Starvation response in *Caenorhabditis elegans*

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To My Beloved Wife Mi-sung
And
Our Lovely Baby Daniel

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Starvation Response in *Caenorhabditis elegans*

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When the supply of environmental nutrients is limited, multicellular animas can make physiological and behavioral changes so as to cope with nutrient starvation. Although starvation response is essential for the survival of animals during nutrient deprivation, uncontrolled or uncoordinated starvation responses could be harmful.

Autophagy, a lysosomal degradation pathway for long-lived proteins and cytoplasmic organelles, is known to be an important starvation response, which

promotes both cell and organism survival by providing fundamental building blocks to maintain energy homeostasis during starvation. Under different conditions, however, autophagy may instead act to promote cell death through an autophagic cell death pathway. Why autophagy acts in some instances to promote survival but in others to promote death is poorly understood. Here I show that physiological levels of autophagy act to promote survival in *Caenorhabditis elegans* during starvation, whereas insufficient or excessive levels of autophagy contribute to death. I find that inhibition of autophagy decreases survival of wild-type worms during starvation. Furthermore, I find that in *gpb-2* starvation-hypersensitive mutants, starvation induces excessive autophagy in pharyngeal muscles, which in turn, causes damage that may contribute to death. These results demonstrate that, depending on level of its activation, autophagy can have either prosurvival or prodeath functions, providing *in vivo* evidence that an uncontrolled starvation response could be harmful to animals.

Thus, it is important that animals ensure that their starvation response is coordinated between individual cells. However, the mechanisms by which animals sense starvation systemically remain elusive. Here I use *gpb-2* mutants to identify molecules and mechanisms that modulate starvation signaling. I found that specific amino acids could suppress the starvation-induced death of *gpb-2* mutants, and that MGL-1 and MGL-2, *C. elegans* homologs of metabotropic glutamate receptors, were involved. MGL-1 and MGL-2 acted in AIY and AIB

neurons respectively. Treatment with leucine suppressed starvation-induced stress resistance and life span extension in wild-type worms, and mutation of *mgl-1* and *mgl-2* abolished these effects of leucine. Theses results suggest that metabotropic glutamate receptor homologs in AIY and AIB neuron may modulate a systemic starvation response in *C. elegans*.

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4. **Kang C** and Avery L (2008) Systemic regulation of starvation response in *Caenorhabditis elegans*. Submitted to ***Genes and Development***.

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LIST OF ABBREVIATIONS

4E-BP	eukaryotic initiation factor 4E-binding protein
ACC	acetyl CoA carboxylase
Ala	alanine
AMPK	adenosine monophosphate-actiaved protein kinase
Arg	arginine
Asp	aspartate
ATG	autophagy-related gene
CaR	extracellular calcium sensing receptor
CPT I	carnitine palmitoyl-CoA transferase I
DAPK	death-associated protein kinase
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase
eEF2	eukaryotic translation elongation factor 2
ERK	extracellular signal-regulated kinase
GAIP	G protein α interacting protein
GFP	green fluorescent protein
Glu	glutamate
Gln	glutamine
Gly	glycine
Gqα	Gq class α subunit
His	histidine
Ile	isoleucine
Leu	leucine
MAPK	mitogen-activated protein kinase
Met	methionine
mTOR	mammalian target of rapamycin
MuRF	muscle Ring-finger protein
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
Pro	proline
RAS	Rous sarcoma kinase
RGS	regulator of G protein signaling
RNAi	RNA interference
S6K1	ribosomal S6 kinase 1
Ser	serine
Thr	threonine
UPS	ubiquitin-proteasome system
Val	valine

CHAPTER I

General Introduction

Since nutrient availability in the environment dramatically fluctuates over time, most animals face periods of food deprivation or starvation frequently through their entire life. When the food supply is limited, animals can make both physiological and behavioral changes so as to cope with nutrient starvation (Figure 1.1). Physiological changes include allocation of stored energy, decrease in metabolic rate and energy expenditure, and increase in protein degradation through the ubiquitin-proteasome system and lysosomal degradation pathways (Finn and Dice, 2006; Wang et al., 2006). All these responses contribute to the generation of sources of metabolic substrates, thereby maintaining energy homeostasis needed for animals to stay alive. Behavioral changes mainly consist of changes in foraging behavior, which increase the chance of an animal finding new food sources (Douglas et al., 2005; Wang et al., 2006).

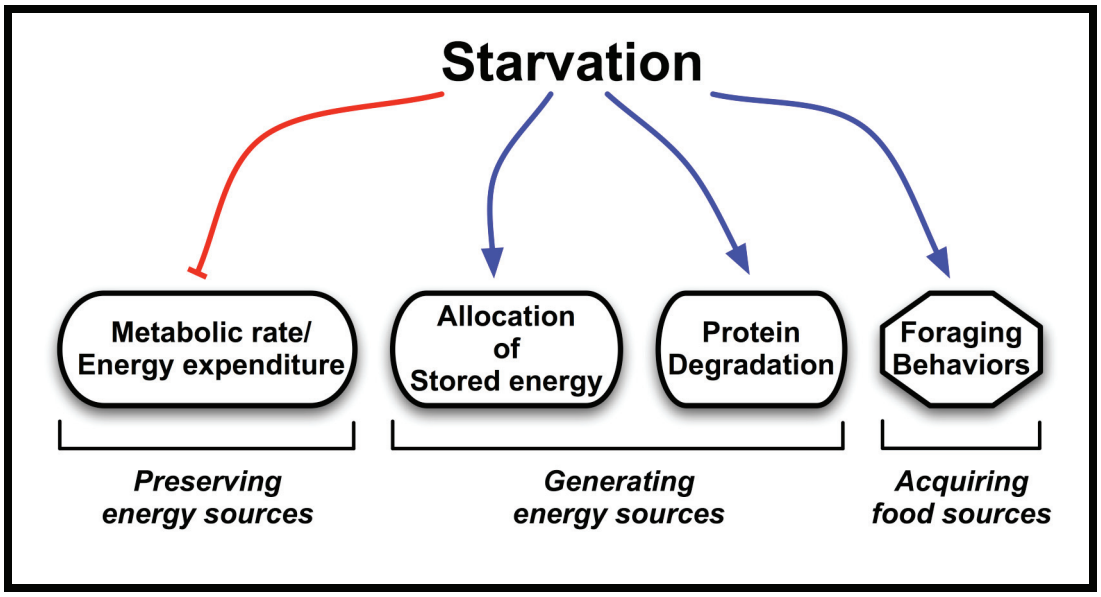


Figure 1.1. Physiological and behavioral changes during starvation

Physiological changes during starvation

During nutrient deprivation, animals make several physiological changes in both cell-autonomous and cell non-autonomous manners, adjusting energy balance (inhibiting anabolic pathways and activating catabolic pathways) for their prolonged-starvation survival. In response to starvation, individual cells decrease rates of general transcription and translation, preserving intracellular metabolites for to support basic functions and maintain viability (Lindsley and Rutter, 2004; Lum et al., 2005). In addition, individual cells increase their degradation of intracellular macromolecules, generating 'emergency' supplies of intracellular

metabolites, which in turn maintain basal cellular activity for their viability (Finn and Dice, 2006; Lum et al., 2005).

Physiological responses to starvation in animals are more complex, and are regulated in part by neuronal and hormonal changes for the precise coordination of the responses. Animals decrease basal metabolic rate by lowering energy demanding processes such as reproduction. Moreover, animals allocate their stored energy to several tissues, for example by increasing lipolysis, generating internal energy sources needed for survival during extreme resource limitation (Bertile and Raclot, 2006; Douglas et al., 2005; Wang et al., 2006).

Metabolic rate changes In response to starvation, individual cells and animals need to stop their growth (and proliferation in the case of individual cells), thereby preserving nutrients and energy, which are exclusively used to maintain basic cellular functions essential for viability. In individual cells, this cessation of growth is achieved through decreases in general transcription and translation, the most energy demanding processes (Lindsley and Rutter, 2004; Lum et al., 2005). It is very well known that mTOR protein kinases modulate transcription, translation, ribosome biogenesis, cell-cycle progression, and eventually cell growth and proliferation. Starvation inhibits the activity of mTOR, which in turn decreases the phosphorylation of its downstream targets, ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1). Decreases in phosphorylation inhibit the activity of S6K1 (a positive regulator of

translation) but activate 4E-BP1 (a negative regulator of translation), resulting in reduced protein synthesis. mTOR also modulates ribosome biogenesis by regulating the production of ribosomal RNA, and transcription by regulating several RNA polymerases (Hay and Sonenberg, 2004). Another kinase, AMP activated kinase (AMPK), senses levels of cellular ATP and inhibits protein synthesis by modulating mTOR and eEF2 in response to low cellular energy conditions (Luo et al., 2005).

In animals, reductions in metabolic rate can be achieved by several mechanisms, of course, including growth regulation by the insulin signaling pathway. In animals, basal and resting metabolism during starvation is decreased, as measured by oxygen consumption. This decrease probably results from decreases in energy demanding processes at the cellular level, for example, lowering protein synthesis, cell proliferation, and growth. In addition, in mammals, starvation reduces body temperature, which reduces metabolic rate (Arrhenius effect). Reduction in body temperature also can affect the activity of animals (torpor). Since reproduction is a hugely energy demanding process but not essential for survival, most animals decrease reproduction during starvation (Rion and Kawecki, 2007; Wang et al., 2006). So far, little is known about the mechanism by which starvation efficiently decreases reproduction.

Allocation of stored energy Many animals store surplus energy as fat when food is plentiful. In response to starvation, animals can derive emergency energy from

fat through fat breakdown, supporting basal cellular activity. So, large energy stores obviously ensure prolonged starvation survival (Bertile and Raclot, 2006; Cahill, 2006). In fact, many animals prepare themselves for expected periods of starvation by increasing fat content. This is clearly exemplified in *C. elegans* dauer formation (Hu, 2007). Fat breakdown is mainly regulated by AMPK through the phosphorylation of its downstream target acetyl CoA carboxylase (ACC). ACC produces malonyl-CoA, a potent inhibitor of carnitine palmitoyl-CoA transferase I (CPT I). CPT I regulates the entry of long-chain fatty acids into the mitochondrial matrix, the rate-limiting step of β-oxidation. AMPK phosphorylates and inactivates ACC, lowering the levels of malonyl-CoA, which eventually leads to fatty acid oxidation (Lindsley and Rutter, 2004).

Protein degradation When fat energy stores are depleted, animals start to break down proteins, generating energetic metabolites to maintain nutrient and energy homeostasis. At the cellular level, this protein breakdown also occurs when intracellular energy is depleted. There are two major protein degradation pathways, the ubiquitin-proteasome system, which is specialized for proteins, and a non-selective lysosomal degradation pathway, autophagy (Finn and Dice, 2006; Lum et al., 2005).

The ubiquitin-proteasome system is a well-known protein degradation pathway, which regulates selective degradation of proteins within the cytosol or nucleus. This system is mediated by a sequential enzyme cascade, involving a

ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3). Briefly, E1 activates ubiquitin and then transfers it to E2. E2 transfers ubiquitin to E3, which modulates covalent attachment of ubiquitin to a target protein. Once tagged, a target protein is recognized and degraded by the 26S proteasome in an ATP-dependent manner (Figure 1.2) (Ciechanover, 1994; O'Connell and Harper, 2007).

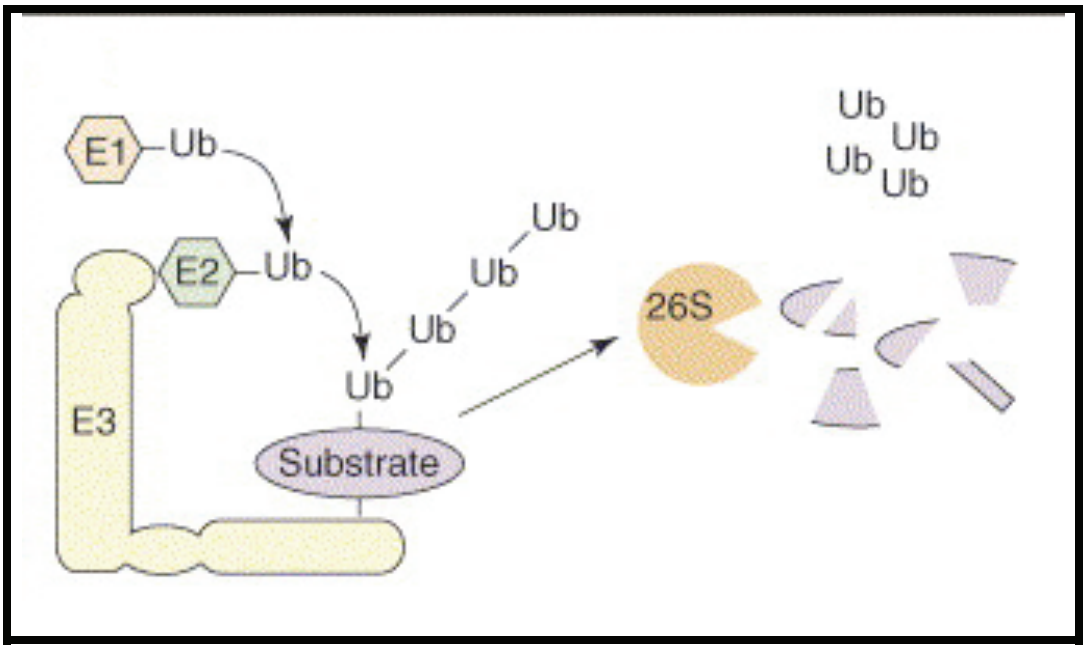


Figure 1.2. Ubiquitin-dependent proteolysis.
Substrates are targeted for 26S proteasome dependent degradation in a three-enzyme cascade. (Adapted from O'Connell and Harper, 2007).

A specific role of the ubiquitin-proteasome system in the degradation of skeletal muscle proteins during starvation period has been well studied. MuRF, muscle RING-finger protein, has a major role in muscle protein turnover during

starvation, which in turn provides energy to animals during relatively short-term starvation (Lecker, 2003; Sandri et al., 2006). It seems that the ubiquitin-proteasome pathway accounts for most muscle protein degradation, based on the fact that inhibition of the lysosomal degradation pathway only decreases muscle protein degradation 25% but that inhibition of the proteasome can eliminate muscle protein degradation. This muscle specific protein degradation is under hormonal control: Decreasing insulin and increasing glucocorticoids induce muscle cells to increase proteolysis (Finn and Dice, 2006).

Autophagy is a well-conserved lysosomal degradation pathway which, in contrast to the ubiquitin-proteasome pathway, can degrade not only proteins but also cytosolic organelles such as mitochondria (Levine and Klionsky, 2004; Mizushima, 2007; Yorimitsu and Klionsky, 2005). It is generally believed that autophagy is non-selective degradation pathway, but recently it has been shown that there are some exceptions (mitophagy and pexophagy) (Yu et al., 2008). Autophagy is initiated by the formation of a double membrane vesicle, the autophagosome, which sequesters cytoplasmic material and subsequently fuses with a lysosome, resulting in the degradation of cytoplasmic material. The products of degradation are recycled to generate macromolecules and ATP so as to maintain cellular homeostasis. From yeast genetics, approximately 20 *ATG* genes were found to be necessary for autophagy, including two ubiquitin conjugation like systems (*ATG12-ATG5* and *ATG8*-PE). Although some *ATG*

genes are well characterized, the molecular function of most *ATG* genes is still under investigation (Figure 1.3) (Levine and Klionsky, 2004).

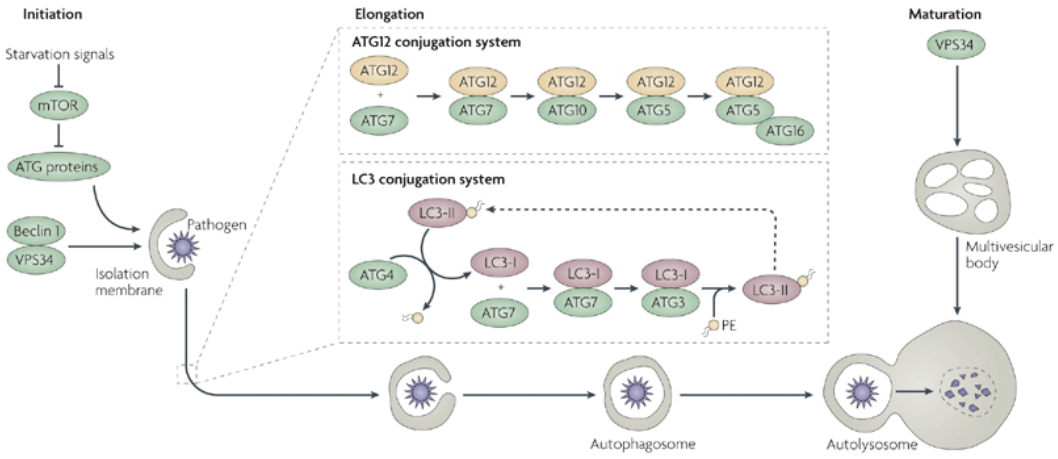


Figure 1.3. Regulation of autophagy by a set of autophagy-related proteins. ATG1 and the class III PI3K complex including Beclin 1 and VPS34 lead to the initiation of autophagy. The elongation of the autophagosome is regulated by two ubiquitin conjugation like systems. (Modified from (Levine and Deretic, 2007)).

Autophagy can be induced by both environmental stress (e.g. nutrient starvation, hypoxia, heat shock) and intracellular stress (e.g. damaged mitochondria, superfluous peroxisomes, protein aggregates, pathogens). Once induced, autophagy plays a protective role to alleviate the harmful effects of intracellular and environmental stress (Cuervo, 2004; Levine and Kroemer, 2008; Mizushima et al., 2008; Shintani and Klionsky, 2004). The best-characterized protective function of autophagy is as a starvation response. When external

nutrients are limited, cells can induce autophagy, thereby generating a source of metabolic substrates to sustain cellular activity needed for survival (Levine and Kroemer, 2008; Lum et al., 2005; Mizushima, 2007). This autophagic response to starvation also has been well studied in various organisms including yeasts, worms, flies, and mice (Levine and Klionsky, 2004). Compared to the ubiquitin-proteasome system, which is specifically induced in muscles during starvation, autophagy can be stimulated in most tissues and cells in response to starvation. This ability makes autophagy a more general and effective starvation response to generate 'emergency' energy than the ubiquitin-proteasome system.

In animals, autophagy can be regulated by hormonal control, especially by the insulin signaling pathway. Food intake increases insulin signaling, which in turn activates Phosphatidylinositol 3-kinases (PI3K)/mTOR kinase. mTOR kinase inhibits autophagy through the phosphorylation of *ATG13*, which reduces the activity of *ATG1*. Inactivation of mTOR by nutrient starvation allows dephosphorylation of *ATG13*, which enhances the association of this protein with *ATG1*, thereby enhancing the activity of *ATG1* and inducing autophagy (Codogno and Meijer, 2005). Other hormonal or neuronal regulations of autophagy in peripheral tissues are not well known.

Behavioral changes during starvation

Animals adjust their behavior in response to starvation. The aims of these adjustments are decreasing energy expenditure by reducing motions that are not essential for survival and increasing the chance of finding new food sources by increasing foraging behavior. For example, consistent with the change in reproduction, most animals don't show mating behavior during starvation, which is obviously a non-essential activity for starvation survival (Wang et al., 2006). This could be regulated by hormonal control, but little is known about the mechanism. Since these behavior changes are context dependent, one should consider the animals' environment and also their internal nutrient status.

Foraging behavior When food is not available, most but not all animals increase their movement to actively search for new food sources at the expense of increased energy expenditure. But in some cases animals decrease their physical activity and eventually enter into dormancy. Which strategy animals use mainly depends on their natural feeding habits. For example, sit-and-wait predators such as snakes feed only a few times in a year. After having a very large meal, they greatly lower their energy expenditure and sometimes enter into dormancy, suggesting that in general they are specialized to preserve energy. This property makes them decrease activity during starvation and wait until food becomes available. However, animals that feed frequently are more likely to increase their activity and search for food because their energy reservoirs are quickly depleted compared to sit-and-wait predators. For example, in rats, food deprivation initially

leads to reduced physical activity and movement. If, however, starvation is prolonged, animals start to increase activity and vigorously search for food. It is known that this foraging behavior is closely associated with depletion of fat stores and the subsequent increase in protein degradation, suggesting that depletion of energy reservoirs induces foraging behavior (Wang et al., 2006). But little is know about the signaling pathway by which foraging behavior is tightly regulated.

Purpose of study and strategy

Although it has been assumed that autophagy is a pro-survival mechanism for both cells and organisms, especially in response to starvation, several recent studies suggest that autophagy also functions as a pro-death mechanism at the cellular level (Baehrecke, 2005; Eskelinen, 2005; Levine and Yuan, 2005; Wang et al., 2005). It has been shown that RNAi knockdown of essential autophagy genes inhibits type II programmed cell death (autophagic cell death) in a variety of cell types under different condition. At the organismal level, the fact that excessive autophagy is associated with severe wasting of denervated myofibers in *runx1* mutants mice suggests that autophagy could be harmful to an organism (Wang et al., 2005). However there has been no direct evidence that autophagy could contribute to the actual death of multicellular organism until recently.

How is it that autophagy performs these seemingly opposite roles with respect to survival and death? Recent studies suggested the intriguing possibility that depending on its level, autophagy could act in either a pro-survival or a pro-death role at the cellular level (Pattingre et al., 2005). To test this possibility at the organismal level, I used *C. elegans* as a model system and proposed dual roles of autophagy in the survival of *C. elegans* during starvation in my graduate studies (Kang et al., 2007).

Another question important in understanding starvation responses at the organismal level is how animals sense starvation systemically. In addition, it is still poorly understood what constituents of food are sensed and how they modulate starvation responses. To identify molecules and mechanisms that modulate starvation response, I used a starvation-hypersensitive *C. elegans* mutant (Kang et al., 2007; You et al., 2006). If some molecules can act as an anti-hunger (food) signal, one would expect treatment with those molecules to rescue the death of starvation-hypersensitive mutants during starvation. After finding those molecules, the next step is finding their molecular targets. Here in my graduate studies, I used a candidate approach to find molecular targets and proposed a model describing how starvation is systemically modulated in *C. elegans*.

CHAPTER II

Materials and Methods

C. elegans strains

Strains were maintained as described at 19 ºC (Brenner, 1974). All worms were maintained and grown on *E. coli* HB101 bacteria. *adEx2066[rol-6(d) lgg-1::GFP]*, an extrachromosomal array that expresses an N-terminal GFP::LGG-1, was a gift from K. Jia and B (Melendez et al., 2003). Levine. *adEx2066* was integrated by γ irradiation, followed by outcrossing seven times to N2, to generate an integration line, *adIs2122[lgg-1::GFP rol-6(df)]*. The following strains were generated using standard genetic procedures: *gpb-2(ad541) I; adIs2122*, *mpk-1(ku1) III; adIs2122*, *gpb-2(ad541) I; mpk-1(ku1) III; adIs2122*, *let-60(n1046sd,am) IV; adIs2122, gpb-2(ad541) I; ced-3(n717) IV; adIs2122, dapk-1(gk219) I; adIs2122, gpb-2(ad541) dapk-1(gk219) I; adIs2122, gpb-2(ad541) I; rgs-2(vs22) X; adIs2122, gpb-2(ad541) dapk-1(gk219) I; rgs-2(vs22)X; adIs2122, gpb-2(ad541) I; mgl-1(tm1811) X, gpb-2(ad541) mgl-2(tm355) I, gpb-2(ad541) mgl-2(tm355) I; mgl-1(tm1811) X, gpb-2(ad541) I; adIs2122, gpb-2(ad541) I;*

mgl-1(tm1811) X; adIs2122, gpb-2(ad541) mgl-2(tm355) I; adIs2122, gpb-2(ad541) mgl-2(tm355) I; mgl-1(tm1811) X; adIs2122, gpb-2(ad541) I; mgl-1(tm1811) X; adEx2241[pttx-3::mgl-1 rol-6(d)], gpb-2(ad541) mgl-2(tm355) I; adEx2242[podr-2b::mgl-2 rol-6(d)], gpb-2(ad541) I; ad2243[pttx-3::egl-1 rol-6(d)], gpb-2(ad541) I; adEx2244[podr-2b::egl-1 rol-6(d)], gpb-2(ad541) I; eat-4(ky5) III, gpb-2(ad541) I; eat-4(ky5) III; mgl-1(tm1811) X, gpb-2(ad541) mgl-2(tm355) I; eat-4(ky5) III, mgl-2(tm355) I; mgl-1(tm1811) X.

RNA interference

RNAi by feeding was performed as described (Kamath et al., 2003) except for a few modifications. Briefly, RNAi clones were inoculated overnight at 37 ºC in LB plus tetracycline at 10μg/ml and ampicillin at 75μg/ml. After 2 hr induction with 0.4 mM IPTG, RNAi clones were seeded onto NG-ampicillin-IPTG plates. L1 animals were transferred to RNAi plates containing bacteria induced to express an RNAi clone or carrying empty RNAi feeding vector as a control and allowed to grow to adulthood. After they had laid eggs, the adult worms were removed from the RNAi plates. The plates were then incubated at 19 ºC for either 21 hr (in wild-type) or 17 hr (in *gpb-2* mutants). L1 worms were collected from RNAi plates and washed three times with M9 solution. The collected L1 worms were used for starvation-survival analyses. The length of the incubation on RNAi plates allowed us to adjust the strength of the RNAi treatment. Since autophagy is necessary for

optimal survival of wild-type during starvation, we anticipated that too effective *bec-1(RNAi)* would also decrease starvation survival of *gpb-2* worms—a pro-survival effect was expected only from a partial knockdown of *bec-1* expression. Indeed, in preliminary exploratory experiments *bec-1(RNAi)* for longer than 17 hours actually decreased starvation survival of *gpb-2* worms.

Molecular biology

cDNA corresponding to the entire coding sequence of *egl-1* was amplified and cloned under cell-specific promoters as indicated. Primer sequences are shown below:

egl-1: 5'GCTCTAGAATGTCCAACGTTTTTGACGTTCAATCT3' and

5'CGCTCGAGTTAAAAAGCGAAAAAGTCCAGAAGACG3'

Template: *egl-1* DNA in pBluescript from Scott Cameron and Malia Potts (UT Southwestern Medical Center).

Expression in AIY and AIB was achieved using the *ttx-3* and *odr-2b* promoters respectively, from Cornelia Bargmann (The Rockefeller University).

cDNA corresponding to the entire coding sequence of *mgl-2* was obtained using SpeI and XhoI restriction enzymes from *mgl-2* cDNA in pBluescript from Isao Katsura (National Institute of Genetics) and cloned under the *odr-2b* promoter.

PCR construction of *mgl-1* with *ttx-3* promoter was achieved as follows: the *ttx-3* promoter region was amplified from a *Pttx-3::mod-1* cDNA::GFP plasmid (from

Cornelia Bargmann, The Rockefeller University) using primers 5'GTCTCATTTAAATTTTCAGAGCTTAAAAATGG3' and 5'TGTTTGCCTCATATTGACACCGAAGACAATTATT3' (PCR#1). cDNA corresponding to the entire coding sequence of *mgl-1* was amplified from *mgl-1* cDNA in pMW118 (from Isao Katsura, National Institute of Genetics) using primers 5'CTTCGGTGTCAATATGAGGCAAACATTTCGGAA3' and 5'TCATAAGAAAGTATCGTGAGCAG3' (PCR#2). PCR#1 and #2 were fused using primers 5'GTCTCATTTAAATTTTCAGAGCTTAAAAATGG3' and 5'TCATAAGAAAGTATCGTGAGCAG3'.

Starvation survival analyses

Starvation survival analyses were performed as described (You et al., 2006) with a few modifications. After collection of L1 worms from RNAi plates or from synchronization by egg preparation, I incubated them in 3 mL of sterilized M9 buffer with or without leucine for the time indicated in the figures at 19˚C. At each time point, an aliquot from each sample tube was placed on a plate seeded with *E. coli* HB101. The number of worms surviving to L4 or adulthood was determined after 3 days further growth at 19˚C. The number from day 1 of starvation was used as control and as the denominator to calculate the percentage of worms recovering after starvation. For initial screening, we used 10.9 mM amino acid. Since we found that high concentrations of leucine decrease survival

of *gpb-2* mutants, we used 3.6 mM leucine in all subsequent experiments except the hormesis experiments, in which we used 7.2 mM leucine.

Bacteria preparation for liquid cultivation

E. coli HB101 was prepared as described (You et al., 2006) with few modifications. An *E. coli* HB101 culture in LB broth of optical density 1.0~1.2 (600 nm) was washed three times with M9 buffer and resuspended in the same volume of M9 buffer. This *E. coli* HB101 suspension was added to the worm liquid culture at a dilution between 1:4 and 1:6 in order to provide food during starvation-survival analyses.

***C. elegans* autophagy analysis**

Autophagy analysis was performed as described (Melendez et al., 2003) with few modifications. For light microscopic analysis of autophagy, starved L1 animals carrying an integrated transgene that expresses a GFP::LGG-1 fusion were collected at each time point. GFP-positive punctate regions were visualized in the pharyngeal muscles of L1 animals using a Zeiss Axioplan 2 compound microscope (Zeiss Corporation, Germany). The percentage of animals with GFP-positive punctate regions was determined. For western blot analysis of autophagy, starved L1 animals carrying an integrated transgene that expresses a GFP::LGG-1 fusion were prepared at each time point and analyzed by immunoblotting using an

anti-GFP antibody. The relative levels of PE-conjugated LGG-1-GFP and LGG-1-GFP were calculated by densitometry using ImageJ (Version 1.36b, National Institutes of Health, Bethesda, MD).

Starvation induced heat-shock resistance analyses

After collection of L1 worms after synchronization by egg preparation, we incubated them in 3 mL of M9 buffer with or without leucine for 0 days (non starved group) or 2 days (starved group). We equally divided each group into two subgroups and gave a heat-shock (35˚, 90 minutes) to one of them (heat-shock subgroup). We calculated the index of starvation induced heat-shock resistance as follows: Index of starvation induced heat-shock resistance = (Number of surviving starved worms with heat-shock / Number of surviving starved worms without heat-shock) – (Number of surviving unstarved worms with heat-shock / Number of surviving unstarved worms without heat-shock).

Starvation induced oxidative stress resistance analyses

After collection of L1 worms after synchronization by egg preparation, we incubated them in 3 mL of M9 buffer with or without leucine for 0 days (non starved group) or 2 days (starved group). We equally divided each group into two subgroups and gave an oxidative stress (H_2O_2 10mM, 40 minutes) to one of them (H_2O_2 group). We calculated the index of starvation induced oxidative stress

resistance as follows: Index of starvation induced oxidative stress resistance = (Number of non arrested starved worms with H_2O_2 / Number of non arrested starved worms without H_2O_2) – (Number of non arrested unstarved worms with H_2O_2 / Number of non arrested unstarved worms without H_2O_2).

Starvation induced lifespan extension analyses

Lifespan analyses were performed as described (Tullet et al., 2008) with a few modifications. After collection of L1 worms after synchronization by egg preparation, animals were allowed to develop at 19˚C. When animals reached adulthood, they were collected and incubated in sterilized M9 buffer with or without leucine for 0 hours (non starved group) or 40 hours (starved group). They were transferred to a plate seeded with *E. coli* HB101, kept at 19˚C, and score every 2 days as dead or alive. Animals that crawled off the plate, exploded, or died as bags of worms were excluded from analysis at the time of death. Lifespans were measured from adulthood. Survival curve P values were calculated by the Mantel-Cox Log Rank test using Prism statistical software (Graphpad).

CHAPTER III

Dual Roles of Autophagy in the Survival of *Caenorhabditis elegans* During Starvation

Abstract

Autophagy is a major pathway used to degrade long-lived proteins and organelles. Autophagy is thought to promote both cell and organism survival by providing fundamental building blocks to maintain energy homeostasis during starvation. Under different conditions, however, autophagy may instead act to promote cell death through an autophagic cell death pathway distinct from apoptosis. Although several recent papers suggest that autophagy plays a role in cell death, it is not known whether autophagy can cause the death of an organism. Furthermore, why autophagy acts in some instances to promote survival but in others to promote death is poorly understood. Here I show that physiological levels of autophagy act to promote survival in *C. elegans* during starvation, whereas insufficient or excessive levels of autophagy contribute to death. I found that inhibition of autophagy decreases survival of wild-type worms during starvation and that muscarinic signaling regulates starvation-induced autophagy, at least in part, through the death-associated protein kinase signaling pathway. Furthermore, I found that in *gpb-2* mutants, in which muscarinic signaling cannot be downregulated, starvation induces excessive autophagy in pharyngeal muscles, which in turn causes damage that may contribute to death. Taken together, my results demonstrate that autophagy can have either pro-survival or pro-death functions in an organism, depending on its level of activation.

Introduction

Autophagy is a well-conserved lysosomal pathway used to degrade long-lived proteins and cytoplasmic organelles. It involves the formation of double-membrane vesicles called autophagosomes that sequester cytoplasmic material, and the subsequent fusion of an autophagosome with a lysosome which results in the degradation of cytoplasmic material (Levine and Klionsky, 2004). Autophagy can be stimulated by both environmental stress (e.g., nutrient starvation, hypoxia, heat shock) and intracellular stress (e.g., damaged mitochondria, superfluous peroxisomes, protein aggregates, pathogens) (Levine and Klionsky, 2004; Mizushima, 2005). Once stimulated, autophagy helps alleviate the harmful effects of intracellular and extracellular stress.

For many years, it has been assumed that autophagy acts as a pro-survival response to starvation at the cell and organism levels. For example, when the supply of environmental nutrients is limited, autophagy can generate a source of metabolic substrates to maintain cellular ATP production, protein synthesis and fatty acid synthesis, thereby sustaining cellular activity needed for the cell to stay alive (Lum et al., 2005). This autophagic response to starvation has been well studied in various organisms including yeast, worms, flies and mice. Loss of autophagy results in death in nitrogen-starved yeast, defects in dauer formation of *C. elegans*, hypersensitivity to starvation in *Drosophila*, and early postnatal

lethality in mice after termination of the placental nutrient supply (Kuma et al., 2004; Melendez et al., 2003; Scott et al., 2004; Tsukada and Ohsumi, 1993). These lines of evidence support the view that autophagy is a survival mechanism for both cell and organism.

However, several recent studies indicate that autophagy also plays a pro-death role at the cellular level. Based on morphological evidence, it has been suggested that autophagy causes a nonapoptotic autophagic cell death during development and in response to toxins or stressful stimuli (Levine and Yuan, 2005). In addition, it was shown that RNAi knockdown of essential autophagy genes inhibits nonapoptotic cell death in a variety of cell types under different conditions (Reef et al., 2006; Shimizu et al., 2004; Yu et al., 2004). Together, these new findings strongly suggest that autophagy has a pro-death function at the cellular level. At the organismal level, excessive autophagy is associated with severe wasting of denervated myofibers in *runx1* mutant mice (Wang et al., 2005), suggesting that autophagy could be harmful to the organism. It is not known, however, whether autophagy can lead to organismal death.

Despite intensive study, it remained elusive until recently how autophagy could perform these seemingly opposite roles as a pro-survival mechanism and a pro-death mechanism. An important clue to understand the mechanism by which autophagy performs dual roles with respect to cell survival came from studying the biological significance of the interaction between Bcl-2 and Beclin 1

(Pattingre et al., 2005). The researchers found that Bcl-2 proteins bind to Beclin-1 and inhibit Beclin 1-dependent autophagy in yeast and mammalian cells. Furthermore, they found that mutants of Beclin 1 that do not bind Bcl-2 demonstrate excessive levels of autophagy and promote autophagy gene-dependent cell death in MCF7 cells. Based on these new findings, a new model for understanding the roles of autophagy in cell survival and cell death presented itself: Physiological levels of autophagy are essential for normal cellular homeostasis and play a pro-survival role, whereas excessive levels of autophagy promote autophagic cell death and play a pro-death role at the cellular level (Pattingre et al., 2005). It is plausible to assume that the same mechanism might cause autophagy to have dual roles in the survival of a multicellular organism. For example, physiological levels of autophagy can act as a pro-survival role in an organism by providing energy during starvation (Kuma et al., 2004), whereas excessive autophagy might act as a pro-death role by causing damage in tissues essential for survival.

In this study, I use *C. elegans* as a model system to evaluate whether autophagy plays dual roles in the survival of a multicellular organism undergoing starvation. I find that insufficient levels of autophagy caused by knockdown of *bec-1* promote the death of worms undergoing starvation. In contrast, *gpb-2* mutants that are hypersensitive to starvation (You et al., 2006) have excessive levels of autophagy during starvation, and knockdown of *bec-1* can rescue the

starvation-induced death of *gpb-2* mutants. Together these findings suggest that autophagy can play both a pro-survival role and a pro-death role at the organismal level depending on its level of activation.

Results

Autophagy is required for optimal survival of worms during starvation

To test the role of autophagy in survival of *C. elegans* during starvation, I used RNA interference (RNAi) to inhibit autophagy. Since animals in which autophagy is completely blocked by mutation are very unhealthy (*unc-51* mutants, data not shown), or die (*bec-1* mutants; (Takacs-Vellai et al., 2005)), I thought that partial inhibition by RNAi would be more appropriate to study the role of autophagy during starvation. I targeted *bec-1* for RNAi. *bec-1* is the *C. elegans* ortholog of yeast *ATG6* and is essential for the autophagy process (Melendez et al., 2003). Depleting *bec-1* function by RNAi decreased the survival of wild-type worms after starvation (Figure 2.1A).

Because the feeding organ, the pharynx, is critical for recovery from starvation (You et al., 2006), I measured autophagy in pharyngeal muscle. I generated a strain carrying an integrated transgene that expressed a GFP-tagged version of *lgg-1*, which is a specific fluorescence marker for autophagy (Melendez et al., 2003; Mizushima, 2004). When autophagy is induced, GFP::LGG-1 changes from its diffuse cytoplasmic cellular localization to form punctate structures that label the preautophagosomal and autophagosomal membranes (Figure 2.1C). I found that starvation induced autophagy in

pharyngeal muscle, and that *bec-1* RNAi treatment decreased the amount of autophagy induced by starvation (Figure 2.1B,C,D).

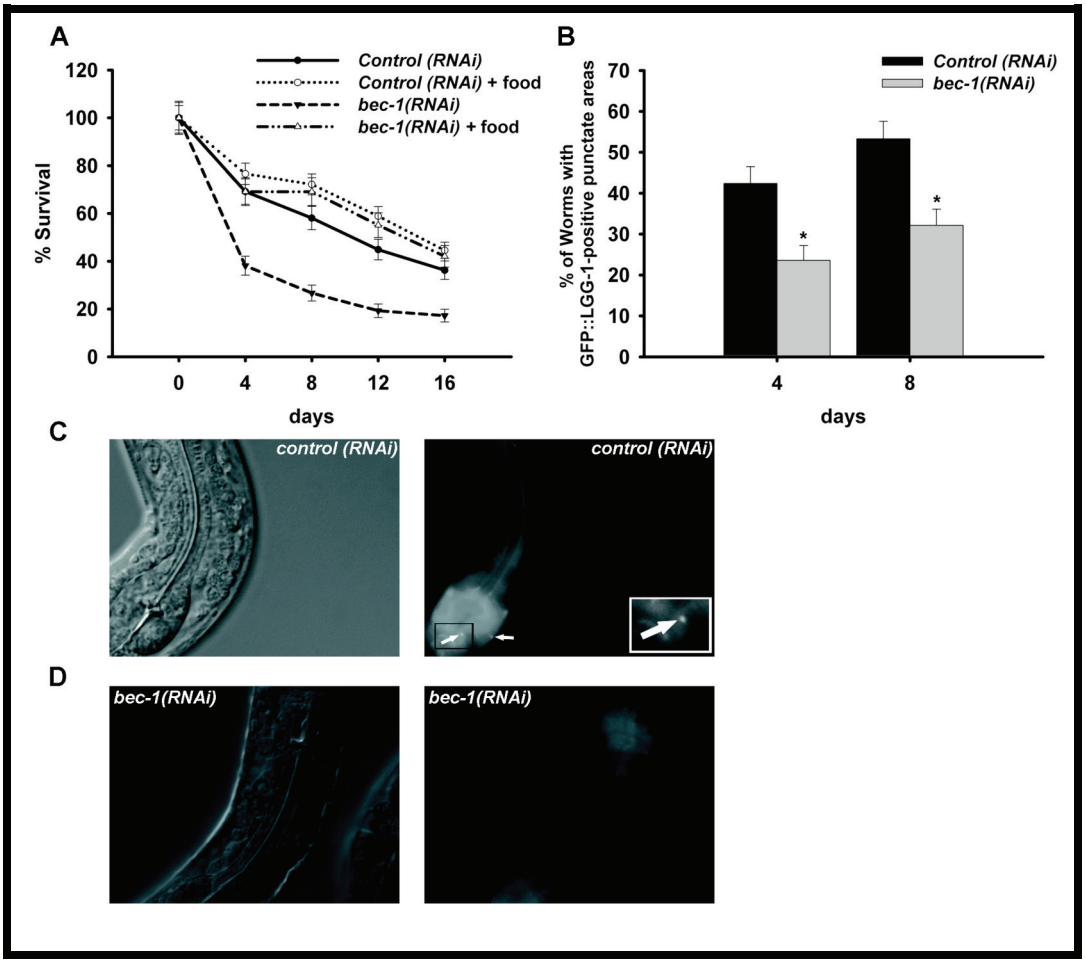


Figure 2.1. Autophagy is required for optimal survival of *C. elegans* during starvation.
Starvation-survival analyses were performed as described in Materials and Methods. (A) Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution. (B) Quantitation of autophagy in the pharyngeal muscles of control

RNAi animals and *bec-1(RNAi)* animals in A. Error bars indicate standard error for proportions. *P<0.001. (C) Representative images of control RNAi animals after 9 days of starvation. Differential interference contrast image of control RNAi animals (left) and green fluorescence image (right). The arrow shows representative GFP::LGG-1-positive punctate structures that label preautophagosomal and autophagosomal structures. In the inset, the area marked by the box is magnified and contrast-enhanced. (D) Representative images of *bec-1(RNAi)* animals after 9 days of starvation. Differential interference contrast image of *bec-1(RNAi)* animals (left) and green fluorescence image (right).

I also tested the effects on the autophagy process of RNAi of another *C. elegans* ortholog of a yeast autophagy gene. RNAi-mediated interference of the *C. elegans* ortholog of yeast *ATG7* decreased survival of wild-type worms after starvation and also reduced autophagy in the pharyngeal muscle (Figure 2.2). This suggests that the effect of *bec-1* RNAi on survival of wild-type worms during starvation is due to defects in autophagy rather than defects in other potential functions of *bec-1*. The survival of *bec-1(RNAi)* worms could be rescued by the addition of food (Figure 2.1A), suggesting that a major cause of death in *bec-1(RNAi)* worms was a lack of nutrients during starvation, probably resulting from a decrease in autophagy.

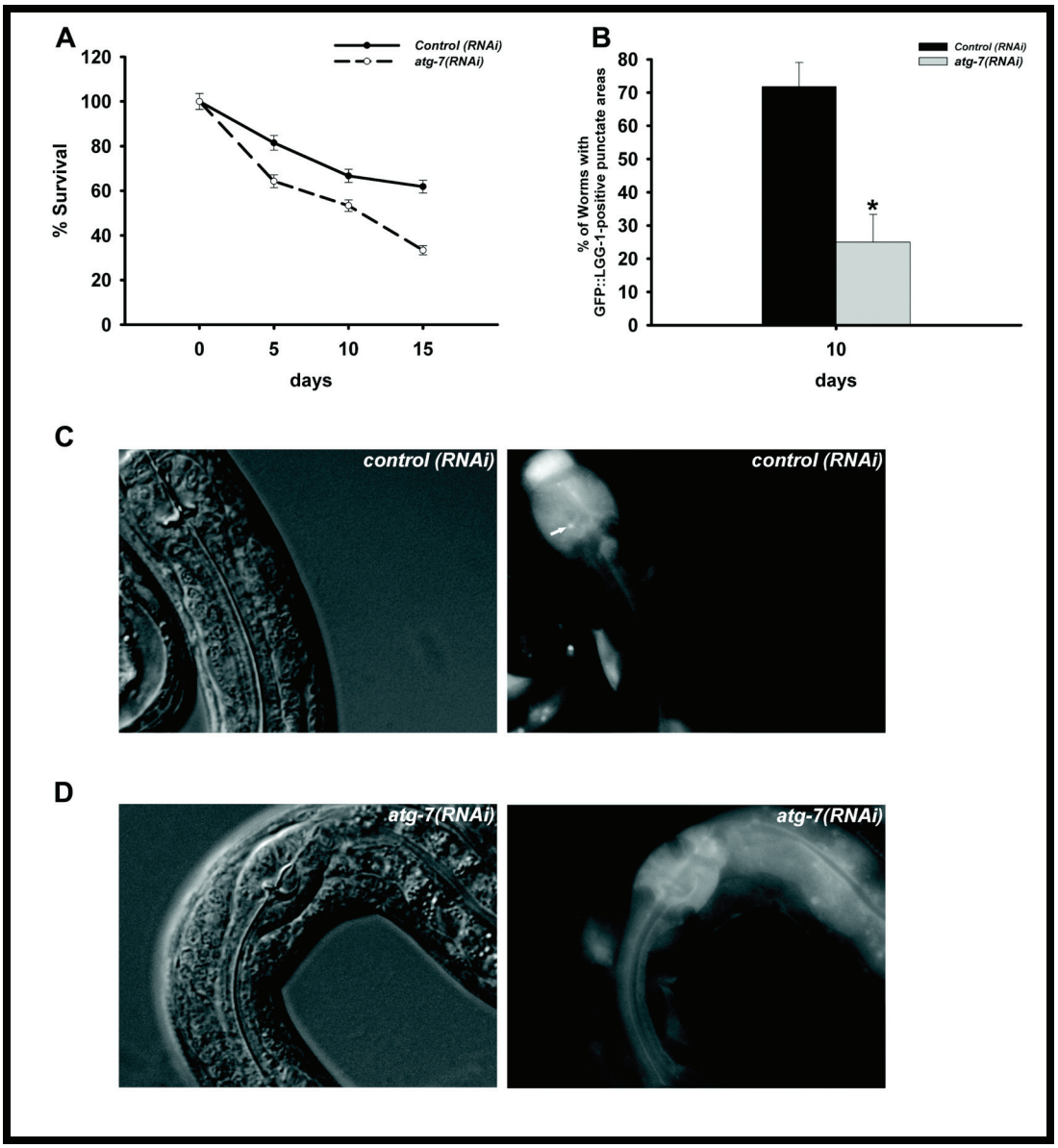


Figure 2.2. The effect of *atg-7* RNAi treatment on the survival of wild-type worms and autophagy in the pharyngeal muscle after starvation.
(A) Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution. *atg-7(RNAi)* decreased the survival of wild-type worms after starvation. Additional trials are in Table 1. (B) Quantitation of autophagy in the pharyngeal muscles of control

RNAi animals and *atg-7(RNAi)* animals in A. Error bars indicate standard error for proportions. *P<0.001. (C) Representative images of control RNAi animals after 10 days of starvation. Differential interference contrast image of control RNAi animals (left) and green fluorescence image (right). The arrow shows representative GFP:LGG-1-positive punctate structures that label preautophagosomal and autophagosomal structures. (D) Representative images of *atg-7(RNAi)* animals after 10 days of starvation. Differential interference contrast image of *atg-7(RNAi)* animals (left) and green fluorescence image (right).

Seeking to understand why starvation kills worms when autophagy is suppressed, I tested whether a decrease in autophagy affected the activity of the pharynx. I found that *bec-1* RNAi treatment decreased pumping rates (Figure 2.3A) and that pumping could be rescued by the addition of food, suggesting that autophagy is required to maintain basal activity of the pharynx during starvation. I next tested whether pumping could predict survival. To determine the importance of basal pumping rates for survival, I counted the pumping rates of individual worms that were still alive immediately after starvation, assessed their survival, then compared the average pumping rate of worms that eventually died to that of worms that survived (Figure 2.3B). Worms that initially had high pumping rates survived better than those that had low pumping rates after starvation—pumping predicts survival with up to 80% accuracy. My finding combined with previous work showing that the pharynx is important for recovery from starvation (You et al., 2006) suggests that maintenance of basal pumping rates may be important for the survival of worms after starvation. Taken together, these results demonstrate

that autophagy is required for the optimal survival of wild-type worms during starvation, perhaps providing an energy source or essential nutrients to maintain cellular activity.

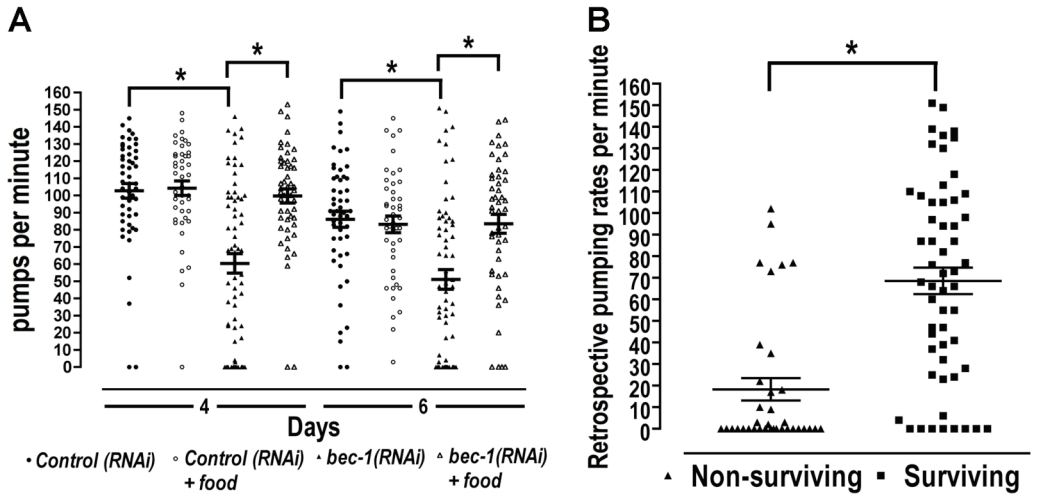


Figure 2.3. Pumping rates in control RNAi animals and *bec-1(RNAi)* animals after starvation.
(A) Each dot represents an individual worm. Horizontal lines represent the average (line) and SEM (error bars). *P<0.0001 (Student's t-test). (B) Retrospective pumping rates of surviving and non-surviving *bec-1(RNAi)* animals after starvation. Each dot represents an individual worm. Horizontal lines represent the average (line) and SEM (error bars). *P<0.0001 (Student's t-test).

Overactivated muscarinic acetylcholine signaling induces excessive autophagy and causes death of worms during starvation

Previously, it is showed that starvation activates mitogen-activated protein kinase in pharyngeal muscles via a muscarinic acetylcholine receptor signaling

pathway (Muscarinic acetylcholine receptor→Gqα→PKC→MAPK) (You et al., 2006). In addition, this starvation signaling induces a change in the physiology of the pharyngeal muscle to increase its activity. However, when the starvation signal is overactivated, as in the case of *gpb-2* (a G protein β subunit involved in RGS-mediated inhibition of Gqα pathway) mutants in which muscarinic acetylcholine receptor signaling cannot be downregulated, starvation instead induces damage in the pharyngeal muscle that leads to aberrant activity and eventual death on recovery from starvation (You et al., 2006).

The environmental cue (starvation) that induces changes in the pharyngeal muscles of worms can also trigger autophagy (Levine and Klionsky, 2004). Furthermore, it is known that overactivated autophagy causes damage at the cellular level (Codogno and Meijer, 2005; Kroemer and Jaattela, 2005; Levine and Yuan, 2005); this is reminiscent of the way overactivated muscarinic signaling causes damage that leads to the malfunction of worm pharyngeal muscle. Based on these similarities, I hypothesized that overactivated muscarinic signaling in *gpb-2* mutants induces unrestrained autophagy, which in turn causes damage to the pharyngeal muscles and eventually contributes to death after starvation.

To test this, I examined the level of autophagy in the pharyngeal muscle of *gpb-2* mutants during starvation. As reported previously (You et al., 2006), *gpb-2* mutants were more sensitive to starvation than wild-type worms (Figure 2.4B).

Moreover, I found that autophagy is excessively induced following starvation in the pharyngeal muscle of *gpb-2* mutants compared to that of wild-type worms as measured by GFP-LGG-1 punctate structures (Figure 2.4A, 2.5). It is unlikely that an increase of GFP-LGG-1 punctate structures in *gpb-2* mutants results from defects in the turnover of autophagosomes because treatment with lysosomal inhibitor (NH_4Cl) increases accumulation of GFP-LGG-1 punctate structures (Figure 2.6). I also confirmed induction of autophagy by detection of the modification of GFP::LGG-1 using immunoblotting (Mizushima, 2004). After starvation, the ratio of phosphatidylethanolamine-conjugated LGG-1 (PE-LGG-1::GFP) to LGG-1 (LGG-1::GFP) is higher in *gpb-2* mutants than in wild-type worms (Figure 2.4C), indicating that autophagy is excessively induced in *gpb-2* mutants compared to wild-type worms following starvation. Death of starved *gpb-2* mutants is likely caused by pharyngeal muscle malfunction, not cell death, because pharyngeal muscle cells of *gpb-2* mutants show no obvious cell death and they are still able to contract after starvation. Furthermore, mutation of *ced-3* could not rescue death of *gpb-2* mutants during starvation (data not shown), suggesting that apoptosis is not involved. To test whether muscarinic acetylcholine receptor signaling is involved in the regulation of autophagy, I used inhibitors of muscarinic signaling such as atropine, a muscarinic acetylcholine receptor antagonist, and U0126, which prevents MAPK activation by inhibiting the upstream kinase MEK (You et al., 2006). Treatment with either atropine or

U0126 reduced autophagy in the pharyngeal muscle of *gpb-2* mutants during starvation (Figure 2.4D,F, 2.5) and also partially rescued the starvation hypersensitivity of *gpb-2* mutants (Figure 2.4E,G). Furthermore, I found that mutation of *mpk-1*, which encodes the MAPK activated by starvation, also decreased autophagy in the pharyngeal muscle of *gpb-2* mutants during starvation (Figure 2.4H, 2.5) and suppressed the starvation hypersensitivity phenotype (Figure 2.4I). Taken together, these data indicate that under starvation conditions, overactivated muscarinic acetylcholine receptor signaling in *gpb-2* mutants causes excessive autophagy. Furthermore, the fact that excessive autophagy is associated with the death of starved *gpb-2* mutants strongly suggests that unrestrained autophagy may contribute to the death. It is important to note that mutation of *mpk-1* rescues the death of *gpb-2* mutants very efficiently, yet is less effective at reducing autophagy than atropine. It is possible that other unknown mechanisms also contribute to the death of starved *gpb-2* mutants.

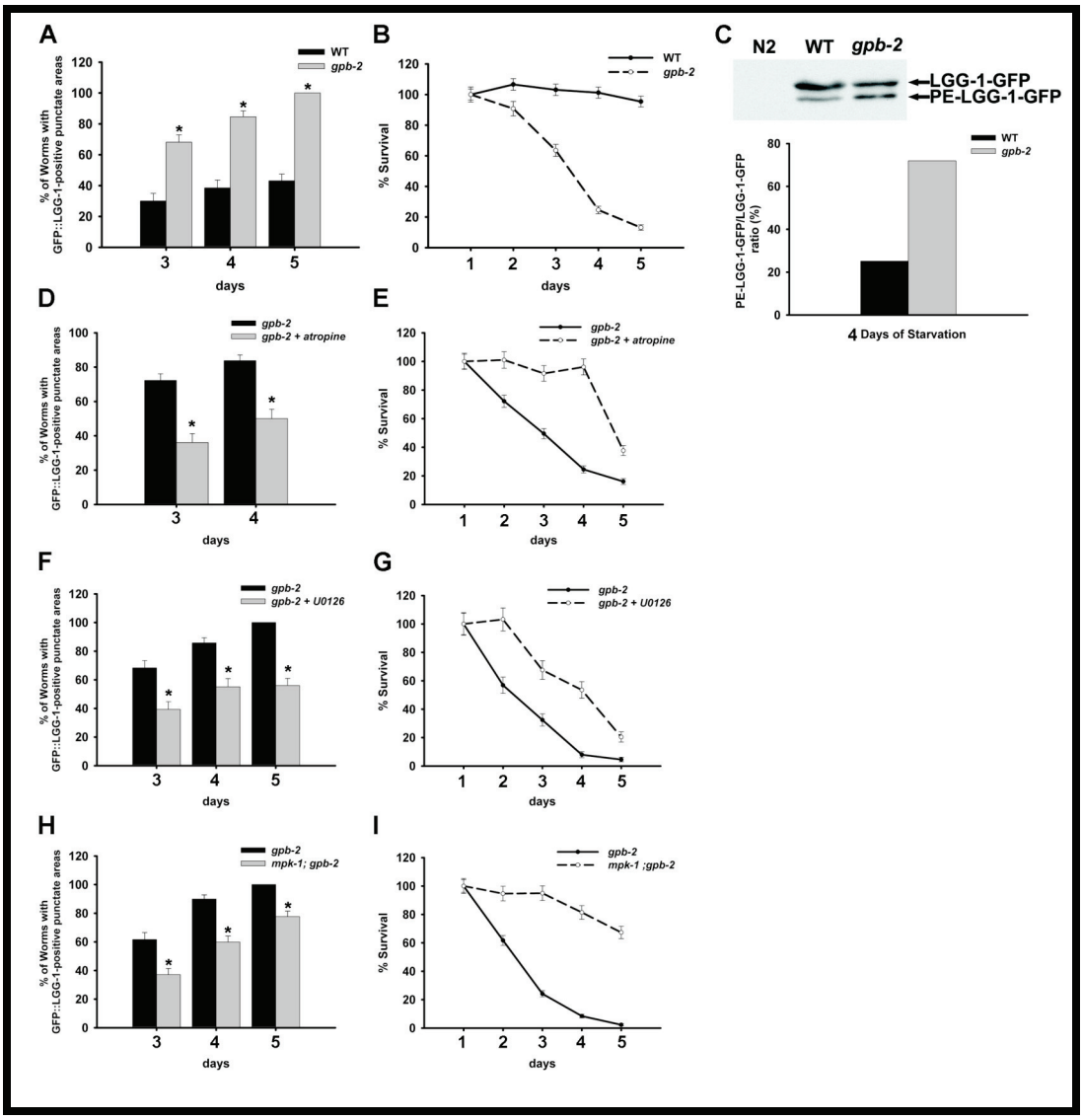


Figure 2.4. Overactivated muscarinic signaling induces excessive levels of autophagy and causes death in *gpb-2* mutants after starvation.
(A, D, F, and H) Quantitation of autophagy in the pharyngeal muscles of indicated genotype after incubation in M9 buffer in the absence of food with or without either atropine (10mM) or U0126 (25μM) for the indicated time. Error bars indicate standard error for proportions. *P<0.0001 for all except 3 days in F and H, for which *P<0.005. (B, E, G, and I) Percent of animals surviving to adulthood

in A, D, F, and H. Error bars are standard errors estimated assuming a Poisson distribution. (C) The relative levels of PE-conjugated LGG-1-GFP and LGG-1-GFP were determined by Western blotting. Starved L1 worms were prepared and analyzed by immunoblotting using an anti-GFP antibody. The arrows indicate LGG-1-GFP and PE-conjugated LGG-1-GFP. The Western blot is representative of what was seen in two independent experiments.

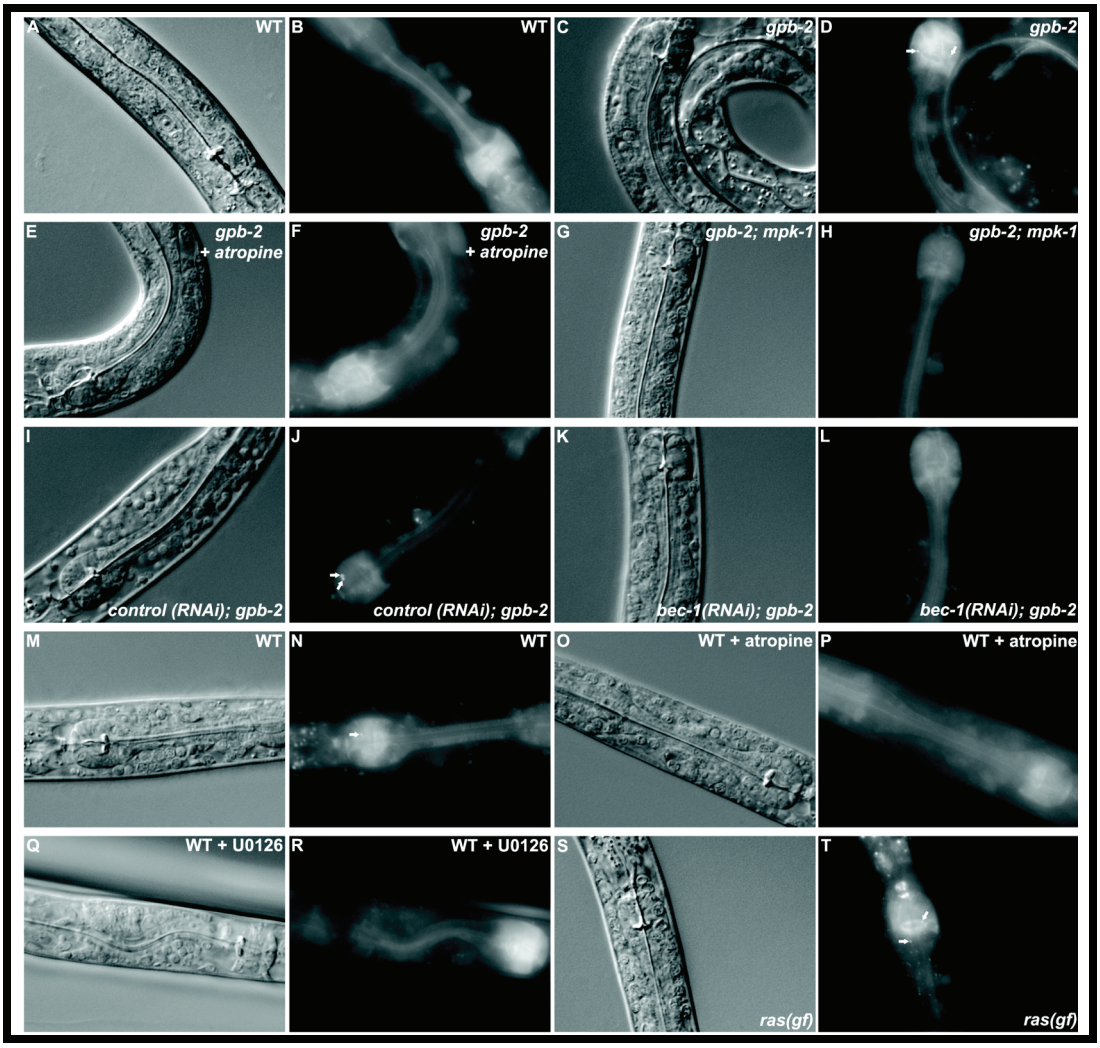


Figure 2.5. Representative images of indicated animals after starvation.
(A-L) Representative images of indicated animals after 2 days of starvation. (A, C, E, G, I and K) Differential interference contrast images of indicated animals. (B, D, F, H, J and L) Green fluorescence images of (A, C, E, G, I and K). (M-T)

Representative images of indicated animals after 8 days of starvation. (M, O, Q and S) Differential interference contrast images of indicated animals. (N, P, R and T) Green fluorescence images of (M, O, Q and S). The arrow shows representative GFP-positive punctate structures that label preautophagosomal and autophagosomal structures.

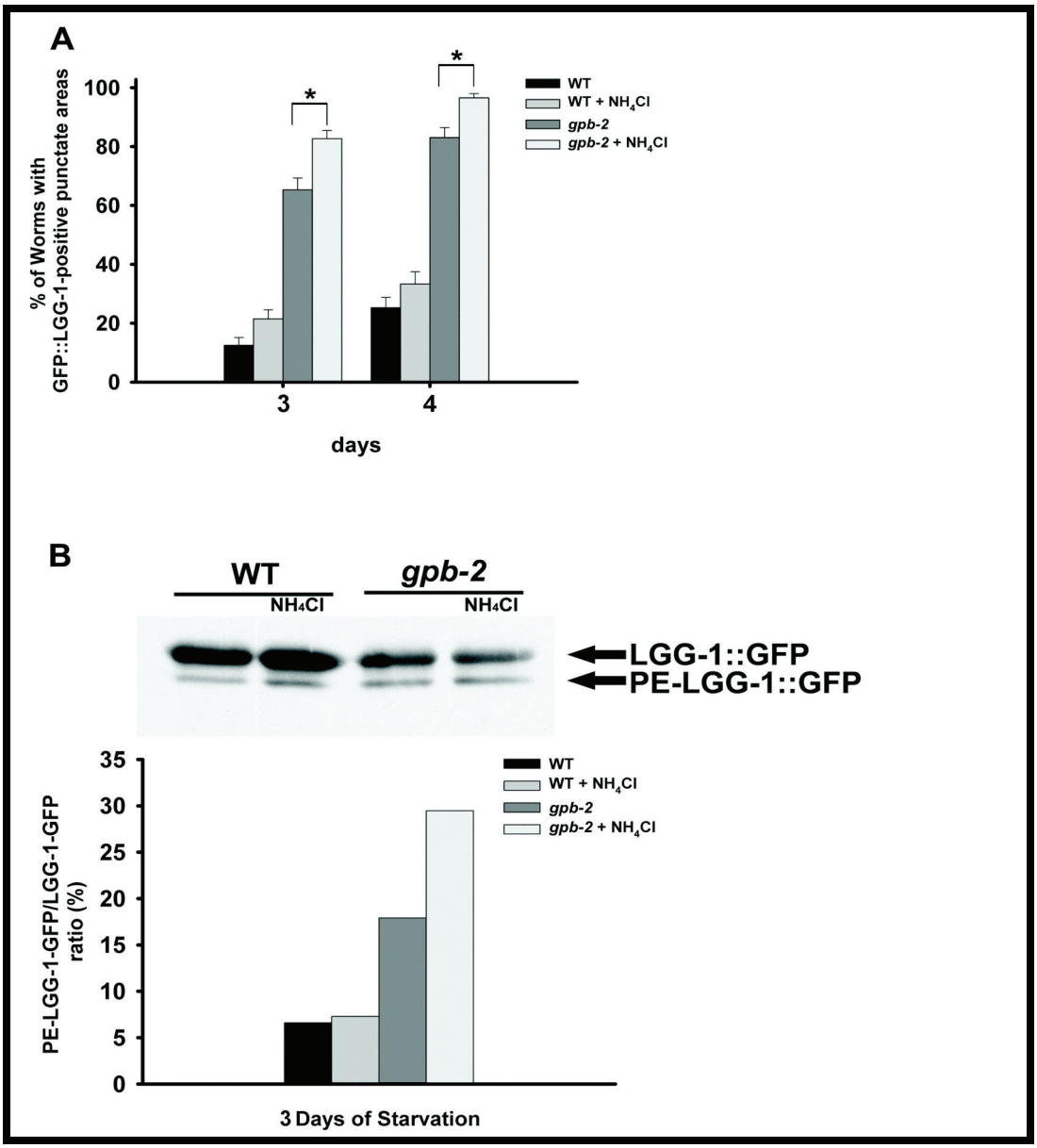


Figure 2.6. The effect of treatment with lysosomal inhibitor (Ammonium chloride) on autophagy in the pharyngeal muscle of wild-type and *gpb-2* mutants after starvation.
(A) Quantitation of autophagy in the pharyngeal muscles of indicated genotype after incubation in M9 buffer in the absence of food with or without ammonium chloride (10mM) for the indicated time. Error bars indicate standard error for proportions. *P<0.001.
(B) The relative levels of PE-conjugated LGG-1-GFP and LGG-1-GFP were determined by Western blotting. Starved L1 worms were prepared and analyzed by immunoblotting using an anti-GFP antibody. The arrows indicate LGG-1-GFP and PE-conjugated LGG-1-GFP. The Western blot is representative of what was seen in two independent experiments.

To identify downstream target genes of muscarinic acetylcholine receptor signaling responsible for the regulation of autophagy, I used RNA interference (RNAi) to reduce the expression of several candidate genes. One of them is *dapk-1*, the *C. elegans* ortholog of death-associated protein kinase (DAP kinase). Previous cell culture studies that showed that DAP kinase mediates autophagy (Inbal et al., 2002), together with the fact that ERK can phosphorylate and activate DAP kinase in cell culture (Chen et al., 2005), led me to hypothesize that muscarinic signaling regulates autophagy through DAP kinase. I found that *dapk-1* RNAi reduces starvation-induced autophagy and partially rescues starvation hypersensitivity of *gpb-2* mutants (data not shown). To verify the RNAi result, I made *dapk-1 gpb-2* double mutants and found that mutation of *dapk-1* also decreases starvation-induced autophagy (Figure 2.7A) and partially rescues death of *gpb-2* mutants (Figure 2.7B). It is unlikely that mutation of *dapk-1* rescues death of *gpb-2* mutants during starvation by the inhibition of apoptosis because

mutation of *ced-3* did not rescue death of *gpb-2* mutants (data not shown). Since mutation of *dapk-1* only partially rescued *gpb-2* mutants, it is likely that other unknown signaling molecules also function downstream of muscarinic signaling to regulate autophagy. In fact, I found that mutation of *rgs-2*, a regulator of G protein signaling, further decreases autophagy in *dapk-1 gpb-2* mutants and rescues death of *dapk-1 gpb-2* during starvation (Figure 2.7C,D). It is possible, however, that RGS-2 negatively regulates muscarinic signaling to decrease autophagy through GOA-1, which is a target of RGS-2 and is known to antagonize muscarinic signaling (Miller et al., 1999). To test this possibility, I examined the activation of MPK-1 in *dapk-1 gpb-2*; *rgs-2* mutants. I found that MPK-1 is activated in *dapk-1 gpb-2*; *rgs-2* mutants as it is in *gpb-2* mutants (Figure 2.7E), suggesting that RGS-2 is unlikely to negatively regulate muscarinic signaling via a feedback loop. A previous study showing that GAIP, the mammalian ortholog of *rgs-2*, functions downstream of ERK to regulate autophagy in cell culture (Ogier-Denis et al., 2000) further supports my hypothesis that RGS-2 functions downstream of muscarinic signaling to regulate autophagy. Thus, these results suggest that DAPK-1 acts, at least in part, downstream of or in parallel to muscarinic signaling in the regulation of autophagy, possibly with RGS-2.

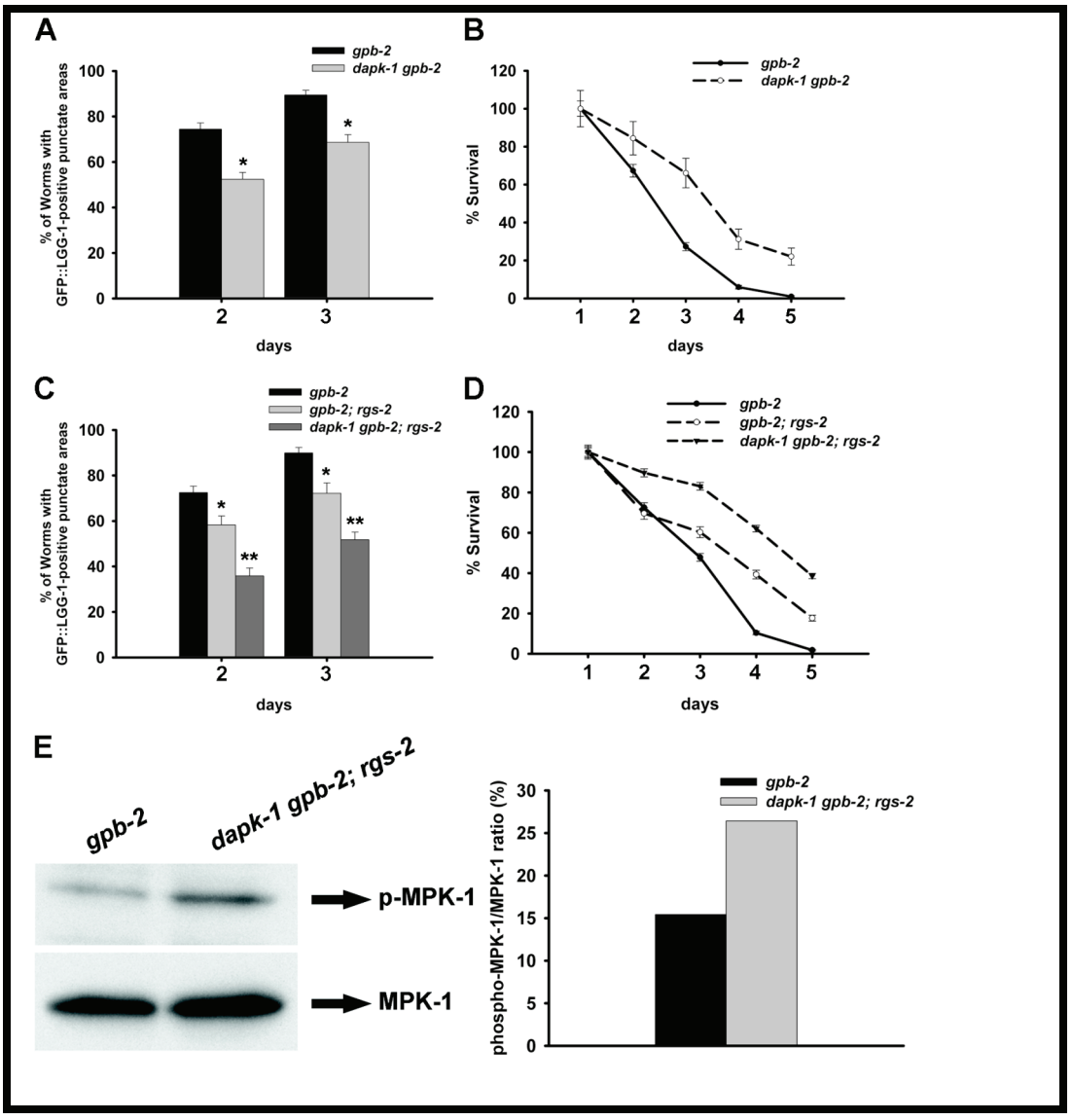


Figure 2.7. DAPK-1 acts downstream of or in parallel to muscarinic signaling in the regulation of autophagy, probably with RGS-2.
(A and C) Quantitation of autophagy in the pharyngeal muscles of indicated genotype after incubation in M9 buffer in the absence of food for indicated time. Error bars indicate standard error for proportions. *P<0.005 and **P<0.0001. (B and D) Percent of worms surviving to adulthood in A and C. Error bars are standard errors estimated assuming a Poisson distribution. (E) The relative levels of phospho-MPK-1 and MPK-1 were determined by Western blotting. Starved L1

worms were prepared 32.5hr after egg preparation and analyzed by immunoblotting using an anti-phospho-MPK-1 antibody and an anti-MPK-1 antibody. The Western blot is representative of what was seen in two independent experiments.

Excessive autophagy causes defects in the pharyngeal muscles of *gpb-2* mutants and contributes to death

To directly examine the role of excessive autophagy in the death of starved *gpb-2* mutants, I inhibited autophagy in these mutants by *bec-1* RNAi. *bec-1* RNAi treatment decreased autophagy in the pharyngeal muscle of starved *gpb-2* mutants (Figure 2.5, 2.8B). It also partially rescued the death of starved *gpb-2* mutants (Figure 2.8A), suggesting that excessive autophagy contributes to the death of the *gpb-2* mutants during starvation conditions. I also verified these results by *atg-7* RNAi (data not shown).

Starvation induces malfunction (hypercontraction) of pharyngeal muscle in *gpb-2* mutants after starvation, so that the worms cannot grind up their bacterial food (You et al., 2006). To investigate the effects of excessive autophagy in *gpb-2* mutants on the ability of pharyngeal muscle to grind bacteria, I used GFP-expressing *E. coli* (You et al., 2006). If worms grind bacteria efficiently, green fluorescence cannot be observed in their intestines. If not, however, the intestines are fluorescent with unground bacteria. As previously shown (You et al., 2006), *gpb-2* mutants cannot grind bacteria after starvation (Figure 2.8C). In contrast *gpb-2* mutants in which *bec-1* expression was reduced by RNAi can grind bacteria

after starvation (Figure 2.8D). Together, these results suggest that excessive autophagy causes defects in the pharyngeal muscles of *gpb-2* mutant and eventually contributes to death after starvation. It is possible, however, that excessive autophagy in other tissues contributes to death of *gpb-2* mutants.

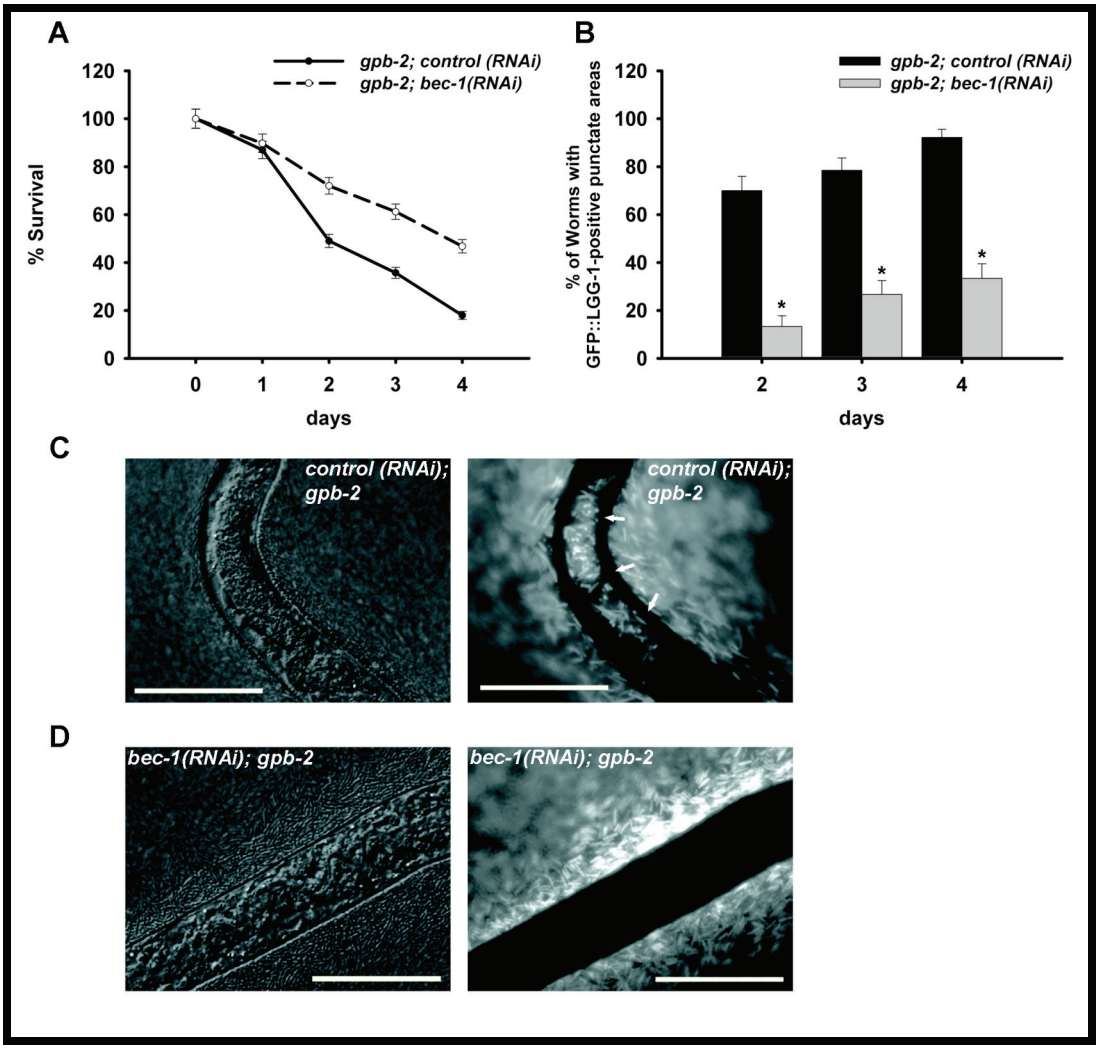


Figure 2.8. Unrestrained autophagy causes defects in the pharyngeal muscles of *gpb-2* mutants and contributes to death after starvation.
(A) Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution. (B) Quantitation of autophagy in A. *bec-1(RNAi)* reduced excessive levels of autophagy in the pharyngeal muscles of *gpb-2* during starvation. Error bars indicate standard error for proportions. *P<0.0001. (C and D) *gpb-2* control RNAi animals (C) and *gpb-2*; *bec-1(RNAi)* animals (D) after 2 days of starvation followed by growth on GFP-expressing *E. coli*. Differential interference contrast image (left) and green fluorescence image (right). Arrows in C indicate unground bacteria in the intestine. Scale bars denote 20μm.

Muscarinic signaling plays a role in regulating autophagy during starvation

My data in *gpb-2* mutants indicate that overactivated muscarinic signaling induces excessive autophagy during starvation. To evaluate whether muscarinic acetylcholine receptor signaling can also regulate autophagy in wild-type worms during starvation, I inhibited muscarinic signaling by treatment with atropine. Treatment with atropine decreased pharyngeal muscle autophagy below basal levels as compared to controls (Figure 2.5, 2.9A), indicating that blocking muscarinic signaling decreases the levels of autophagy in wild-type worms during starvation. Treatment with atropine also decreased survival of wild-type worms after starvation (Figure 2.9B), confirming that physiological levels of autophagy are required for optimal survival of wild-type worms under starvation. The survival of wild-type worms treated with atropine could be rescued partially by the addition of food (Figure 2.9B), suggesting that the effect of atropine on survival of wild-type worms during starvation may be due to decreases in

autophagy rather than drug toxicity only. I also found that treatment with U0126 reduced pharyngeal muscle autophagy and decreased survival of wild-type worms after starvation to lesser extent than treatment with atropine (data not shown). In contrast, a Ras gain-of-function mutation (which has a Gly to Glu substitution at codon 13 and mimics the effect of overactivated muscarinic signaling on MAPK) induced excessive autophagy in the pharyngeal muscles during starvation (Figure 2.5, 2.9C). It also decreased survival of worms after starvation (Figure 2.9D). Thus, overactivated muscarinic signaling increases the levels of autophagy above physiological levels during starvation. Taken together, these data suggest that muscarinic acetylcholine receptor signaling positively regulates autophagy in wild-type worms under starvation and is important for the optimal survival of starved wild-type worms.

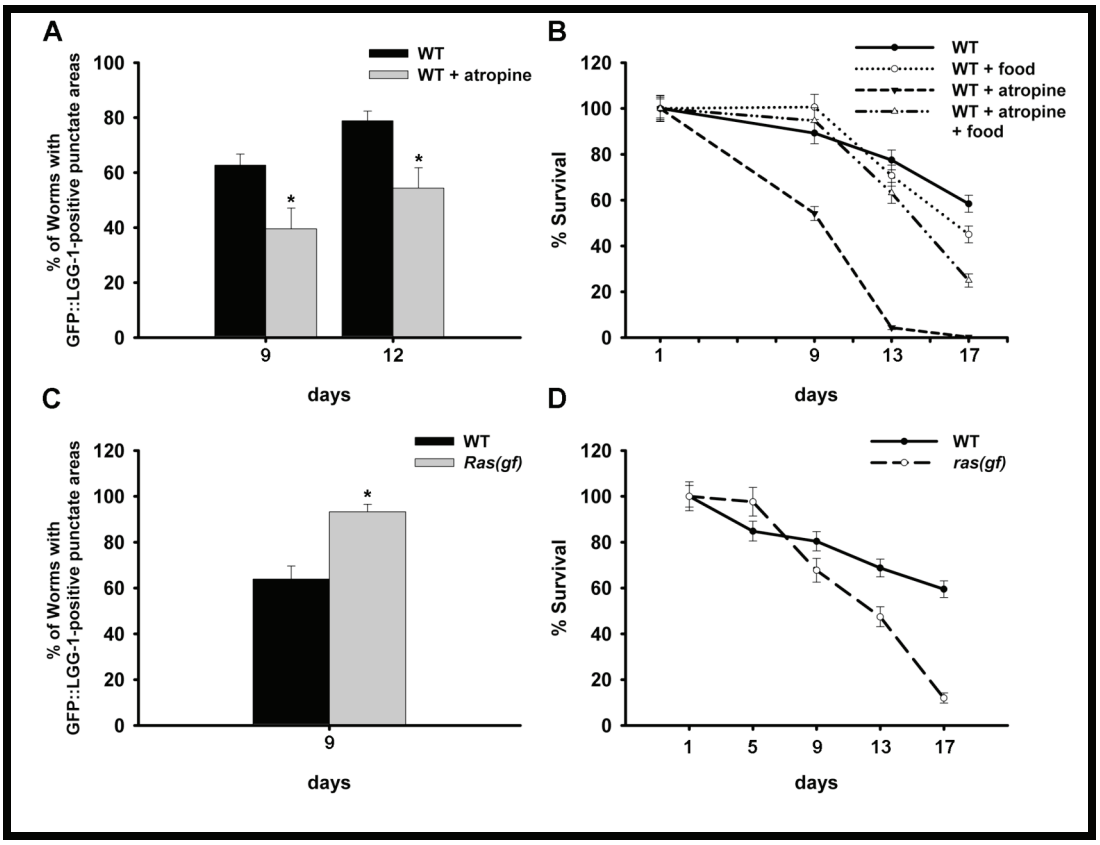


Figure 2.9. Muscarinic signaling positively regulates autophagy during starvation.

(A) Quantitation of autophagy in the pharyngeal muscles of wild-type animals treated with atropine (10mM) after incubation in M9 buffer in the absence of food for the indicated time. (C) Quantitation of autophagy in the pharyngeal muscles of *ras(gf)* animals. Error bars indicate standard error for proportions. *P<0.01. (B and D) Percent of worms surviving to adulthood in A and C. Error bars are standard errors estimated assuming a Poisson distribution.

Discussion

Dual roles of autophagy in the survival of a multicellular organism during starvation

My results demonstrate that physiological levels of autophagy are required for optimal survival of *C. elegans* during starvation and that muscarinic acetylcholine receptor signaling is important for the induction of physiological levels of autophagy. In contrast, insufficient or excessive levels of autophagy render *C. elegans* hypersensitive to starvation. These results lead me to conclude that autophagy can play opposing roles promoting survival or death at the organismal level during starvation, depending on its level (Figure 2.10). The mechanism by which autophagy plays dual roles with respect to survival was not well understood until recently. As described in the Introduction, previous studies suggested the intriguing possibility that it is the level of autophagy that is critical in deciding between a pro-survival or a pro-death role (Pattingre et al., 2005). My results provide in vivo evidence that a similar model may apply at the whole organism level: physiological levels of autophagy are pro-survival, whereas insufficient or excessive levels of autophagy are pro-death.

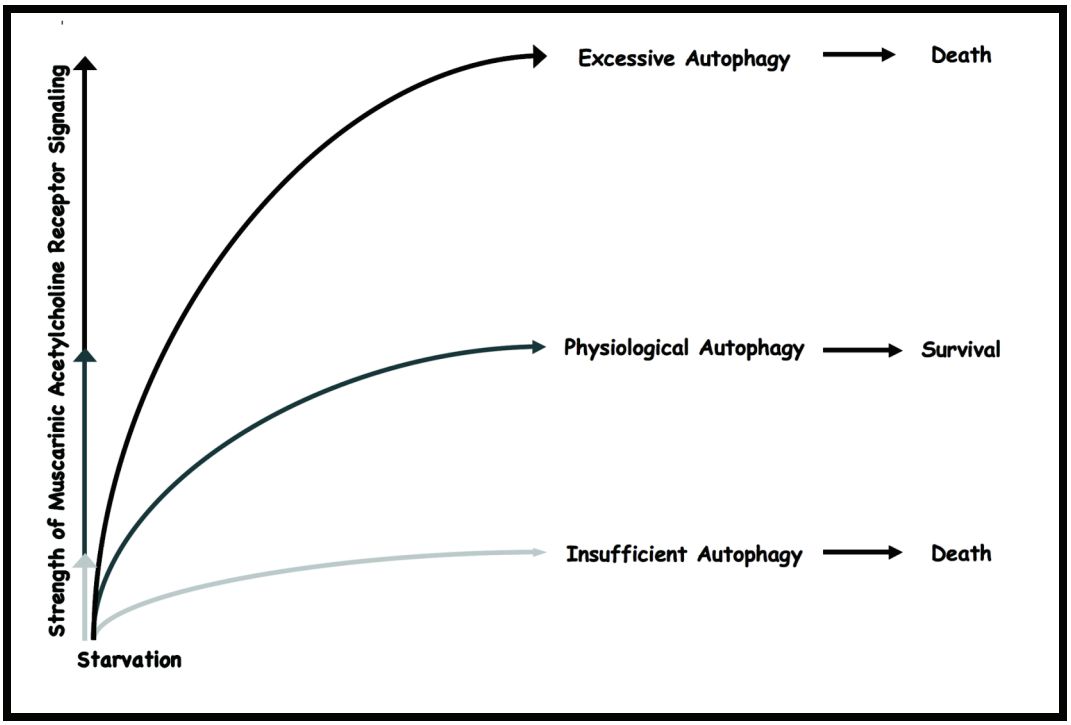


Figure 2.10. Model of dual roles of autophagy in the survival of *C. elegans*.

The muscarinic acetylcholine pathway as an autophagy-inducing signaling pathway

During the past decade, molecular mechanisms involved in the regulation of autophagy have been discovered (Codogno and Meijer, 2005; Levine and Klionsky, 2004; Yorimitsu and Klionsky, 2005). Despite this significant progress, the signaling pathway involved in the regulation of autophagy at the organismal level has been extensively characterized only in the context of autophagy-inhibiting signals. It is well known that the type I PI3K-mTOR signaling pathway negatively regulates autophagy at both the cellular and organismal levels

(Codogno and Meijer, 2005; Scott et al., 2004; Shintani and Klionsky, 2004). Although several pathways such as eIF2α kinase, ERK1/2, and DAP kinase pathways have been implicated as autophagy-inducing signaling pathways at the cellular level (Inbal et al., 2002; Pattingre et al., 2003; Talloczy et al., 2002), little is known about autophagy-inducing signaling pathways in the multicellular organism. My results demonstrate that the muscarinic acetylcholine pathway functions as an autophagy-inducing signaling pathway in the pharyngeal muscles of *C. elegans* during starvation. I found that during starvation, overactivation of muscarinic signaling in *gpb-2* mutants causes excessive autophagy in the pharyngeal muscles, whereas inhibition of muscarinic signaling either by treatment with inhibitors or by mutation decreases autophagy in the pharyngeal muscles. Furthermore, I found that either mutation of *dapk-1* or *rgs-2* reduces starvation-induced autophagy and partially rescues the death of *gpb-2* during starvation, suggesting that DAPK-1 and perhaps RGS-2 may act, at least in part, downstream of or in parallel to muscarinic signaling in the regulation of autophagy. My results combined with the previous study showing that starvation activates muscarinic acetylcholine signaling in the pharyngeal muscles of *C. elegans* (You et al., 2006) suggest that the muscarinic acetylcholine signaling pathway positively regulates autophagy in response to starvation, probably through DAP kinase and RGS-2.

Previous studies showed that the muscarinic acetylcholine signaling pathway increases pumping rates to enhance feeding response by altering pharyngeal muscle physiology as a response to starvation (You et al., 2006). This change may render worms more competent to digest food when food becomes available once again. Because it is obvious that this change is an energy-demanding process, the question remains how energy is provided to increase pumping rates during starvation. Since it is known that autophagy can provide energy to maintain energy homeostasis (Kuma et al., 2004; Lum et al., 2005), one intriguing possibility is that autophagy in the pharyngeal muscles induced by the muscarinic acetylcholine signaling pathway may provide the energy needed to increase pumping rates. In fact, my result that shows reduced autophagy by *bec-1* RNAi decreases pumping rates during starvation supports this possibility.

Several lines of evidence indicate that autophagy may play important roles in tumor development (Cuervo, 2004; Kondo et al., 2005; Levine, 2006; Levine and Yuan, 2005). Autophagy can act to suppress cancer development by removing damaged organelles and promoting autophagic cell death. On the other hand, autophagy can contribute to cancer development by providing an energy source to cancer cells located far from the blood supply, where nutrients are extremely limited. My observation that overactivation of the Ras signaling pathway induced autophagy, combined with previous studies showing that Ras signaling is frequently overactivated in various cancers, leads to the intriguing

hypothesis that autophagy activated by overactivated Ras signaling may contribute to the survival of cancer cells under nutrient-limited conditions. However, this hypothesis must be viewed with caution because Ras signaling is known to inhibit autophagy in other systems and autophagy can function as a tumor suppressor depending on the circumstances (Kondo et al., 2005; Levine, 2006; Levine and Yuan, 2005). Nevertheless, it would be interesting to test if inhibition of autophagy affects the survival of cancer cells in the central area of the large tumor masses, where the nutrient supply is limited.

CHAPTER IV

Systemic Regulation of Starvation Response in *Caenorhabditis elegans*

Abstract

When the supply of environmental nutrients is limited, multicellular animals can make both physiological and behavioral changes so as to cope with nutrient starvation. Although physiological and behavioral effects of starvation are well known, the mechanisms by which animals sense starvation systemically remain elusive. Furthermore, what constituent of food is sensed and how it modulates starvation response is still poorly understood. In this study, I use a starvation-hypersensitive mutant to identify molecules and mechanisms that modulate starvation signaling. I found that specific amino acids could suppress the starvation-induced death of *gpb-2* mutants, and that MGL-1 and MGL-2, *C. elegans* homologs of metabotropic glutamate receptors, were involved. MGL-1 and MGL-2 acted in AIY and AIB neurons respectively. Treatment with leucine suppressed starvation-induced stress resistance and life span extension in wild-type worms, and mutation of *mgl-1* and *mgl-2* abolished these effects of leucine. Taken together, my results suggest that metabotropic glutamate receptor homologs in AIY and AIB neuron may modulate a systemic starvation response, and that *C. elegans* senses specific amino acids as an anti-hunger signal.

Introduction

During nutritional deprivation, individual cells can respond to starvation by modulating intracellular signaling, which in turn induces a starvation response and thereby enhances their survival (Levine and Kroemer, 2008; Levine and Yuan, 2005; Lum et al., 2005). An important starvation response of individual cells is a change of metabolism (inhibiting anabolic pathways and activating catabolic pathways), so as to generate metabolic substrates to maintain basal cellular activities (Lum et al., 2005). In multicellular organisms, however, the situation is complicated by the need to induce behavioral changes in addition to physiological changes, and more importantly by the danger that uncoordinated starvation responses in individual cells could be harmful to the organism. In *Caenorhabditis elegans*, for example, I have previously reported that excessive autophagy in pharyngeal muscle causes its malfunction, which eventually prevents the recovery of worms from starvation (Kang et al., 2007). Thus, it is important that multicellular organisms ensure their starvation response is coordinated between individual cells, and therefore it is plausible to assume that there are mechanisms by which animals sense starvation systemically.

Since animals cannot synthesize several amino acids, so-called “essential acids”, they must ingest these amino acids from external food sources to maintain homeostasis (Gietzen and Rogers, 2006). This fact leads to the intriguing

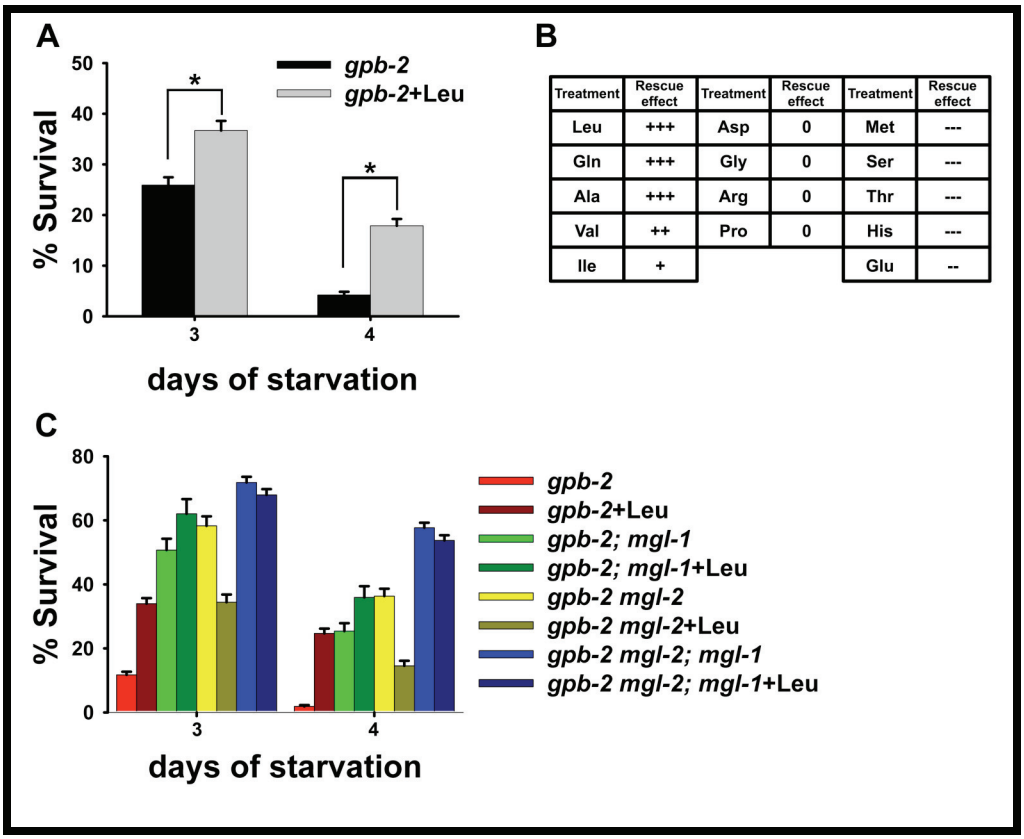
possibility that amino acids act as a food or anti-hunger signal. Indeed, treatment with amino acids can inhibit starvation-induced autophagy in cultured mammalian cells (Codogno and Meijer, 2005). Furthermore, at the organismal level, intracerebroventricular administration of leucine causes a decrease in food intake in the rat (Cota et al., 2006), suggesting that the possibility could be true. But it is not known whether amino acids modulate a starvation response in multicellular organisms.

Results and Discussion

To examine the effect of amino acids on starvation signaling in *Caenorhabditis elegans*, I screened several amino acids for suppression of the death of the starvation-hypersensitive *gpb-2* mutant. Our lab previously showed that starvation signaling is overactivated in this background (Kang and Avery, 2008; Kang et al., 2007; You et al., 2006). I found that treatment with a subset of amino acids including leucine, glutamine, alanine, valine, and isoleucine can rescue the death of *gpb-2* mutants during starvation, whereas other amino acids didn't rescue or worsened survival (Figure 3.1A,B). Given the specificity of the rescue effect, it is unlikely that amino acids act as a carbon source to maintain nutrient homeostasis; rather it suggests that amino acids might act as signaling molecules that modulate a starvation response (Hereafter, I use leucine as a representative amino acid).

It has recently been shown that a broad spectrum of amino acids can be sensed by class 3 G-protein-coupled receptors, which include the extracellular calcium sensing receptor (CaR)(Klionsky et al.), heterodimeric taste receptors and GPRC6A (Conigrave and Hampson, 2006). MGL-1 and MGL-2 metabotropic G-protein-coupled glutamate receptors are the closest homologs of CaR in the *C. elegans* genome (Dillon et al., 2006). Thus, I hypothesized that MGL-1 and MGL-2 were involved in the modulation of amino acid response and starvation

response. To test this, I made *gpb-2; mgl-1*, *gpb-2 mgl-2* and *gpb-2 mgl-2; mgl-1* mutants. Mutation of either *mgl-1* or *mgl-2* partially rescued death of *gpb-2* mutants during starvation, and double mutation caused an additive effect, suggesting that *mgl-1* and *mgl-2* modulate a starvation response in a parallel manner (Figure 3.1C). Mutation of either *mgl-1* or *mgl-2* didn't abolish the response to leucine, while mutations of both *mgl-1* and *mgl-2* made *gpb-2* mutants resistant to leucine treatment, suggesting that both *mgl-1* and *mgl-2* are involved.



Treatment	Rescue effect	Treatment	Rescue effect	Treatment	Rescue effect
Leu	+++	Asp	0	Met	---
Gln	+++	Gly	0	Ser	---
Ala	+++	Arg	0	Thr	---
Val	++	Pro	0	His	---
Ile	+			Glu	--

Figure 3.1. A subset of amino acids rescues the starvation hypersensitivity of *gpb-2* mutants, and *mgl-1* and *mgl-2* are involved in the process.
Starvation survival analyses were performed as described in Materials and Methods. (A) Treatment with leucine partially rescues the starvation

hypersensitivity of *gpb-2* mutants. Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food with or with leucine for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution, and similar results were obtained in three independent experiments. (*) $P<0.001$ (chi-squared test of independence). (B) Summary of the rescue effect of amino acids on the survival of *gpb-2* mutants during starvation. Plus, zero, and minus signs indicate positive, no, and negative effects on survival respectively. (C) Mutation of either *mgl-1* or *mgl-2* can rescue death of *gpb-2* mutants during starvation, and double mutation causes an enhanced rescue effect. Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food with or without leucine for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution, and similar results were obtained in three independent experiments.

One of the causes of death in *gpb-2* mutants during starvation is excessive autophagy in pharyngeal muscle, which causes malfunction of the pharynx, the *C. elegans* feeding organ (Kang et al., 2007). To test whether *mgl-1* and *mgl-2* are involved in the modulation of autophagy in pharyngeal muscle of *gpb-2* mutants during starvation, I generated mutant strains carrying an integrated transgene that expressed a GFP tagged version of LGG-1, a specific marker for autophagy (Kang et al., 2007; Klionsky et al., 2008; Melendez et al., 2003). I found that mutation of either *mgl-1* or *mgl-2* decreased the excessive levels of autophagy in pharyngeal muscle, and double mutation showed an additive effect on decreasing autophagy, suggesting that *mgl-1* and *mgl-2* can modulate autophagy in a parallel manner (Figure 3.2). Treatment with leucine also decreased excessive levels of autophagy during starvation, consistent with the hypothesis that amino acids act as anti-hunger signals. However, leucine had no effect on levels of autophagy

when *mgl-1* and *mgl-2* were both mutated, suggesting that they are necessary for the modulation of autophagy by amino acids.

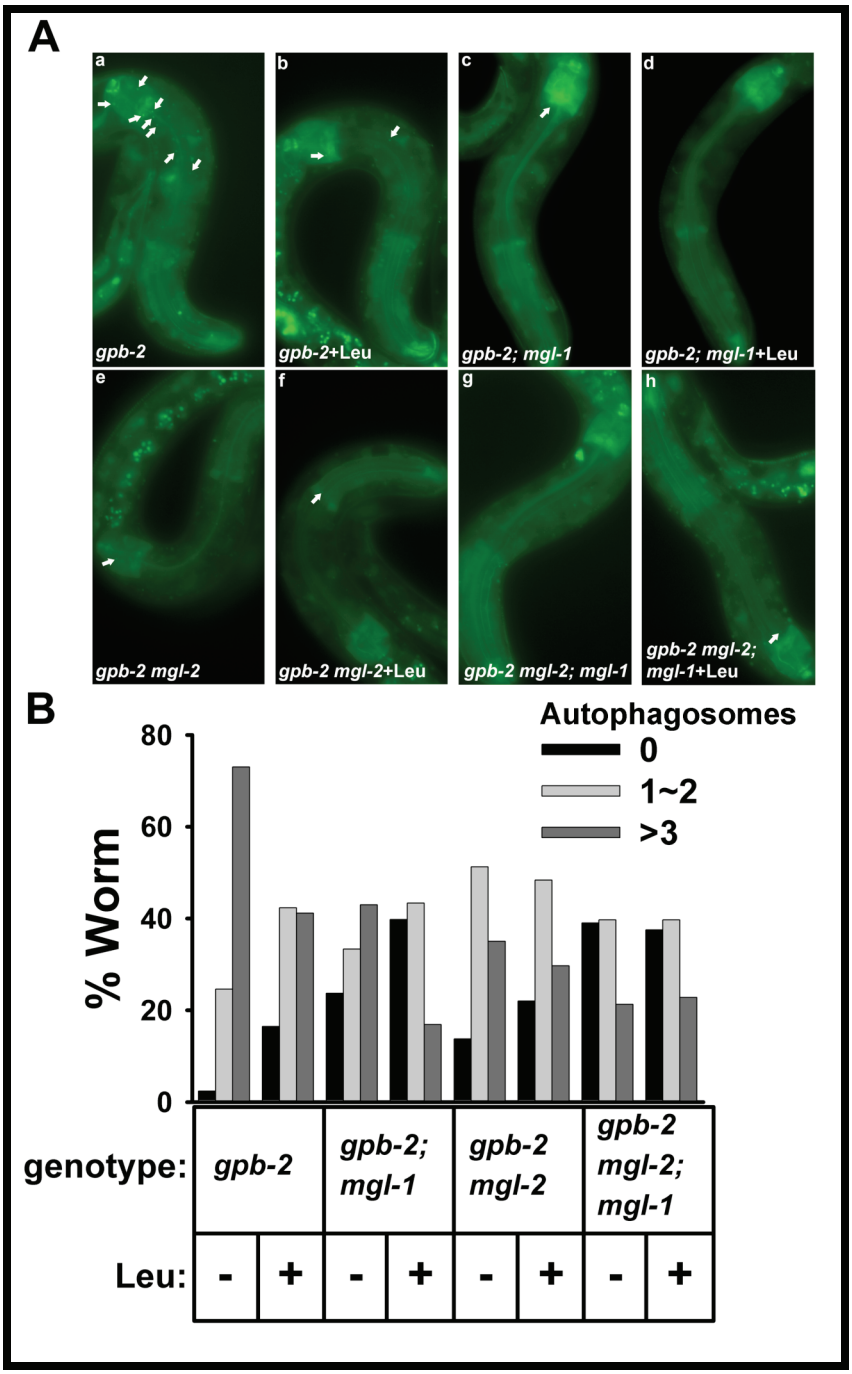


Figure 3.2. *mgl-1* and *mgl-2* modulate autophagy in the pharyngeal muscles of *gpb-2* mutants during starvation.
(A) Representative images of indicated mutants with or without leucine after 3 days of starvation. The arrows show representative GFP::LGG-1 positive punctate structures that label preautophagosomal and autophagosomal structures. (B) Quantification of autophagy in the pharyngeal muscles of worm of the indicated genotype after 3 days of starvation with or without leucine. Data come from three independent experiments (n=80~141).

It has been reported that *mgl-1* and *mgl-2* are mainly expressed in a limited number of neurons (www.wormbase.org). These expression patterns, together with the results showing that *mgl-1* and *mgl-2* can modulate levels of autophagy in pharyngeal muscles (Figure 3.2), lead to the intriguing hypothesis that *mgl-1* and *mgl-2* can modulate a starvation response in a cell non-autonomous manner. To test this, I examined whether expression of *mgl-1* or *mgl-2* in specific neurons could restore the starvation-sensitivity of *gpb-2; mgl-1* and *gpb-2 mgl-2* mutants. Among the neurons where *mgl-1* and *mgl-2* are expressed, AIY and AIB are particularly interesting because it has recently been reported that AIY and AIB neurons control food- and odor-evoked behaviors (Chalasani et al., 2007). I found that AIY specific expression of *mgl-1* and AIB specific expression of *mgl-2* could restore the starvation-sensitivity of *gpb-2; mgl-1* and *gpb-2 mgl-2* mutants respectively, supporting our hypothesis that *mgl-1* and *mgl-2* act in AIY and AIB neurons respectively to modulate a starvation response (Figure 3.3A,B).

Based on homology, *mgl-1* encodes a group II glutamate receptor, expected to inhibit adenylyl cyclase activity, which is likely to decrease neuronal

activity, whereas *mgl-2* encodes a group I glutamate receptor, expected to stimulate phospholipase C and likely increase neuronal activity (Dillon et al., 2006). To test whether *mgl-1* and *mgl-2* modulate starvation response by regulating the activity of AIY and AIB neurons respectively, I killed AIY and AIB neurons by using AIY and AIB specific expression of *egl-1*, the BH3-only protein that promotes cell death (Chang et al., 2006; Conradt and Horvitz, 1998).

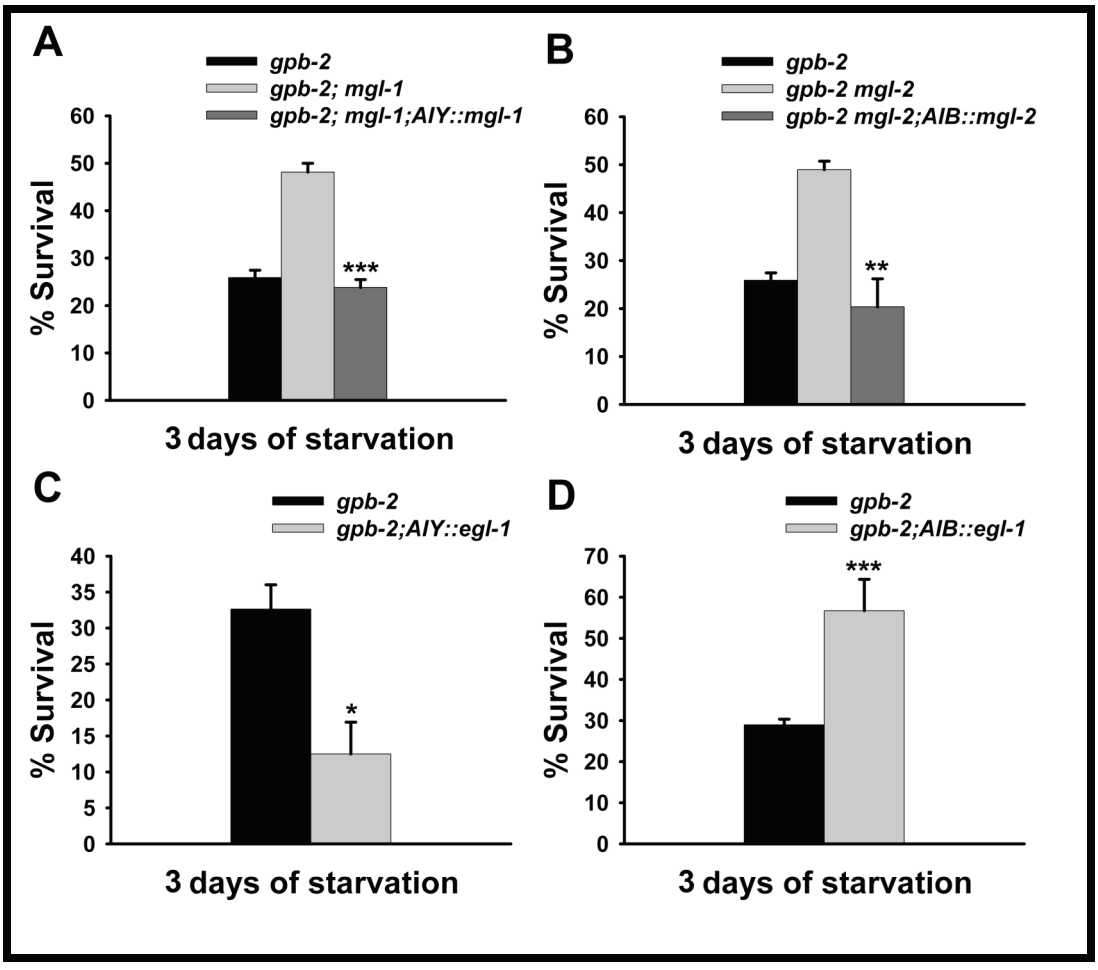


Figure 3.3. *mgl-1* and *mgl-2* mainly act in AIY and AIB neurons respectively. (A-D) Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution, and similar results were obtained in three independent experiments. (*) $P<0.05$, (**) $P<0.01$, (***) $P<0.001$ (chi-squared test of independence). (A and B) AIY specific expression of *mgl-1* and AIB specific expression of *mgl-2* restore the starvation-sensitivity of *gpb-2; mgl-1* and *gpb-2 mgl-2* respectively. Data for *gpb-2* single mutants in A and B are from Fig. 1A. (C and D) AIY specific expression of *egl-1* exacerbates death of *gpb-2* mutants during starvation, whereas AIB specific expression of *egl-1* rescues it.

I found that AIY specific expression of *egl-1* exacerbates the starvation sensitivity of *gpb-2* mutants and that AIB specific expression of *egl-1* rescues *gpb-2* mutants (Figure 3.3C,D), suggesting that changes in the neuronal activities of AIY and AIB are sufficient to modulate the starvation response. These results, together with recent findings showing that AIY is activated by food- or odor presentation and AIB is activated by food- or odor removal, suggest that AIY inhibits the starvation response, whereas AIB activates it.

Since *mgl-1* and *mgl-2* are similar to metabotropic glutamate receptors, it is possible that glutamate neurotransmission is involved in the modulation of *mgl-1* and *mgl-2*. To test this possibility, I looked at the effect of *mgl-1* and *mgl-2* on starvation survival in an *eat-4* mutant background. *eat-4* encodes a vesicular glutamate transporter, and glutamate neurotransmission is impaired in *eat-4* mutants (Lee et al., 1999), including transmission from the known presynaptic partners of AIY and AIB (Chalasani et al., 2007). If amino acids modulate the

activity of *mgl-1* and *mgl-2* through glutamate neurotransmission, one would expect mutation of *eat-4* to abolish the effects of *mgl-1*, *mgl-2*, and leucine. However, this is not the case. *mgl-1, mgl-2*, and leucine have pronounced effects on starvation survival in the absence of *eat-4*. This suggests that glutamatergic neurotransmission is not necessary for the activation of MGL-1 and MGL-2. We suggest, rather, that amino acids can directly modulate the activity of *mgl-1* and *mgl-2* (Figure 3.4). An alternative possibility, however, is that other vesicular glutamate transporters function redundantly with *eat-4*, and therefore they modulate the activity of *mgl-1* and *mgl-2*.

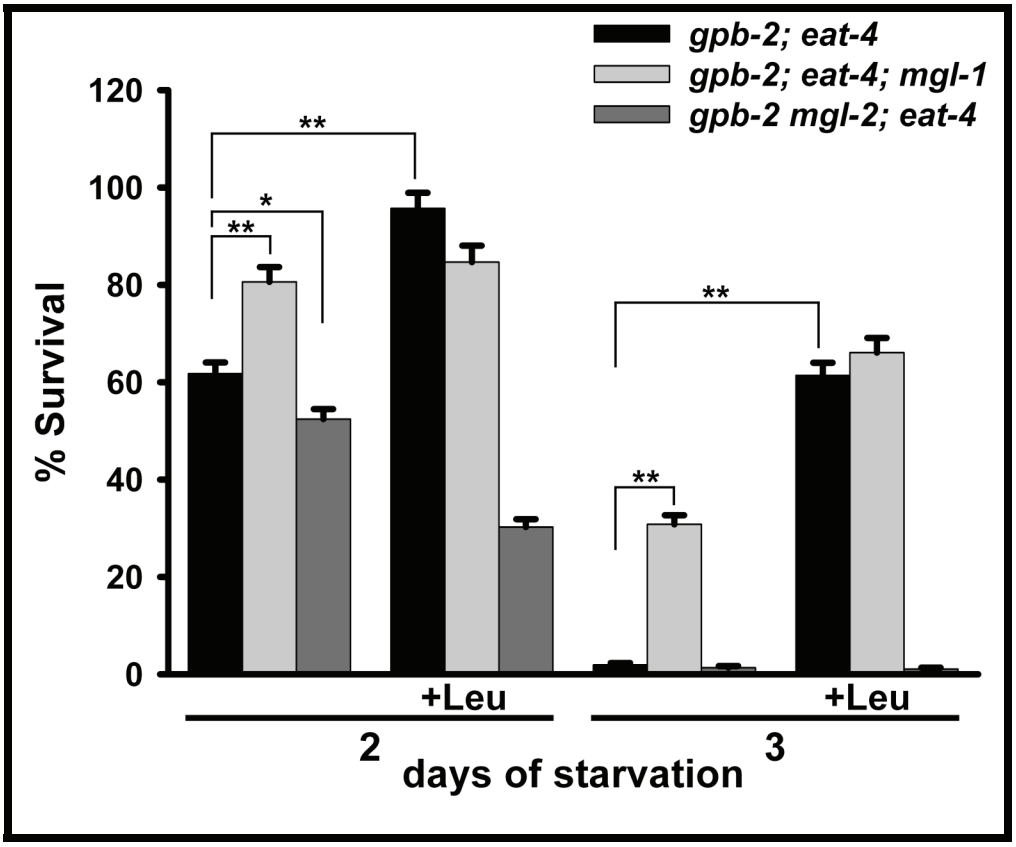


Figure 3.4. The effects of *mgl-1*, *mgl-2*, and leucine on starvation survival in an *eat-4* mutant background.
Percent of worms surviving to adulthood on NGM plates with HB101 bacteria after incubation in M9 buffer in the absence of food with or with leucine for the indicated time. Error bars are standard errors estimated assuming a Poisson distribution, and similar results were obtained in three independent experiments. (*) P=0.015, (**) P<0.0001 (chi-squared test of independence). *mgl-1*, *mgl-2*, and leucine affect starvation survival in the absence of *eat-4*. (*eat-4* also affects survival in some backgrounds– compare with Figure 3,1C – but since *eat-4* is broadly expressed, this effect cannot be simply interpreted.)

My data in *gpb-2* mutants indicate that amino acids act as an anti-hunger signal and that *mgl-1* and *mgl-2* are involved in the systemic starvation response. To evaluate whether treatment with amino acids can also suppress starvation response in wild-type worms and whether *mgl-1* and *mgl-2* are involved in that process, I took advantage of the concept of starvation-induced hormesis. Hormesis indicates beneficial effects of low-doses of treatments known to be harmful at higher doses (Gems and Partridge, 2008). It has been reported that short-term starvation (1~2 days) increases oxidative-stress resistance and extends lifespan in *C. elegans* (Cypser et al., 2006; Weinkove et al., 2006). I confirmed that starvation induces heat-shock resistance, oxidative stress resistance, and lifespan extension in wild-type worms, and found that treatment with leucine partially suppressed these effects of starvation, suggesting that amino acids act as an anti-hunger signal in wild-type worms (Figure 3.5A, B, C). More interestingly, I didn't observe the effect of leucine on the starvation-induced heat-shock resistance, oxidative stress resistance, and lifespan extension in *mgl-2; mgl-1*

mutants, suggesting that *mgl-1 and mgl-2* are likely involved in wild-type amino acid responses, as they are in the *gpb-2* starvation response (Figure 3.5A, B, D).

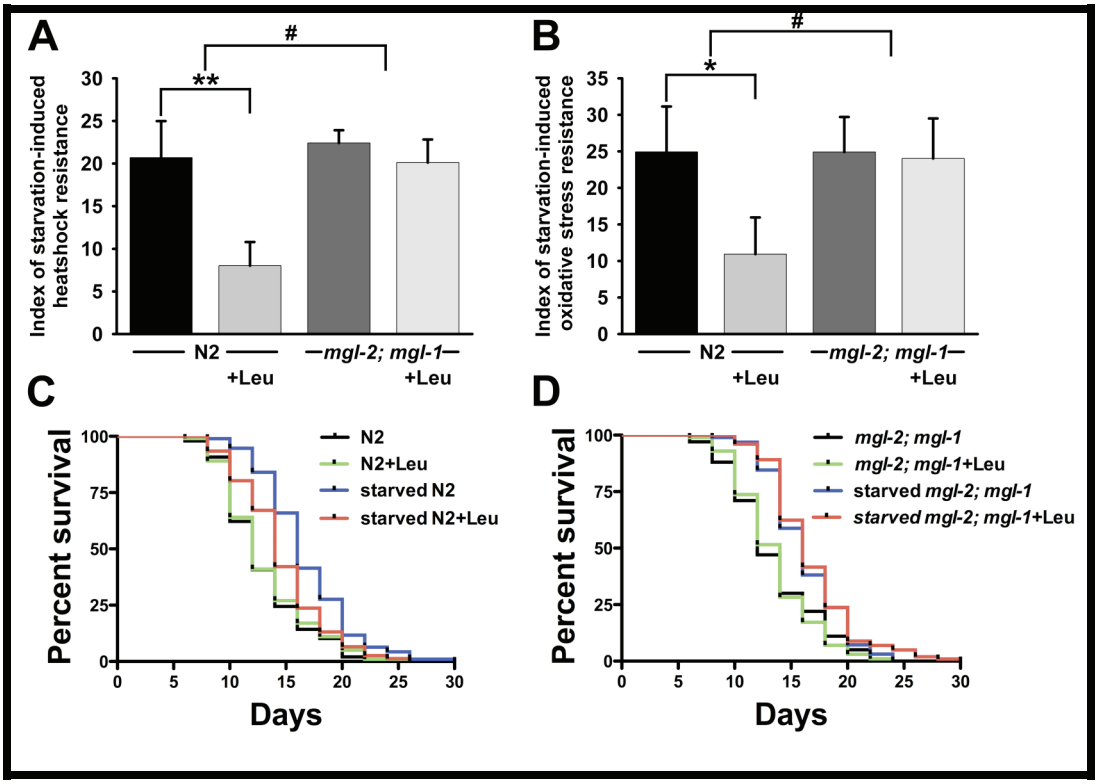


Figure 3.5. Starvation induces hormesis, treatment with leucine partially suppresses it in wild-type worms, and the effect of leucine is abolished in *mgl-2; mgl-1* mutants.
(A and B) Indexes of starvation induced heat-shock resistance and oxidative stress resistance were calculated as described in Materials and Methods. Starvation induces heat-shock resistance and oxidative stress resistance, treatment with leucine partially suppresses it in wild-type worms, and the effect of leucine is not observed in *mgl-2; mgl-1* mutants. Data come from more than four independent experiments. (*) P<0.05 (**) P <0.01 (Student's t-test, paired) (#) P<0.05 (Student's t-test, unpaired). (C and D) Life span curves represent combined data from two independent experiments. Starvation extends life span, and treatment with leucine partially suppresses the lifespan extension in wild-type worms

(starved N2, 16.74±0.04 v.s. starved N2 with leucine, 14.61±0.05, P<0.005, Mantel-Cox Log-rank test). Effect of leucine is not observed in *mgl-2; mgl-1* mutants (starved *mgl-2; mgl-1*, 16.23±0.03 v.s. starved *mgl-2; mgl-1* with leucine, 16.73±0.04).

It is reasonable to think that the stress response should be coordinated in multicellular organisms, compared to individual cells because uncoordinated stress response in individual cells within multicellular organisms could impair the function of tissues, and eventually be harmful for the survival of organisms. However, little is known about whether the stress response previously thought to be cell-autonomous can be systemically regulated. A recent study elegantly showed that, in *C. elegans*, the heat shock response is systemically regulated through the activity of AFD (Prahlad et al., 2008), which was previously known to senses ambient temperature (Mori, 1999). By analogy, my study suggests that the starvation response can be systemically regulated through *mgl-1* and *mgl-2*, presumably regulating the activity of AIY and AIB interneurons (Figure 3.6), which were previously known to modulate behaviors in response to food- or odor presentation or removal (Chalasani et al., 2007).

Since both AIY and AIB are amphid interneurons, it is unlikely that they directly sense environmental amino acids. It is possible that AIY and AIB receive synapses from unknown sensory neurons that directly sense environmental amino acids, so as to modulate a systemic starvation response depending on that information. However, it is also possible that AIY and AIB directly respond to

internal amino acids, previously absorbed from the environment. The latter possibility is plausible based on the fact that, in rodents, a sensory role for either taste or odor is not involved in the sensing of essential amino acids, and it is suggested that a chemosensor for essential amino acids is in the brain (Gietzen and Rogers, 2006). More importantly our *eat-4* mutation data suggest that glutamate neurotransmission is not involved in the regulation of *mgl-1* (AIY) and *mgl-2* (AIB) in response to amino acids, suggesting the possibility that *mgl-1* and *mgl-2* may directly sense internal amino acids as in the case of extracellular calcium sensing receptor. I can't exclude the possibility that unknown sensory neurons use a vesicular glutamate transporter other than *eat-4* or that MGL-1 and MGL-2 respond to a different transmitter. It would be interesting to test if AIY and AIB could directly sense amino acids by calcium-imaging.

In summary my data suggest that *mgl-1* and *mgl-2* are involved in the two related systemic processes, amino acid response and starvation response. My current model (Fig 5) suggests that the amino acid signal, which may be a component of the food signal, activate AIY neurons and inhibit AIB neurons by modulating the activities of MGL-1 and MGL-2, respectively. AIY then inhibits the starvation response, whereas AIB activates it. Given the fact there is no direct connection between AIY/AIB neurons and pharyngeal muscle, it is reasonable to think that specific peptides are secreted from AIY and AIB neurons, which act on several tissues to modulate the systemic starvation response. It is also possible

that AIY and AIB neurons regulate specific downstream neurons, which are then responsible for the secretion of specific peptides to modulate the systemic starvation response. Further experiments are needed to elucidate whether the systemic signal is indeed one or more neuropeptides, and if so which peptides regulate the systemic starvation response downstream of AIY and AIB. My model system, the starvation-hypersensitive *gpb-2* mutants, might be helpful to find such peptide signals.

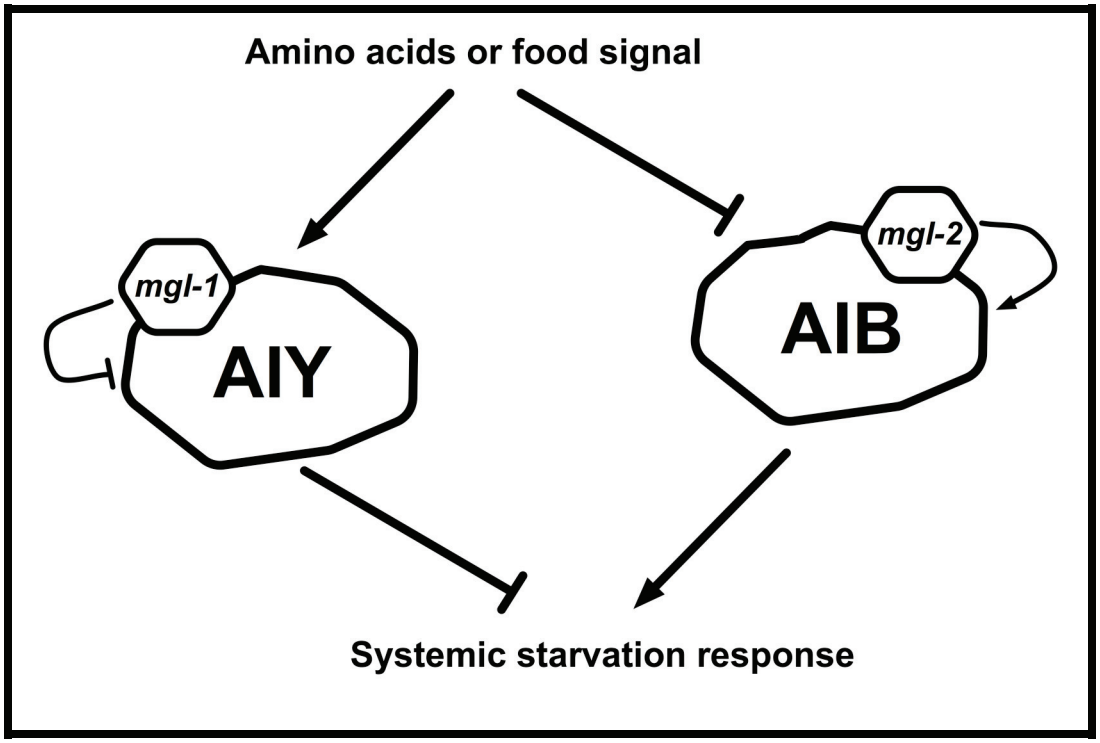


Figure 3.6. Model of the systemic regulation of starvation response in *C. elegans*.
Amino acids or food signal modulates the activity of AIY and AIB neurons, probably through MGL-1 and MGL-2 respectively, which in turn regulate the systemic starvation response.

CHAPTER V

Conclusions and Future Directions

Dual roles of autophagy in the survival of *Caenorhabditis elegans*

In this study, I showed that physiological levels of autophagy promote optimal survival of *C. elegans* during starvation, whereas insufficient or excessive levels of autophagy render *C. elegans* hypersensitive to starvation. I also showed that muscarinic acetylcholine receptor signaling induces physiological levels of autophagy through DAP kinase and RGS-2 during starvation. These data provide *in vivo* evidence that levels of autophagy, presumably regulated by muscarinic signaling, are critical at the organismal level in deciding between the prosurvival and prodeath roles (Kang et al., 2007).

Based on the ability of autophagy to promote cell survival in response to metabolic stress, it has been suggested that autophagy may contribute to tumor development by providing an energy source to tumor cells located far from the blood supply where nutrients are extremely limited (Botti et al., 2006; Kondo et al., 2005; Levine, 2006; Levine and Yuan, 2005). Recent findings by White and colleagues showing that autophagy promotes cell survival in solid tumors support this hypothesis (Degenhardt et al., 2006). In this regard, my observation that the overactivation of the Ras signaling pathway induced autophagy, combined with the fact that Ras signaling is frequently overactivated in various cancers, leads to the intriguing hypothesis that autophagy activated by overactivated Ras signaling

may provide a survival advantage to cancer cells in the central area of the large tumor masses until vascular support can be established (Kang et al., 2007).

Autophagy is also believed to suppress tumors because monoallelic loss of *beclin 1* is frequently associated with human cancer, and because mice with heterozygous disruption of *beclin 1* are tumor-prone (Qu et al., 2003). Recent findings support the view that autophagy acts as a tumor suppressor mechanism by limiting genome damage and chromosomal instability (Karantza-Wadsworth et al., 2007; Mathew et al., 2007).

These two seemingly contradictory functions of autophagy suggest the possibility that autophagy can act either as cancer's friend or foe, depending on the progression of the tumor. Until vascular support is established (and thus nutrient limitation is resolved), autophagy provides a temporary survival advantage to tumor cells where they suffer from metabolic stress. After vascularization, autophagy instead suppresses tumor progression by limiting genome damage and chromosomal instability, and possibly by causing autophagic cell death. At this stage, other selective pressures drive cancer cells to gain additional mutations that impair the autophagy process and further tumor progression. In fact, recent studies (Gozuacik and Kimchi, 2006; Inbal et al., 2002; Reef et al., 2006) showing that well-known tumor suppressor genes (DAPK1 and $p19^{ARF}$) can induce autophagy suggest the possibility that mutations in these tumor suppressor genes may decrease the level of autophagy, thereby

inhibiting the tumor suppressor activity of autophagy and leading to further tumor progression. With respect to this possibility, it would be interesting to examine the timing of mutations that can affect the autophagy process during tumor progression.

In *C. elegans*, *gld-1* mutants are established as an *in vivo* model for germline tumor. Mutations in *gld-1* cause lethal germline tumors, shortening the lifespan of mutant worms. Recently, it has been shown that mutations of *daf-2* and *eat-2* confer resistance to these tumors (Pinkston et al., 2006). These findings, together with the fact that mutations of *daf-2* and *eat-2* also affect levels of autophagy, lead to the intriguing possibility that autophagy may be involved in the effect of *daf-2* and *eat-2* mutations on *C. elegans* a germline tumor. It will be interesting to test whether autophagy acts in either a positive or negative role in the *C. elegans* germline tumor model.

Systemic regulation of starvation response in *Caenorhabditis elegans*

In this study, I showed that the starvation response in *C. elegans* can be systemically regulated through *mgl-1* and *mgl-2*, presumably regulating the activity of AIY and AIB interneurons. I also showed that a subset of amino acids can act as an anti-hunger (food) signal in *C. elegans*. These are very well

exemplified by hormesis experiments, in which starvation induced stress responses should reflect responses at the organismal levels.

For systemic regulation of starvation response by AIY and AIB, it is reasonable to think that specific peptides or hormones act in the system, probably regulating specific receptors on several tissues. The starvation-hypersensitive *gpb-2* mutants will be very useful to find such peptide/hormone signals and receptors. Using powerful *C. elegans* forward genetic screens, mutations that abolish the rescue effect of leucine on *gpb-2* mutants during starvation can be found. This experiment may provide some clue about peptide/hormone signals or receptors involved in systemic regulation of the starvation response. It is also possible to perform a candidate approach if I can find specific genes (especially for receptor candidates), which satisfy the following criteria. (1) It should be expressed in many tissues. (2) Its potential ligand should be an internal cue. (3) Mutant worms show a starved phenotype. Finding such peptides, hormones or receptors would be interesting for a better understanding of the systemic starvation response.

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