measured by fluorescence spectroscopy (using Spex Fluoromax fluorescence spectrometer, Edison, NJ, USA). For each 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, 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 belonged 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.

**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, postfixed 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 a Reichert-Jung Ultracut-E type ultramicrotome, stained with lead citrate and examined using a JEM100CX II electron microscope.

**Results**

Herein I summarize results of my PhD research:

Examination of C. elegans autophagy genes:

- *unc-51/ATG1, bec-1/ATG6, lgg-1/ATG8* and *atg-18/ATG18* C. elegans orthologs of yeast autophagy genes were expressed in neurons. Except *unc-51/ATG1* each genes also expressed in the hypodermis, muscle and in the gut.
- Autophagosome formation was compromised in *bec-1(ok691)* and *unc-51(e369)* mutant animals.

Role of autophagy genes in excitotoxic neurodegeneration:

- Cell death induced by *mec-4(u231)* gain-of-function mutation was age and temperature-dependent.
• **unc-51(e369)** and **bec-1(ok691)** loss-of-function mutations and **lgg-1** RNAi treatment reduced neuronal demise in **mec-4(u231)**, **deg-1(u506)** and **deg-3(u662)** gain-of-function mutant animals.

• **unc-51(e369)** loss-of-function mutation and **bec-1** RNAi treatment suppressed 6-OHDA induced neurodegeneration.

• **unc-51(e369)** and **bec-1(ok691)** loss-of-function mutations and **lgg-1** RNAi treatment did not influence expression pattern and level of the **mec-4** degenerin gene.

• **unc-51(e369)** and **bec-1(ok700)** loss-of-function mutations suppress touch insensitivity in **mec-4(u231)** and **deg-1(u506)** gain-of-function degenerin mutant animals.

• Starvation and RNAi silencing of **let-363/TOR2** reduced the survival of neurons in **mec-4(u231)** gain-of-function mutant background.

Role of autophagy genes in the regulation of cell size determination:

• Loss-of-function mutations of **unc-51** and **bec-1** autophagy genes reduced body length in **C. elegans**.

• **unc-51(e369)** and **bec-1(ok691)** loss-of-function mutations reduced body volume in **C. elegans**.

• **unc-51(e369)** loss-of-function mutation reduced gut cell size in **C. elegans**.

• **unc-51** and **bec-1** mutant animals had wild-type cell numbers.

• Sma (small) phenotype of loss-of-function mutations of autophagy genes **unc-51** and **bec-1** were epistatic over the Lon (Long) phenotype of loss-of-function mutations of **daf-2** (insulin/IGF-1 pathway)

• Sma (small) phenotype of loss-of-function mutations of autophagy genes **unc-51** and **bec-1** were epistatic over the Lon (Long) phenotype of loss-of-function mutations of **lon-1** and **lon-2** (TGF-β pathway).

• Sma (small) phenotype of loss-of-function mutations of autophagy genes **unc-51** and **bec-1** were epistatic over the Lon (Long) phenotype of overexpression of **dbl-1** (TGF-β pathway).

Role of autophagy genes in the regulation of aging:

• Mutational inactivation of autophagy genes **unc-51**, **bec-1** and **atg-18** shortened lifespan in **C. elegans**.
• **unc-51(e369)** and **bec-1(ok691)** loss-of-function mutations accelerated AGE pigment accumulation in *C. elegans*.

• Age associated locomotory defects occurred with earlier times of onset in **bec-1(ok691)** and **atg-18(gk378)** mutant animals than in wild-type animals.

• Short lifespan phenotype of loss-of–function mutations of autophagy genes **unc-51** and **bec-1** were epistatic over the long-lived phenotype of loss-of–function mutation of **daf-2** (insulin/IGF-1 pathway).

• Short lifespan phenotype of loss-of–function mutations of autophagy genes **unc-51**, **bec-1** and **atg-18** was epistatic over the long-lived phenotype of **let-363 RNAi** treatment.

• Short lifespan phenotype of loss-of–function mutations of autophagy genes **unc-51**, **bec-1** and **atg-18** was epistatic over the long-lived phenotype of loss-of–function mutation of **eat-2** (caloric restriction).

• Short lifespan phenotype of loss-of–function mutations of autophagy genes **unc-51**, **bec-1** and **atg-18** was epistatic over the long-lived phenotype of **atp-3 RNAi** treatment.

### Conclusions

Each autophagy genes we examined was expressed in tissues playing profound role in the regulation of neurodegeneration, regulation of cell size and aging. Effect of the inactivation of **unc-51/ATG1** and **bec-1/ATG6**, the *C. elegans* orthologs of yeast autophagy genes, have never been studied in the function of autophagy itself. Presence of abnormal autophagic vacuoles in **unc-51(e369)** and **bec-1(ok691)** mutant animals indicate that BEC-1 and UNC-51 mediate normal autophagosome formation.

Inactivation of **unc-51/ATG1**, **bec-1/ATG6** and **lgg-1/ATG8** *C. elegans* autophagy genes partially suppresses degeneration of neurons with toxic ion channel variants. Whether autophagy genes interact with the necrosis process itself or alternatively may affect the activity, or presence of ion channels needs clarification. Autophagy genes did not influence expression pattern and level of the **mec-4** degenerin gene. Disruption of GFP expression in the head dopamine neurons by 6-OHDA was significantly suppressed in **unc-51(e369)** mutant and **bec-1(RNAi)** animals. This implies that suppression of necrotic death by deregulation of autophagy genes was not merely a consequence of a reduction in the quantity of toxic degenerin proteins. BEC-1 and UNC-51 deficiency restored
mechanosensitivity in degenerin mutants. I find this result very important as any therapy based on the modification of autophagic activity is expected to maintain the function of neurons. I also demonstrated that the TOR-kinase mediated signaling pathway, a nutrient sensing system that downregulates the autophagy gene cascade, protected neurons from undergoing necrotic cell death, whereas nutrient deprivation promoted necrosis. Together, these data imply that function of autophagy genes contributes to neurodegenerative cell death.

Wild-type *C. elegans* strains display a characteristic body length of 1.2 mm. I examined *unc-51* and *bec-1* loss-of-function mutant nematodes and found that they showed a marked shortening in mean body size. I also monitored body volume, which was also markedly reduced as compared to wild-type. The mean longitudinal diameter of gut cells in *unc-51* mutant animals also showed reduction. *unc-51* and *bec-1* mutant animals had wild-type cell numbers as revealed by counting different cell types expressing reporters labeled with green fluorescent protein. Together my data indicated that the reduced body size of *unc-51* mutants was due to a decrease in cell size. Thus, autophagy genes, or at least some of them, are required for normal cell growth. I found that *daf-2(e1370), lon-1(e185)*, and *lon-2(e678)* mutants as well as DBL-1-overexpressing nematodes, which as single mutant animals are each long displayed small or wild-type body size when they also carried a loss-of-function mutation in *unc-51* or *bec-1*. In other words, *unc-51* and *bec-1* are epistatic to *daf-2, dbl-1, lon-1* and *lon-2* to influence body size. In summary my data indicate that the insulin/IGF-1 and TGF-b signaling pathways may interact with the UNC-51 and BEC-1 autophagy genes to control cell size in *C. elegans*.

RNA interference- (RNAi) mediated downregulation of certain autophagy genes has already implicated the autophagic process in the survival of *C. elegans*. Depleting *BEC-1/Atg6* suppressed the long-lived phenotype of *daf-2* mutants in *C. elegans*. 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. In my study I demonstrated that mutational inactivation of autophagy genes shortens life span in *C. elegans*. 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. I found that *bec-1, unc-51* and *atg-18* mutants accumulated lipofuscin more rapidly during the course of life than wild-type animals. Autophagy gene mutant animals became paralyzed at earlier times of onset than the wild type. Together these data suggest that the
rate at which tissue deterioration occurs is accelerated in autophagy deficient mutant nematodes, as compared with wild-type animals. My major finding here is to show that autophagy genes are required for life-extension in various long-lived mutant strains. Increased longevity in nematodes with reduced mitochondrial activity, TOR signaling and inherent caloric restriction was completely suppressed by inhibiting autophagy genes. While 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. Together, our results favor the view that autophagy might play a central role in animal aging.

Autophagy is the only intracellular process that can degrade damaged macromolecules or organelles even in bulk quantity. Autophagy is therefore unique, not replaceable by any other intracellular processes. In accordance with this my results show that inactivation of autophagy genes have widespread effects on cell function. Autophagy thence also seems to be a common effector mechanism of several conserved signaling pathways from among which I have shown insulin/IGF-1, TGF-β and TOR signaling.
Publications related to PhD thesis
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