University of Nevada, Reno

The Role of Autophagy in ADL-Mediated Responses in *C. elegans*

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of the requirements for the degree of

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by

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Abstract

Autophagy is a ubiquitous cellular degradative process crucial in times of stress and starvation. Although much is known about the mechanisms of autophagy, not much is known about how the process of autophagy is coordinated between cells and tissues in multicellular organisms. The nematode *Caenorhabditis elegans* is an excellent multicellular model organism to study how autophagy is regulated and coordinated in response to environmental conditions. Recent work in the lab has identified a chemoreceptor gene, *srh-234*, of which the expression in a single neuron (called ADL) is reduced in starved animals. Starvation is known to induce autophagy in *C. elegans*, and our preliminary findings suggest that mutants defective in autophagy reduce *srh-234* expression, similarly as during starvation. Using these findings, we decided to investigate whether autophagy regulates this *srh-234* chemoreceptor in the ADL neuron in a cell-autonomous (ADL) or non-cell-autonomous manner (in a remote tissue, such as the intestine). We used a tissue-specific and RNAi enhanced method to knockdown certain autophagy genes in either the intestine or the ADL neuron, and examined *srh-234* expression levels in ADL neurons. Our findings indicate that autophagy genes selectively knocked down in the intestine do not appear to alter *srh-234* expression in ADL neurons, while knockdown of autophagy genes in ADL itself (i.e. *atg-7, bec-1,* and *daf-15)* results in a significant down regulation of *srh-234* expression. Thus, loss of autophagy reduces the expression of *srh-234* in a cell-autonomous manner, suggesting that the regulation of *srh-234* and therefore ADL-mediated responses is likely not dependent on other tissues.

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Introduction

Autophagy and its relationship to disease

Autophagy is a self-degradative process crucial for balancing energy sources in time of extracellular and intracellular stress from nutrient deficiency and starvation. This mechanism uses autophagosomal-lysosomal pathways to sequester and recycle various misfolded or aggregated cytoplasmic proteins and damaged organelles. By doing so, cell survival is possible under starvation conditions. In addition to cellular survival, this survival mechanism can promote lifespan extension, and development. Interestingly, studying autophagy can help us better understand many neurodegenerative diseases, and aging (Mijaljica, Prescott, & Devenish, 2010). For instance, Alzheimer's disease accumulate amyloid-beta's and this accumulation is dependent on autophagy. Besides neurodegenerative diseases, autophagy is also related to cancer in which autophagy can act as a tumor suppressor by preventing an accumulation of damaged cells and proteins. However, as a mechanism of cell survival, it can also promote the growth of established tumors (Yang, Chee, Huang, & Sinicrope, 2011). Thus, understanding the mechanisms underlying autophagy is important for understanding multiple diseases. Moreover, it is unclear how the process of autophagy is coordinated across multiple cells/tissues in a multicellular organisms. In this thesis, I will use the nematode *Caenorhabditis elegans* (*C. elegans*) as a multicellular model organism to study how autophagy induced in a remote tissue (the intestine) may affect responses in a single sensory neuron type (called ADL), which could provide insight into how autophagy is coordinated between different tissues and cells in a multicellular organism.

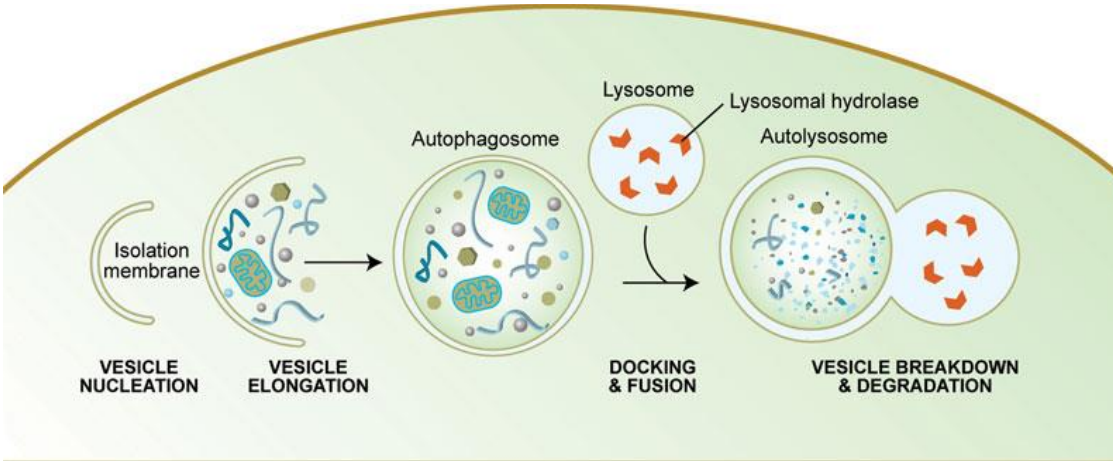

Figure 1. Steps of autophagy. The process begins with the formation of an isolation membrane. The vesicle grows, forming an autophagosome and eventually surrounds the organelles and cytoplasm around it. Lysosome or endosomes then fuse with the autophagosome turning it into an autophagolysosome. Finally, the sequestered material is degraded in and the vesicle is broken down and degraded. (Meléndez & Levine, 2009).

C. elegans as a model organism to study the autophagy process

Autophagy occurs in all organisms, including *C. elegans*. *C. elegans* are microscopic roundworms (nematodes) that are considered to be excellent model organisms. They share many commonalities with other organisms and are low-maintenance. It has a short life cycle with developmental stages, small genome size for eukaryotic organism, and has digestive, nervous, and muscular system. Due to the short life span, this increases the speed of experiments as well as progress. The transparent body makes it exceptional for studying cellular differentiation, staining in various cells and organs for observation, and also autophagy (Riddle, Blumenthal, & Meyer, 1997). *C. elegans* are also non-parasitic and only feeds on bacteria such as *Escherichia coli* (*E. coli*). They also have a sequenced genome which is advantageous to studying how genes

work together for growth, development, and mechanisms of the organism. Overall, *C. elegans* are simple and cost-efficient to study. Autophagy in *C. elegans* is essential for "lifespan extension, reproductive development, programmed cell death, necrotic cell death, neurodegenerative diseases, cell size regulation, neurotransmitter receptor trafficking, and dauer development" (Dwivedi, Sung, Shen, Park, & Lee, 2011). Moreover, recent studies have shown that *C. elegans* provide direct genetic evidence with humans that autophagic machinery protects against neurodegenerative and muscular diseases caused by aggregated-prone proteins (Meléndez & Levine, 2009).

Starvation induces autophagy and dauer diapause

Like in other animals, when *C. elegans* L1-larvae face harsh environmental conditions with a scarce food supply or an area of over population, they will enter diapause at the L3-larval stage and halt their development (also known as dauers) (Cassada & Russell, 1975). Even though they are arrested in development, they will continue to seek for food and favorable conditions. However, even when food source is located, they will not resume development if the population density is too high (Wolkow & Hall, 2015). A study has shown that reducing the activity of *daf-2* which encodes insulin/IGF-1 receptor causes an animal to enter dauer development even in favorable conditions. When autophagy activity of *daf-2* mutants are simultaneously depleted, a defect in dauer formation occurs and the animal die within a few days (Kovacs & Zhang, 2010). Thus, autophagy plays a significant role in the ability to survive during starvation conditions in *C. elegans.*

Autophagy in cell death as a survival mechanism

Aside from environmental conditions such as starvation, internal factors are also issues important to autophagy. Cell death is essential in development and homeostasis. It removes damaged or old cells either by programmed cell death (apoptosis) or by necrosis. Even though the relationship between apoptosis and autophagy is not fully understood, *C. elegans* are excellent to study this relationship. If the mechanism for apoptosis is impaired, autophagy induces physiological germ cell death (Jenzer, Simionato, & Legouis, 2015). For this reason, autophagy is classified as a type II programmed cell death. Autophagy also contributes to cellular destruction during necrosis by up-regulating in the early phases. It synergizes with lysosomal pathways in order to remove the unwanted cells (Samara, Syntichaki, & Tavernarakis, 2008). Thus, aside from the removal of undesirable cells, pathways in an animal is essential to survival.

The relationship between insulin/TOR signaling and autophagy

The role of autophagy in lifespan extension can be seen by mutations in the insulin signaling pathway as well as through dietary restrictions. Autophagy works with DAF-16 and FoxO, which are transcription factors that regulate insulin signaling and alter key tissues to extend lifespan (Alic, et al., 2014). However, autophagy may not be sufficient enough by itself to extend lifespan. Autophagy provides the raw material for new macromolecular synthesis while DAF-16/FoxO program cells to recycle the material into life-extending proteins (Hansen, et al., 2008). In addition to insulin signaling, another pathway is important for autophagy. The TOR pathway is a major control point that is downstream of growth factor receptor signaling, ATP levels, and insulin signaling

(Glick, Barth, & Macleod, 2010) (see Figure 2). Two main functions of the TOR signaling pathway is protein synthesis and autophagy. Translation initiation factor 4E-binding protein (4E-BP) and ribosomal-protein S6 kinase (S6K) are components of the translation machinery that stimulates protein synthesis (Hansen, et al., 2008). By inhibiting protein synthesis, extension in lifespan occurs. Potential longevity of the organism could be due to the dietary restriction from autophagy. Thus, TOR inhibition

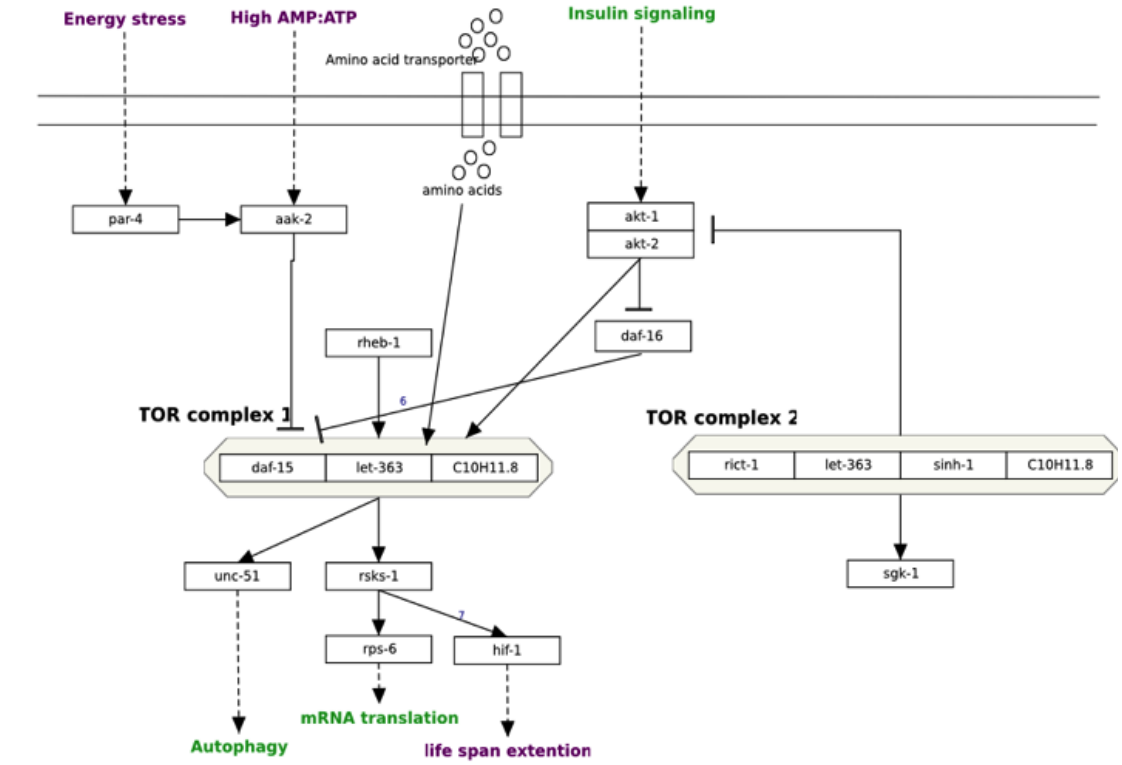

Figure 2. TOR signaling pathway. TOR interacts with *daf-15* and *let-363* shifts metabolism to accumulate fat and extends lifespan. As described earlier, DAF-16 regulates *daf-15* to extend the lifespan. The TOR complex plays a role in autophagy with its down regulation of *let-363* and *unc-51*. (Giesbertz, Pico, Digles, & Hanspers, 2013).

elicits autophagy and down-regulating TOR signaling appears to extend lifespan. Thus, like insulin signaling, the TOR pathway is important in lifespan regulation, because it correlates with autophagy in times of food limitation.

Starvation-dependent regulation of an ADL-expressed chemoreceptor

The lab previously showed that the candidate chemoreceptor, *srh-234*, genes is specifically expressed in a single sensory neuron, called the ADL neuron (Gruner, et al., 2014). The ADL neuron is considered to be a general sensor neuron for sensing food conditions, and is likely capable of different behavioral tasks. It helps with detection of chemicals for avoidance, social feeding behavior with population density, and various other functions. The lab previously showed that sensory inputs from the presence of food and starvation rapidly alters the expression of *srh-234* (Gruner, et al., 2014). Since the expression of this *srh-234* chemoreceptor is altered by starvation, and since starvation induces autophagy, we decided to explore whether changes in the autophagy process changes the expression of *srh-234*. Preliminary results suggest that mutations in the autophagy gene, *unc-51,* reduces the expression of *srh-234* in ADL neurons, similar as during starvation conditions. Thus, the autophagy process regulates the expression of the ADL-expressed *srh-234* chemoreceptor, but the mechanisms are unclear.

RNAi, "revolutionary development" and SID-1 Method

Mutations in many autophagy genes are lethal. For instance, mutations in the *let-363* gene encoding for the TOR kinase, causes early larval lethality. To circumvent this lethality, RNA interference (RNAi) can be used to knock down specific genes in specific tissues. RNAi is a biological process that has extensive potential in gene suppression. By potentially silencing mRNA, it can inhibit gene expression temporarily. Lowering gene expression is also called "knocking down." In *C. elegans*, RNAi can be induced *in vivo* by introducing double-stranded RNA (dsRNA), usually by expressing dsRNA against specific genes in *E. coli* culture, which then can spread throughout the body of the worm as well as transmitted to its progeny. The SID-1 is a transmembrane protein required for systemic RNAi in *C. elegans*, it also transports dsRNA in and out of cells (Hunter, et al., 2006). It enhances the uptake of siRNA which increase siRNA-mediated gene silencing efficacy (Duxbury, Ashley, & Whang, 2005). SID-1 method is a genetic tool to knock down RNAi against specific genes in specific tissues, and provides an excellent method to knock down target genes in specific tissues or cells for our study. Experiments with RNAi are typically sensitive to various conditions. The health of *C. elegans*, freshness of bacterial culture with RNAi constructs and IPTG in LB plates, temperature for maintaining specimen, and the developmental stage of worms should all be handled with care. Any inconsistencies or carelessness could affect results significantly.

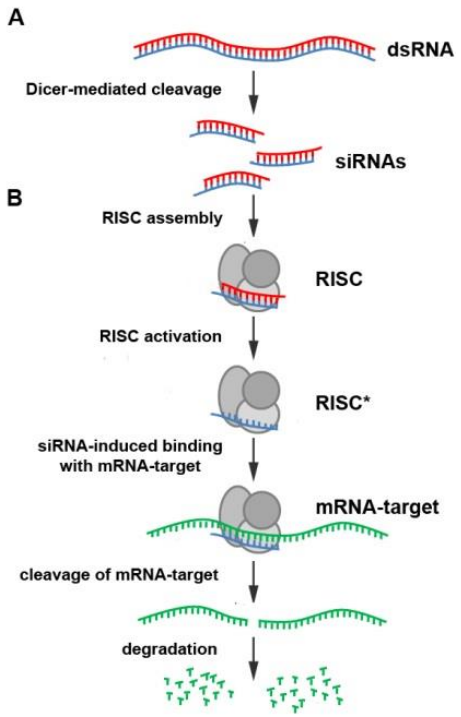

Figure 3. Steps of RNAi. The Dicer enzyme cleaves a portion off the end of the dsRNA. The RNAi induced silence complex (RISC) is a multi-protein that incorporate siRNA to be used as a template for mRNA. Since a portion of RNA is cleaved, mRNA cannot function properly and degrades the RNA (Petrova, Zenkova, & Chernolovskaya, 2013).

Hypothesis of Thesis

The goal is to investigate whether loss of autophagy in a remote tissue such as the intestine can alter the responses of a chemoreceptor gene, *srh-234*, in a single ADL sensory neuron using the *C. elegans* model system. This may provide insight into how autophagy is coordinated between different tissues and cells. To do so, certain autophagy genes will be knocked down by tissue-specific RNAi feeding, either in the intestine or ADL neurons, and subsequently I will examine the *srh-234p::*GFP expression in ADL neurons. We hypothesize if autophagy is systemic or coordinated between different

tissues, a knock down of autophagy genes specifically in the intestine would alter responses (*srh-234* expression) in the ADL neuron.

Methods

C. elegans strains and maintenance

We used the wild-type variant N2 Bristol *Caenorhabditis elegans* strain. To enhance RNAi in specific tissues, we generated two strains. One strain used is VDL254 (*sid-1(pk3321) him-5(e1490) V; oyIs56 III; Ex[ges-1p::sid-1::SL2::mCherry (pMG66) + rol-6] #1*) which targets the cells in the intestine *(*aka *intestine::sid-1*) The other strain is VDL255 (*sid-1(pk3321) him-5(e1490) V; oyIs56 III; Ex[sre-1p::sid-1::SL2::mCherry (pMG57) + rol-6] #3*) which targets the ADL neuron (aka *ADL::sid-1)*. The worms were grown and maintained at 20°C.

RNAi feeding of autophagy genes

To enhance RNAi in specific tissues, we fed transgenic animals carrying either *ADL::sid-1* or *intestine::sid-1* to bacterial RNAi clones directed against different autophagy genes. To induce RNAi, I used nematode growth media (NGM) plates containing Isopropyl β-D-1-thiogalactopyranoside (IPTG) as well as ampicillin. Experiment procedures are similar to previously described (Timmons, 2000). Briefly, five L3-L4 staged hermaphrodites of each transgenic line (*ADL::sid-1* or *intestine::sid-1*) were placed on each plate. After reaching adulthood, animals were transferred to another plate with the same RNAi food and allowed to lay eggs. Of note, *C. elegans* are

hermaphrodites and can self-fertilize. Once the progeny reached the young adult stage, these animals were analyzed for expression of *srh-234p::GFP*.

All RNAi bacterial clones were grown overnight from the RNAi library from Biosciences on Luria Broth (LB) plates with tetracycline and ampicillin as antibiotics. A single colony from the bacterial culture was then grown between 4-6 hours and removed from incubation during the exponential growth stage of *E. coli*. 50µL of the diluted RNAi food was placed on LB plates to grow for another 8-18 hours at room temperature. We used the L4440 empty vector and *hlh-3* as negative and positive controls, respectively, for the *ADL::sid-1* experiments. Each strain was send to the Nevada Genomic Center for nucleotide sequencing to confirm that the RNAi clone contains the expected gene. The following is the list of RNAi clones used in this study: *atg-7, atg-9, bec-1, daf-15, hlh-3, let-363, lgg-1, sqst-1,* and *unc-51.*

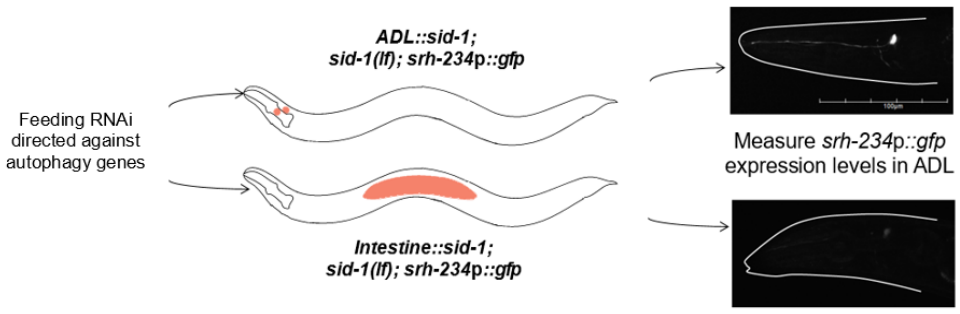

Figure 4. RNAi in targeted tissues. *C. elegans* are fed RNAi that are directed against autophagy genes, in order to knock down autophagy genes specifically in the ADL neuron and in the intestine, but not in any other tissues. *srh-234*p::GFP expression levels are then measured to see if specific autophagy genes alter ADL-mediated responses (Gruner, et al., 2014).

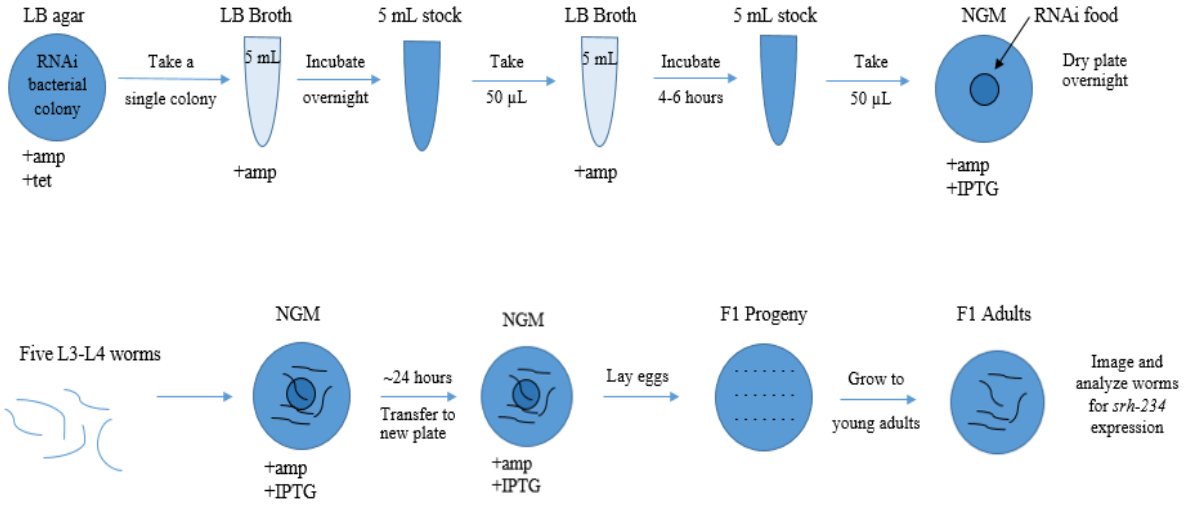

Figure 5. The RNAi feeding protocol: RNAi bacterial clones are extracted from the RNAi library. Single colonies were transferred to LB to grow overnight, 50µL is used to incubate in new broth for bacteria to reach exponential growth to seed NGM plates. Five L3-L4 animals are transferred to the plate to grow until adults. Next, adults are transferred to new plates and grown until the young adult stage, followed by *srh-234p*::GFP expression analysis.

Analysis of *srh-234* expression levels after RNAi feeding

After RNAi feeding of autophagy genes, animals were placed on microscope slides with 0.1% agarose and sodium azide (NaN_3) in order to paralyze animals for imaging. Expression levels of *srh-234* in ADL neurons were measured using Volocity software to analyze the fluorescent levels emanating from the *srh-234p*::GFP reporter in each animal. The exposure level was set to 2 m/s to properly display the green fluorescence emanating from the reporter in the ADL neurons, and we used the same exposure time in all images. Arbitrary units were used from the pixel intensity of fluorescence levels in images and statistical analysis of the mean fluorescence of *srh-234p*::GFP was used. The average mean is compared to the RNAi control, L4440, with the use of Mann-Whitney non-parametric test to check for statistical significance between the different RNAi clones.

Results and Discussion

Autophagy is a crucial process for the survival of many organisms, including *C. elegans*. In a multicellular organism, autophagy needs to be precisely controlled in order to coordinate an appropriate survival response (Glick, Barth, & Macleod, 2010). To study how autophagy is coordinated in a multicellular organism, we used *C. elegans* as a model system to study how loss of autophagy (by knocking down autophagy genes with RNAi) in the intestine can alter the expression in remote ADL neuron using the *srh-234p::*GFP expression reporter as a read out. We hypothesized that loss of autophagy in the intestine alters the expression of *srh-234p::*GFP expression levels in the ADL neuron. In order to test this hypothesis, we used RNAi to knock down genes involved in the autophagy process in animals carrying either the *ADL::sid-1* or *intestine::sid-1*, and subsequently measured the expression levels of the ADL-expressed chemoreporter gene, *srh-234.* However, our findings differed from our hypothesis.

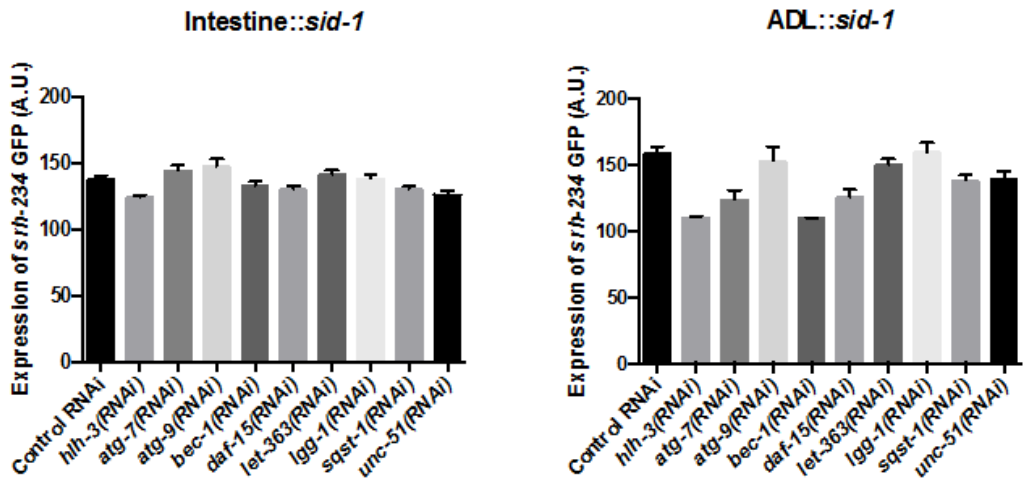

Figure 6. Expression of *srh-234p::GFP* when autophagy genes are knocked selectively in either the intestine or ADL neurons, but not in other tissues. *atg-7, atg-9, bec-1, daf-15, let-363, lgg-1, sqst-1, and unc-51*were all tested with the control *L4440*. The data gathered is a collective sample over multiple trials at n>100. *atg-7, bec-1,* and *daf-15* are significantly different, as determined by Mann-Whitney non-parameter statistical tests (see Appendix A-C).

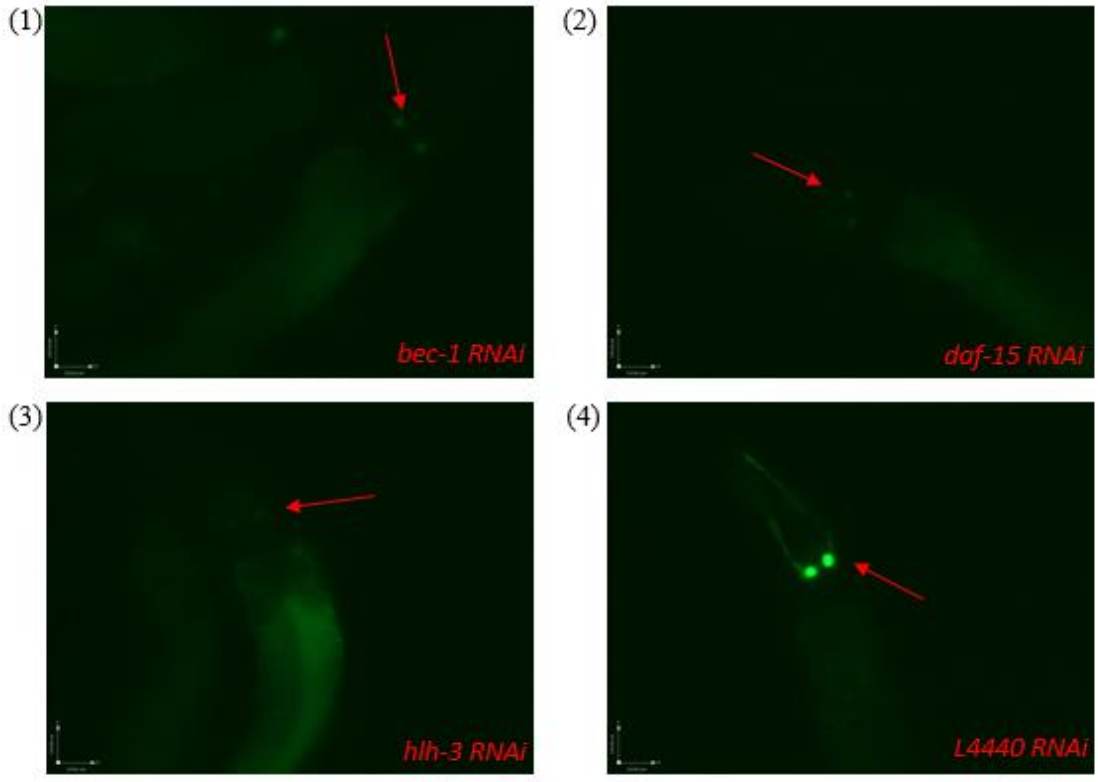

Figure 7. Images of *srh-234p::*GFP expression after RNAi feeding in animals carrying *ADL::sid-1* (selective knockdown of autophagy genes in ADL). In panels 1-3, RNAi down-regulation of *atg-7, bec-1,* and *daf-15* exhibit similar phenotypic expressions with the negative control *hlh-3.* A distinct difference can be seen in comparison to the positive control *L4440* (4). Images were taken at 2 m/s exposure

As seen in Figures 6 and 7, inactivation (or knockdown) of autophagy genes in the intestine by RNAi does not significantly differ from the control RNAi, while knockdown of autophagy genes, *atg-7, daf-15,* and *bec-1*, specifically in ADL lowers the expression of *srh-234*p::GFP (*(see Appendix 1-4 for statistics)*. This suggests that normal autophagy is required in a cell-autonomous manner in ADL neurons to properly regulate *srh-234* expression. Knockdown of other autophagy genes in animals carrying the *ADL::sid-1*, such as *sqst-1* and *unc-51* only display a minor reduction in *srh-234p*::GFP expression. These findings are consistent with our previous findings that mutations in

unc-51 reduce the expression of srh-234p::GFP, but our data provides further insight into the tissue requirement of this autophagy-dependent regulation of srh-234p::GFP. In contrast, knockdown of autophagy genes specifically in the intestine (*intestine::sid-1*) do not appear to significantly alter the expression of *srh-234p*::GFP expression levels when compared to the control RNAi. One possibility for not finding a significant change expression levels of *srh-234* in these *intestine::sid-1* RNAi feeding experiments that RNAi may have not completely knocked down the autophagy genes. For these particular RNAi experiments, we only had a negative control but did not have a positive control. Another possibility is that the RNAi effect may not be potent enough. RNAi experiments are highly affected by levels of IPTG and environmental conditions such as temperature, or even different developmental stages of *C. elegans*.

In summary, autophagy is correlated to starvation as it shares similar effects on ADL chemoreceptor, *srh-234*. To examine the role of autophagy in ADL-mediated responses, we used RNAi to target specific genes in the tissues, ADL::*sid*-1 and Intestine::*sid*-1. Our findings indicate that genes knocked down in the intestine does not alter *srh-234::*GFP and *atg-7, bec-1,* and *daf-15* down-regulates *srh-234* expression only in the ADL suggesting cell autonomy. Overall, studying autophagy in *C. elegans* is a great way to understand the autophagy process in humans as well.

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Appendix

Table Analyzed	ADL::sid-1
Column C	*atg-7(RNAi)*
vs.	vs.
Column A	Control RNAi
Mann Whitney test	
P value	0.0037
Exact or approximate P value?	Exact
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,C	936 , 54
Mann-Whitney U	33
Difference between medians	
Median of column A	151.3, n=38
Median of column C	119.6, n=6
Difference: Actual	-31.68
Difference: Hodges-Lehmann	-28.32

Appendix 1. Mann-Whitney test for *atg-7* (RNAi) with control RNAi, L4440.

Table Analyzed	ADL::sid-1
Column E	*bec-1(RNAi)*
vs.	vs.
Column A	Control RNAi
Mann Whitney test	
P value	< 0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,E	2350 , 971
Mann-Whitney U	25
Difference between medians	
Median of column A	151.3, n=38
Median of column E	107.3, n=43
Difference: Actual	-43.99
Difference: Hodges-Lehmann	-41.68

Appendix 2. Mann-Whitney test for *bec-1* (RNAi) with control RNAi, L4440. P<0.0001, tests highly significant.

Table Analyzed	ADL::sid-1
Column F	*daf-15(RNAi)*
vs.	vs.
Column A	Control RNAi
Mann Whitney test	
P value	< 0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,F	1837 , 864
Mann-Whitney U	234
Difference between medians	
Median of column A	151.3, n=38
Median of column F	108.8, n=35
Difference: Actual	-42.46
Difference: Hodges-Lehmann	-31.94

Appendix 3. Mann-Whitney test for *daf-15*(RNAi) with Control RNAi, L4440. P<0.0001, test highly significant

Table Analyzed	ADL::sid-1
Column J	*unc-51(RNAi)*
vs.	vs.
Column A	Control RNAi
Mann Whitney test	
P value	0.0579
Exact or approximate P value?	Exact
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,J	1099 , 279
Mann-Whitney U	174
Difference between medians	
Median of column A	151.3, n=38
Median of column J	141.7, n=14
Difference: Actual	-9.565
Difference: Hodges-Lehmann	-14.71

Appendix 4. Mann-Whitney test for *unc-51* (RNAi) with control RNAi, L4440.