STARVATION-SIGNALING IN THE NEMATODE CAENORHABDITIS ELEGANS USING REGULATOR OF G-PROTEINS GPB-2

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DEDICATION

I would like to thank the members of my Graduate Committee, my beautiful and intelligent wife, Caroline, my family, and all the friends and colleagues that supported me throughout this process.

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by

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During starvation, *C. elegans* adjust their behavior in order to survive. Using the starvation-sensitive *gpb-2(ad541)* loss-of-function mutant, components in a starvation-signaling pathway were identified. The goals of the studies presented here were to identify neurons that propagate a starvation signal, and to identify genes that regulate fat storage in the gut during starvation. Starvation in *gpb-2(ad541)* worms is lethal, and this lethality can be induced by arecoline, an acetylcholine receptor antagonist. Starvation sensitivity in *gpb-2(ad541)* worms is inhibited by atropine, an acetylcholine receptor antagonist. Previous work suggests that cholinergic signaling propagates a starvation signal in the pharynx of the worm, and the MC

neurons are responsible for sending that signal. By ablating the MC neurons in newly hatched L1 worms, I aimed to prevent starvation-induced lethality due to the *gpb-2(ad541)* background. Several genes have also been identified to act downstream of *gpb-2* in the regulation of fat in the gut. Both *flp-20* loss-of-function and *mgl-2* loss-of-function mutations rescue the starvation-induced lethality of *gpb-2(ad541)*, while introduction of a *gcy-28* loss-of-function mutation restores lethality. When fat was assayed using Oil Red O, it was found that GCY-28, a receptor-type guanylate cyclase, is necessary to maintain fat levels during starvation. GCY-28 is expressed in various head neurons and throughout the gut, and GCY-28 may play a role in regulating how gut cells store fat.

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PRIOR PUBLICATIONS

Pollok, R. (2013) *Transcription factor zfp-1 enhances cell death defects in C. elegans*. Worm Breeder's Gazette 19(3).

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LIST OF DEFINITIONS

AIB - A pair of neurons in *C. elegans.*

bec-1 - Necessary for autophagy in *C. elegans.*

BME - β-mercaptoethanol.

cha-1 - Encodes choline acetyltransferase.

ddH_2O - Double-distilled water.

eat-2 - Encodes a nicotinic acetylcholine receptor post-synaptic to the MC neuron.

eat-11 - Eat mutant that was later annotated as *gpb-2.*

eat-16 - An RGS protein that inhibits *egl-30.*

egl-10 - An RGS protein that inhibits *goa-1.*

egl-30 - Encodes $G_q\alpha$.

EGTA - Ethylene glycol tetraacetic acid.

FLP - Flippase for a FLP-FRT recombination.

flp-20 - FMRFamide like neuropeptide.

FRT - Flippase recognition target.

Gα - Alpha subunit of a heterotrimeric G protein.

Gβ - Beta subunit of a heterotrimeric G protein.

Gβ-ɣ - A complex of the beta and gamma heterotrimeric G protein subunits.

gcy-28 - Receptor type guanylate cyclase.

GDP - Guanosine diphosphate.

GDP-Gα - Alpha subunit of a heterotrimeric G protein complexed with guanosine diphosphate.

G_o - A family of G proteins.

$G_o\alpha$ - An alpha subunit of a heterotrimeric G protein in the G_o family.

goa-1 - *C. elegans* $G_o\alpha$.

gpb-2 - A Gβ protein that acts as a RGS.

GPCR - G protein-coupled receptor.

G_q - A family of G proteins.

$G_q\alpha$ - An alpha subunit of a heterotrimeric G protein in the G_q family.

GTP - Guanosine triphosphate.

GTP-Gα - Alpha subunit of a heterotrimeric G protein complexed with guanosine triphosphate.

hlh-30 - A transcription factor that regulates fat utilization.

I2 - A pair of neurons in the pharynx of *C. elegans*.

L1 - The first larval stage of *C. elegans.*

L2 - The second larval stage of *C. elegans.*

L3 - The third larval stage of *C. elegans.*

L4 - The fourth larval stage of *C. elegans.*

lf - Loss of function.

MAPK - Mitogen activated protein kinase.

MC - A pair of neurons in the pharynx of *C. elegans.*

mgl-1 - Encodes a metabotropic glutamate receptor.

mgl-2 - Encodes a metabotropic glutamate receptor.

MRWB - Modified Ruvkun's witches brew, this is a buffer that fixes *C. elegans* for staining.

mxl-3 - A transcription repressor that inhibits fat utilization.

N2 - The reference strain of *C. elegans*.

NGM - Nematode growth media.

PBS - Phosphate buffered saline.

RecCasp - Recombinant caspase.

RecGFP - Recombinant GFP.

RGS - Regulator of G proteins

RNAi - RNA interference.

unc-17 - Encodes a synaptic vesicle acetylcholine transporter.

unc-43 - Encodes a calmodulin protein kinase II.

CHAPTER ONE
Introduction

Starvation Signaling in *C. elegans*

What is starvation?

The study of starvation is of great interest, but research into starvation has unique challenges. Broadly speaking, starvation occurs when an organism obtains fewer nutrients than it needs to maintain itself. Clearly this definition of starvation fails to describe the realities found in nature. A feast and famine cycle requires a different metabolic response compared to a latent nutrient deficiency over time. Studying the different permutations of starvation can be time consuming and ethically dubious, depending on the model system of choice, but these considerations lend strength to using *Caenorhabditis elegans* to research starvation.

There are obvious reasons to use *C. elegans* to study starvation, such as their fast doubling time and short lifespan, but a less obvious reason is their invariant cell lineage. In wild type *C. elegans*, there are 1090 somatic cells generated, and it is possible to track each cell through its entire lifecycle. Since *C. elegans* are translucent, it is also possible to manipulate the animals in a cell specific manner using novel techniques. By stimulating or killing various cells, it can be determined where and how a cell is involved in various responses, such as a starvation response. By taking advantage of this unique trait in *C. elegans*, further insight into starvation can be provided that could one day be translated into humans.

The genetics of feeding

A major component of studying starvation is paying attention to how it changes behavior and, even in a simple organism like *C. elegans*, behavior can be very difficult to monitor. Relatively obvious behaviors, such as movement velocity, turning, and pharyngeal pumping, all play a role in the *C. elegans* starvation response. Despite the simplicity of the described behaviors, it is incredibly difficult to get a consistent result, as lighting, temperature, food, and growth substrate all change each response by a variable amount. When the focus is shifted to feeding, the number of behaviors and phenotypes are limited to the pharynx of *C. elegans*, allowing for much more controlled experiments.

The pharynx is the feeding organ of *C. elegans* (Figure 1). It is divided into the corpus, followed lengthwise by the isthmus and the terminal bulb (Albertson and Thomson 1976). Visible pumping of both the corpus and the terminal bulb serve to pull bacteria into the grinder, located in the terminal bulb. The bacteria is then broken up and passed into the gut. In this relatively simple organ, there are a variety of phenotypes that can be observed with just these few behaviors. Fast pumping, slow pumping, uncoordinated pumping, and stuffed pharynx are all scorable differences (Avery and Horvitz 1989). The pharynx is almost completely neuronally isolated from the remainder of the worm. When the pharynx is dissected out, it will still function normally for a short time (Avery et al. 1995). The relative isolation of the pharynx in *C. elegans* allows the study of pumping behavior, which is regulated by only a small number of neurons (Figure 1).

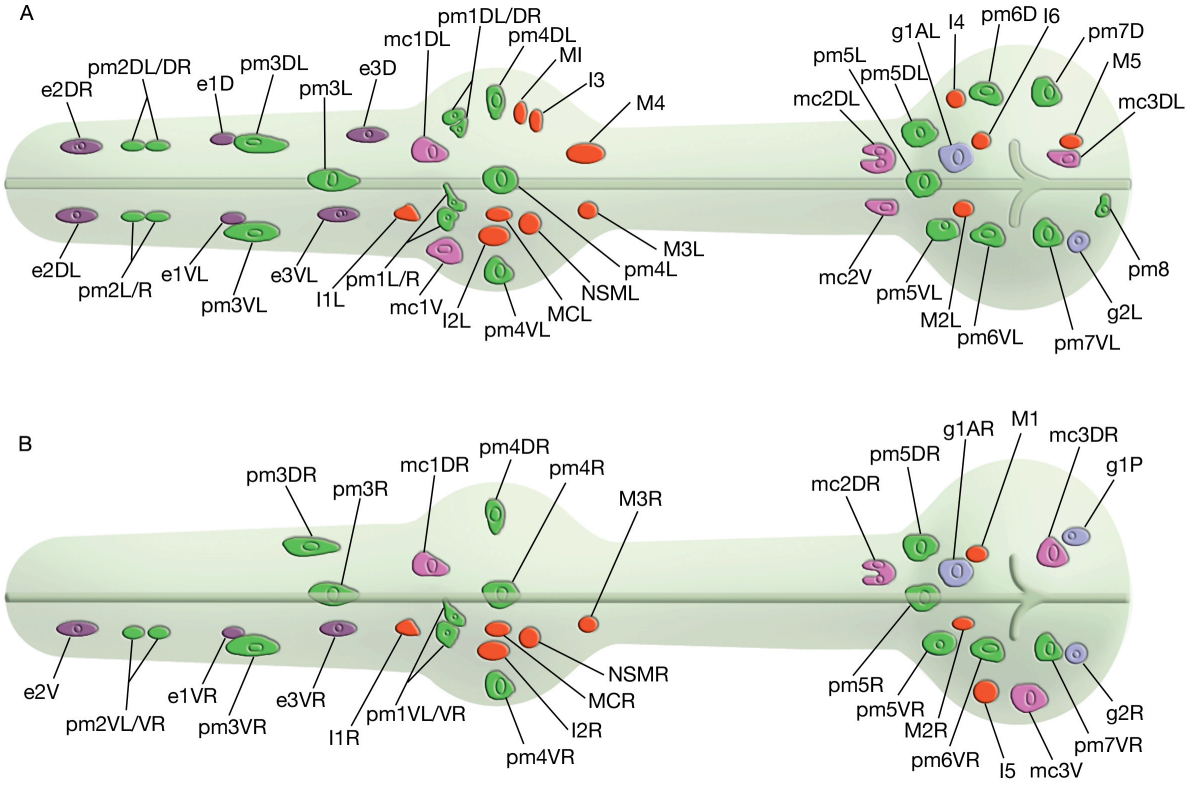


Figure 1. Pharynx of an N2 *C. elegans* with the nuclei identified. The pharynx is positioned with the anterior to the left, posterior to the right, dorsal up, and ventral down. The pharynx is bisected so that the left side of the worm is in panel A and the right side in panel B. Red nuclei are neurons. The MC neurons (MCL and MCR) are located in the anterior bulb on the posterior side. Reprinted with permission from Wormatlas.org (Altun and Hall 2009).

If a killed cell causes a phenotype, and if an identical phenotype can be obtained through mutagenesis of a gene, it is reasonable to hypothesize that the mutagenized gene is necessary for that cell to function properly. This is the idea behind an experiment Leon Avery performed to search for *C. elegans* mutants defective in eating. Leon killed each pharyngeal neuron with laser ablation and identified the resulting phenotypes (Avery and Horvitz 1989). By mutagenizing *C. elegans* with a constitutive pharyngeal pumping phenotype, Leon screened for worms defective in pumping or with abnormal pharynxes

(Avery 1993). Some of the resulting mutations from this screen were functionally related to neuron ablations, such as *eat-2* and ablated MC neurons. *eat-11* was isolated in this set of experiments and, while the phenotype does not match that of a neural ablation, it has become the basis for much of my work.

gpb-2 is a regulator of RGS proteins

eat-11 was independently identified in a screen for suppressors of *unc-43(gf)* (Robatzek and Thomas 2000). UNC-43 is a calmodulin protein kinase II and, in a gain-of-function mutant, *C. elegans* move much more slowly (Reiner et al. 1999). Mutations in G_o and G_q signaling have also been shown to change the rate at which *C. elegans* move, and Robatzek believed that *unc-43* was involved with G_o/G_q signaling (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996). Many of the mutants recovered from the *unc-43(gf)* suppressor screen were in the G_o/G_q pathways, and G_q mutants were found to be suppressors of *eat-11* (Brundage et al. 1996). Robatzek further characterized *eat-11*, and found that it is an ortholog of human $G\beta_5$ (Robatzek et al. 2001). With the characterization of *eat-11* as a Gβ protein, it was designated as *gpb-2*.

G_o/G_q signaling is processed through heterotrimeric G proteins that are activated by G protein coupled receptors (GPCRs). The heterotrimeric G protein is composed of a Gα, a Gβ, and a Gɣ subunit (Neves et al. 2002). When a seven membrane-spanning GPCR encounters its ligand, it acts as a guanine-nucleotide exchange factor (GEF) (Preininger and Hamm 2004). The active GEF exchanges a GDP bound to the Gα subunit with a GTP, and GTP-Gα then dissociates from Gβɣ. The GTP-Gα and Gβɣ complexes can each further affect

intercellular signaling. GTP-Gα can eventually auto-hydrolyze into GDP-Gα, but hydrolysis is accelerated by regulators of G proteins (RGS) (Neves et al. 2002). GDP-Gα, which cannot interact with effectors, then binds to free Gβɣ. Once GDP-Gα associates with Gβɣ, the newly reformed heterotrimeric G protein is ready for further cell signaling.

The most varied subunit in heterotrimeric G proteins is Gα, with fewer Gβ and Gɣ subunits. In *C. elegans*, 21 Gα, two Gɣ and, including *gpb-2,* two Gβ proteins have been identified (Jansen et al. 1999; Cuppen et al. 2003). The composition of a heterotrimeric G protein determines its function, and the specific Gα subunit used determines its classification that leads to identification of the signaling pathway. G_o and G_q signaling is specified by the Gα proteins GOA-1 and EGL-30, respectively (Mendel et al. 1995; Ségalat et al. 1995; Brundage et al. 1996; Lackner et al. 1999). All identified heterotrimeric G proteins in *C. elegans* include the Gβ subunit GPB-1 (Zwaal et al. 1996). GPB-2 does not function in the canonical heterotrimeric G protein complex, but is still active in G protein signaling (Robatzek et al. 2001).

GPB-2 acts to enhance GTPase activity in RGS proteins that inhibit both G_oα/GOA-1 and G_qα/EGL-30 in *C. elegans* (Robatzek et al. 2001) (Figure 2). GPB-2 binds to a Gɣ-like (GGL) domain, and the RGS proteins EGL-10 and EAT-16 contain these domains (Snow et al. 1998). EGL-10 targets GTP-G_oα, and EAT-16 does the same for GTP-G_qα, stopping activation of target effectors. Strong mutations in *gpb-2* prevent inhibition of both G_oα and G_qα, but the *gpb-2(ad541)* allele inhibits G_oα, but not G_qα. *C. elegans* with a *gpb-2(lf)* allele move with deeper body bends, their pharynxes pump irregularly, and they are hypersensitive to arecoline treatment. In transgenic *C. elegans* with a gain-of-function *gpb-2* mutation, these

phenotypes are reversed from the loss-of-function mutants. Arecoline is an agonist for muscarinic cholinergic receptors, and the role of muscarinic signaling in starvation helped identify the starvation sensitive phenotype in *gpb-2(lf) C. elegans* (You et al. 2006).

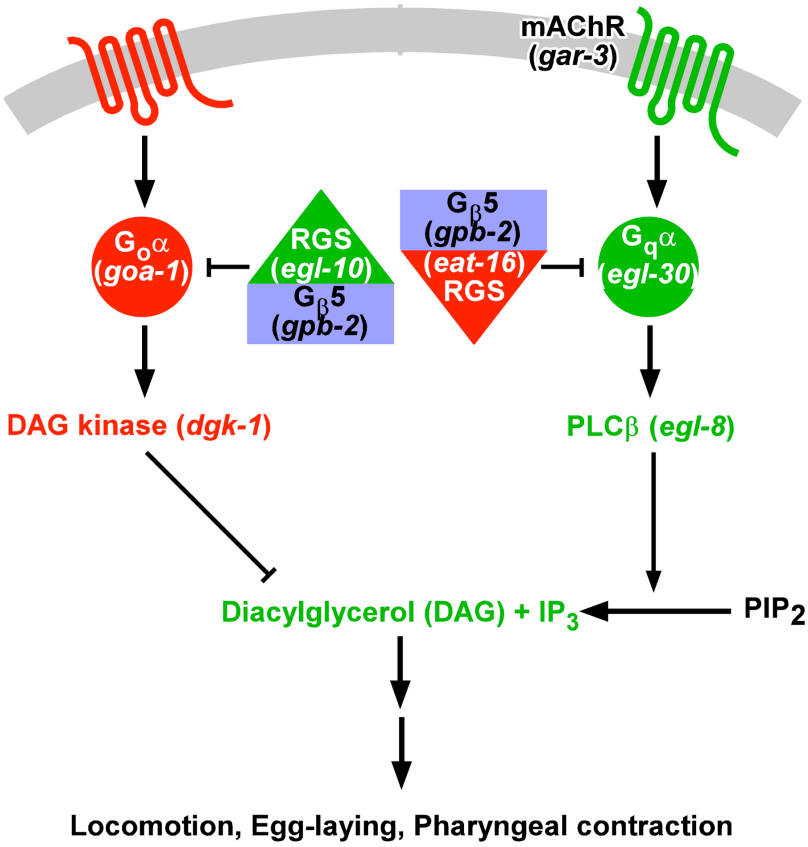


Figure 2. GPCR signaling and regulation in *C. elegans*. *gpb-2* stabilizes both EAT-16 and EGL-10, inhibiting signal transduction through the G_q and G_o pathways. The *gpb-2(ad541)* allele fails to stabilize EAT-16, causing enhanced G_q signaling. Reprinted under Creative Commons from Wormbook.org (Avery and You 2012).

Cholinergic muscarinic signaling propagates a starvation response

Young You tested *gpb-2(ad541) C. elegans* for starvation sensitivity and found that when starved immediately after hatching, worms would die within a few days (You et al. 2006). Mitogen-activated protein kinase (MAPK) signaling is downstream of cholinergic muscarinic activation (Berkeley et al. 2001), and MAPK is known to play a role in starvation response (Roberts and Fink 1994). Dr. You hypothesized that *gpb-2(ad541) C. elegans* might be particularly vulnerable to starvation since G_q is hyperactivated, and she found this to be the case. Because cholinergic muscarinic receptors activate G_q signaling, and G_q signaling propagates a starvation response, acetylcholine is likely an important neurotransmitter involved in starvation response. By exploiting acetylcholine's role in starvation, it is possible to further identify components in starvation signaling.

In moderation, autophagy enhances survival of *C. elegans* during starvation

The *lgg-1::gfp* reporter construct fluoresces when autophagosomes are present, and can show the location in a worm in which autophagy is taking place (Meléndez et al. 2003). When control worms are starved, there is some autophagy present in the pharynx of the worm (Kang et al. 2007). This autophagy provides energy that allows the worm to continue functioning in environments lacking available food sources. When worms are starved and autophagy is inhibited, such as with *bec-1(RNAi),* the worms are more sensitive to starvation and are likely to die.

The starvation-sensitivity in *gpb-2(ad541) C. elegans* is partially due to excessive autophagy in the pharynx (Kang et al. 2007). When *gpb-2(ad541)* is in the presence of the

lgg-1::gfp autophagy reporter, there is increased fluorescence in the pharynx during starvation. The *gpb-2(ad541); lgg::gfp-1* worm also has less fluorescence in the pharynx, indicating reduced autophagy during starvation, when treated with the cholinergic antagonist atropine. When autophagy is inhibited in *gpb-2(ad541)* with *bec-1(RNAi)*, the worms survive starvation better. Thus, one of the mechanisms that sensitizes *gpb-2(ad541)* worms to starvation is autophagy.

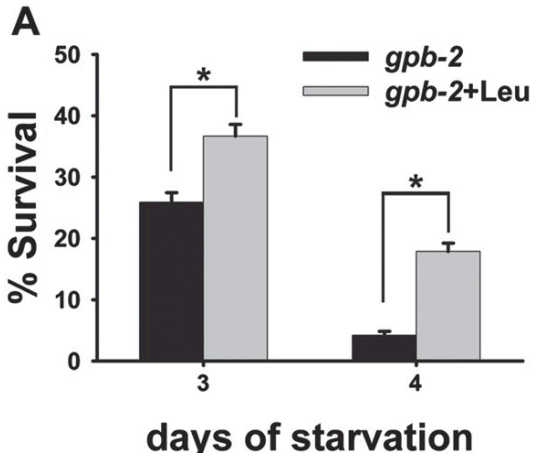
Autophagy needs to be tightly regulated during starvation in *C. elegans* to maximize the lifespan of the worm (Kang et al. 2007). Too little autophagy during starvation causes the worms to run out of energy, and the animals cannot survive. Too much autophagy, and the worm inflicts too much damage on itself to recover when it is reintroduced into food. *gpb-2* is one regulator of starvation that helps modulate the starvation response, tuning the starvation response to the worm's environment.

Environmental leucine inhibits the starvation response in *C. elegans*

External sources of nutrients, such as leucine, can regulate a starvation response in *C. elegans* (Kang and Avery 2009). Autophagy is a mechanism that mammalian cells in culture utilize to survive, but it can be inhibited by the addition of leucine. When starvation-sensitive *gpb-2(ad541)* worms are starved in the presence of leucine, their survival is also increased (Figure 3). Using the *lgg-1::gfp* reporter, *gpb-2(ad541)* worms experience less autophagy when they are starved in the presence of leucine.

Amino acids can be sensed by class 3 G-protein-coupled receptors (Conigrave and Hampson 2006), and the closest relatives of class 3 GPCRs in *C. elegans* are the

metabotropic glutamate receptors (*mgl)* (Dillon et al. 2006). It was found that the presence of leucine inhibits *mgl-2* and that, during starvation without any supplements, *mgl-2* enhances a starvation response in the *gpb-2(ad541)* background (Kang and Avery 2009). When *mgl-2* expression is restored in the AIB neuron in the *gpb-2(ad541) mgl-2(tm355)* background, starvation sensitivity is restored, indicating that *mgl-2* is necessary in the AIB neuron.



B

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. Addition of leucine can rescue starvation-induced lethality in *gpb-2(ad541) C. elegans*. (A) After 3 days of starvation, 25% of *gpb-2(ad541)* worms survive, but this number is increased when the media is supplemented with leucine. After a more stringent 4-day starvation, leucine significantly rescues survival in *gpb-2(ad541*). (B) Worms were starved for 3 days in M9 buffer supplemented with an amino acid. Some amino acids, such as leucine and glutamine, have a strong rescue effect on starvation. Other amino acids rescue starvation, while some exacerbate the lethality. Reprinted with permission from CSHL Press: Genes & Development (Kang and Avery 2009).

gcy-28 regulates foraging in *C. elegans* and may act in the *mgl-2* signaling pathway

The receptor type guanylate cyclase *gcy-28* is expressed in various head neurons and throughout the body of *C. elegans*. One particular phenotype of *gcy-28(lf)* is in foraging for food, and the foraging behavior depends on expression of *gcy-28* in the AWC neurons. Other work done in our lab has shown that the FMRFamide signaling molecule *flp-20* is secreted by the AIB neuron in *C. elegans,* which is regulated by *mgl-2*. Both *flp-20* and *mgl-2* loss-of-

function mutants can rescue starvation-sensitive *gpb-2(ad541).* AWC and AIB neurons are in the same signaling pathway, and we hypothesized that *gcy-28* could also be expressed in the intestine as a downstream target that is regulated by *flp-20.*

CHAPTER TWO
Materials and methods

General methods and strains

Unless otherwise noted, *C. elegans* were grown and maintained according to standard practices (Stiernagle 2006) at 20°C. The nematode growth media (NGM) was adjusted to contain 2% agar, 200 µg/mL streptomycin sulfate, 10 µg/mL nystatin (Avery 1993). Injections were done using standard protocols (Merritt and Seydoux 2010). Plates were typically seeded with HB101 (Boyer and Roulland-Dussoix 1969). Strains used include N2, DA2440 *gpb-2(ad541) I; cha-1(p1152) IV,* CB245 *unc-17(e245) IV,* PR1152 *cha-1(p1152) IV,* DA2441 *gpb-2(ad541) I;* and *unc-17(e245) IV,* DA674 *gpb-2(ad541) unc-29(e1072am) I,* DA541 *gpb-2(ad541) I,* CX713 *gcy-28(ky713) I*, FX2411 *gcy-28(tm2411) I*, DP38 *unc-119(ed3) III*.

Laser ablation

All laser ablations were done on *C. elegans* staged between 0 and 2 hours after hatching. Worms were grown on a NGM plate seeded with DA837 bacteria. Plates were rinsed with 500 µL M9 buffer, and media was transferred to unseeded NGM plate. Worms were mounted onto a microscope slide with 4% agar dissolved in M9 buffer with 50 mM sodium azide. Target neurons were located and ablated, and worms were removed from the slide within 15 minutes.

C. elegans were starved after laser ablation in either solid media or in buffer. When starved on solid media, worms were simply transferred to plates composed of 4% agarose dissolved in M9 buffer with worm picks. When starved in buffer, 96-well plates were used. Each well was filled with 40 µL of M9 buffer. 10 µL of M9 buffer was applied to the worms on the slide, and the worm was then aspirated into the appropriate well on the 96-well plate. All unused wells were filled with water, and the plates were sealed with parafilm.

Genomic DNA preparation

A freshly starved 60 mm plate of *C. elegans* was used. 1 mL of water was added to the plate and aspirated into a 1.5 mL tube. The supernatant was briefly spun down and aspirated down to 100 µL. It was then frozen for at least 5 minutes at -80°C. 500 µL of TEN/10% SDS, 2.5 µL 20 mg/mL proteinase K, and 1 µL β-mercaptoethanol (BME) was then added, and the solution as allowed to incubate at 65°C for 1 hour. After incubation, 600 µL Phenol:Chloroform:IAA was added, and the solution was shaken by hand for 3 minutes. It was then spun down for 5 minutes at 16,000 × *g*, and the aqueous layer was transferred to a fresh 1.5 mL tube. 1 mL of ice-cold ethanol was added, and the sample was spun down for 15 minutes at 4°C. The ethanol was aspirated, and the sample was allowed to air dry. The genomic DNA was then dissolved in 400 µL water or TAE buffer.

Transgenic reporter gene construction through PCR fusion

Reporter gene fusion was performed as described by Boulin, et al. 2006. A target promoter region and a reporter were both amplified from template DNA. A 3' linker was

fused to the target promoter using designed primers. A 5' linker, complementary to the promoter's 3' linker, was fused to the reporter. Both promoter and reporter products, with complimentary 3' and 5' linkers, respectively, were then used as template material for a final PCR reaction. The final result was stitched together as a single linear piece of DNA, which was then ready to inject into worms.

pador-1::GFP **construction**

pador-1::GFP was made through PCR fusion. *pador-1* (the promoter of *ador-1*) was amplified from N2 genomic DNA using PrimerA_T21B4_4 and PrimerB3_T21B4_4_95_67. GFP was amplified from pPD95.67 using PrimerC_95_67 and PrimerD_95_67. Products were fused together using PrimerA*T21B4_4 and PrimerD*_95_67. *pador-1::GFP* was injected at a concentration of 100 ng/μL into N2 *C. elegans* with no injection marker.

Combinatorial cell killing with the Gateway system

MC specific expression was attempted using a modified Gateway system developed by the Hubbard lab (Voutev and Hubbard 2008). Plasmids used were developed by the Hubbard lab and provided by Addgene (Table 2). Transformations are used in combination with ccdB suicide selection and varying antibiotics to selectively isolate the desired recombination event. pDONR 221 and pGC180 are used to drive expression of FLP with a designed promoter. pDONR 221, pGC188, and pGC247 are used to join a promoter and gene together with a FRT-flanked stop site (Figure 4).

The promoters *prig-5* and *ptyra-2* were amplified from genomic DNA using primers rig5promF, rig5promR, tyra2promF, and tyra2promR. Gateway sites were added to *prig-5* using primers B1rig5F and B2rig5R. Gateway sites were added to *ptyra-2* using primers B1tyra2F and B2tyra2R. Gateway entry vectors for both *prig-5* and *ptyra-2* were created using pDONR221. The *mCherry* reporter was amplified with Gateway sites from a *pmyo-2::mCherry* vector using primers B4mCherryF and B3mCherryR. The Gateway entry vector for *mCherry* was created using pGC188.

Combinatorial killing using recombinant caspase

Recombinant GFP (RecGFP) and recombinant caspase (RecCasp) function by expressing two complimentary proteins using different promoters. When one member of a protein pair is expressed, it is not functional but, when its partner is present, the protein is brought together with a leucine zipper and function is restored. All of the recombinant proteins were constructed by the Chalfie lab and provided by Addgene. RecGFP is built into the TU#710 and TU#711 plasmids. RecCasp is built into the TU#813 and TU#814 plasmids.

The promoters were validated by attaching them to GFP using PCR stitching reactions. The promoters were first amplified from genomic N2 DNA. The primers used to initially amplify the promoters of interest were flp21prom F, flp21prom R, flp2prom F, flp2promb R, tyra2proma F, tyra2proma R, prab3_F, and prab2_R. GFP was amplified from the plasmid pPD95.69 using the primer pPD9569_gfp_R and a forward primer specific to a different promoter for a stitching PCR reaction. The primers designed for stitching PCR were

pflp21_gfp_stitch_F, pflp2_gfp_stitch_F, ptyra2_gfp_stitch_F, prig5_gfp_stitch_F, and prab3_gfp_stitch_F.

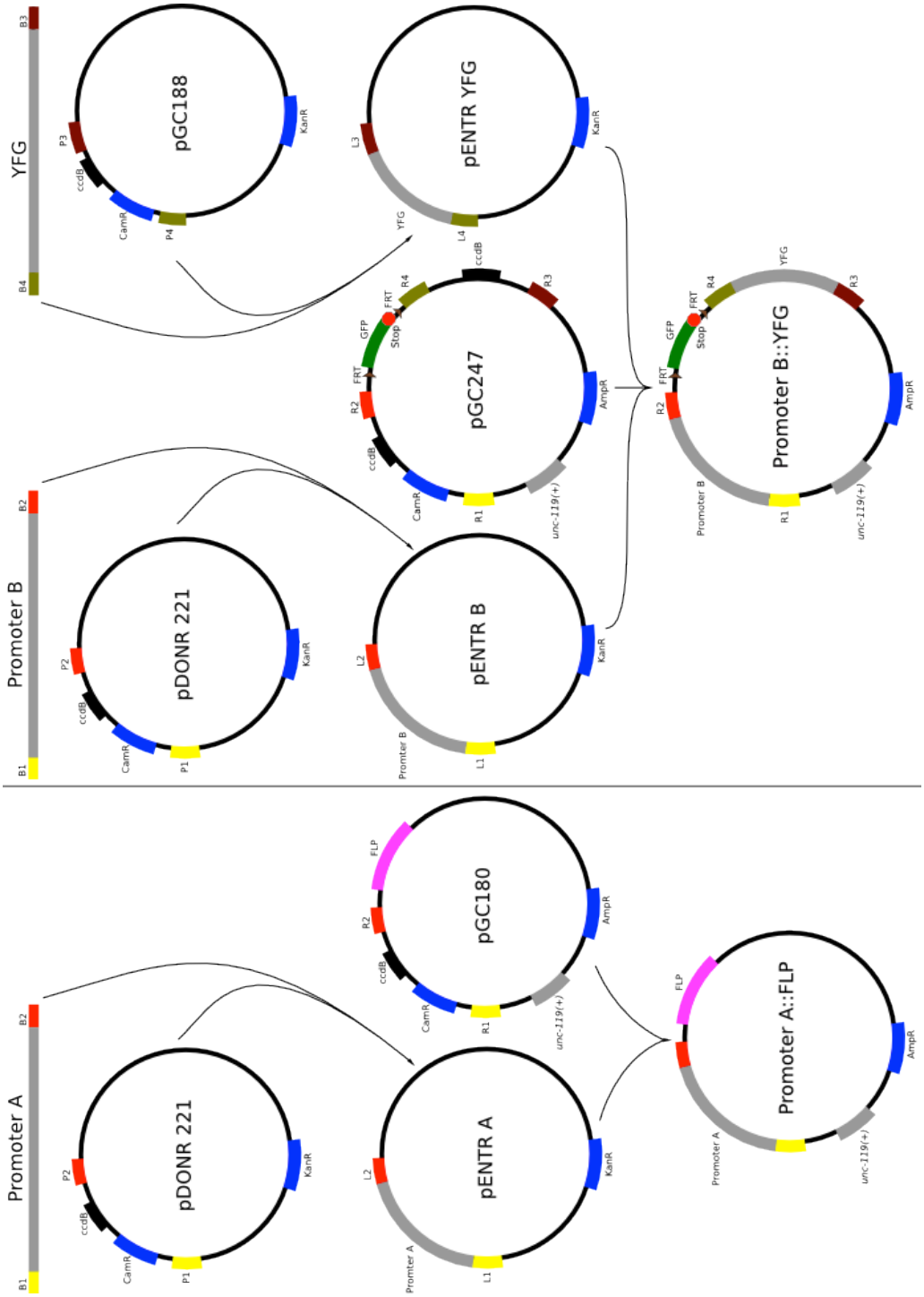


Figure 4. Using a Gateway recombination strategy, two different promoters can selectively drive expression of a target gene. pDONR 221 and pGC180 were used to generate a promoter that drives expression of FLP. A new copy of pDONR 221 and pGC188 accept a second promoter and a gene of interest.

L1 egg preparation

L1 worms were isolated by dissolving worms, including gravid mothers, in a buffered hypochlorite solution (Stiernagle 2006). A single 100 mm NGM plate should be covered in thousands of worms, with the bacteria nearly exhausted. The plate was washed with ddH_20, and the water was collected into a 15 mL conical tube. The solution containing the worms was briefly spin down, and the liquid was aspirated until only 3 mL remained. 200 µL 10 M NaOH and 800 µL of commercial bleach was added to the worms. I then manually applied force to the tube by repeatedly banging the tube tip first onto a solid surface until most worms are visibly sheared. The time required for shearing varies depending on the freshness of the bleach. After worms were dissolved, they were rinsed with ddH_2O, and the buffer was aspirated after a 1 minute spin at 200 × *g*. This rinse step was performed twice. A third rinse was performed using M9 buffer, but 3 mL of buffer was saved after the final aspiration. The capped tube was then placed on a rocker in an incubator, whose temperature depends on the experiment, overnight.

***C. elegans* starvation assays**

Unless otherwise noted, starvation assays were done with worms starved in M9 buffer. Worms were prepared using an L1 egg preparation and, when they were placed on an incubating rocker, starvation begun. At various time points, aliquots of worms were removed using a P200 micropipette (volume dependent on particular experiment) and placed on a seeded NGM plate. Worm survival was determined by counting worms that were growing a predetermined number of days later. For the N2 reference strain, 48 hours after being placed

on food was sufficient to observe whether a worm will survive. Some worm strains grew much slower, and the time chosen to check viability can vary.

Oil Red O Staining

Worms were synchronized using L1 egg preparations. When the worms were at the appropriate age, they were rinsed with 500 µL of M9 buffer and transported to a 1.5 mL microcentrifuge tube. The worms were rinsed three times with PBS buffer to remove all bacteria (all rinses and washes were spun at ≤ 200 × *g* in a picofuge for a few seconds unless otherwise indicated). After the third wash, ~60 µL of worms + PBS were left at the bottom of the tube. At this stage, worm tubes were spinning on a Thermoscientific rotator. The length of time worms are left spinning is a optional starvation step that is set for each experiment. 60 µL of 2X MRWB buffer (500 µL 2X MRWB: 40 µL 2 M KCl, 4 µL 5 M NaCl, 70 µL 100 mM EGTA, 2 µL 100 mM spermine, 2 µL 100 mM spermidine HCl, 100 µL 150 mM PIPES, 28 µL H_2O, 1 µL BME, 250 µL 4% formaldehyde) was added, and the worm tubes were spinning on the rotator for one hour. The surface tension should keep the solution with worms at the bottom of the tube, allowing the worms to fall continuously through the buffer. During the incubation in MRWB, a working Oil Red O solution of 60% stock solution (0.2 g of solid Oil Red O dissolved in 40 mL isopropanol) and 40% H_2O was prepared and placed on a rocker. When the worm incubation in MRWB was complete, it was centrifuged at 14000 × *g* for one minute and the buffer was aspirated. They were washed once with PBS and then incubated in 1 mL 60% isopropanol for 15 minutes on the rotator. The working Oil Red O solution was filtered with a 0.22 micron filter and syringe. The first 1 mL of filtered solution

(mostly isopropanol with little Oil Red O) was discarded. Isopropanol was removed from the worms, and 1 mL of the filtered Oil Red O working solution was added. The worms were incubated for 1 hour on the rotator. The worms were then washed with 1 mL of PBS containing 0.01% Triton X, rendering them ready for examination under a microscope.

Image analysis of Oil Red O stained *C. elegans*

All images were captured using a Zeiss AxioCam MRm monochrome digital camera. To increase contrast in the images, a green filter was applied to make the red stain appear dark gray, as opposed to a light gray. In order to correct differences in the bright field and to increase contrast, the background was automatically removed using ImageJ (Abramoff et al. 2004) and the Calculator Plus plugin (Rasband and Landini) (Figure 5A). To remove the background, first the microscope light source was warmed up for 15 minutes before images were taken. A photo with no sample was captured with the light and with the light source blocked. These images were then processed using a macro written in ImageJ (Figure 6). The images were then manually stitched together and cropped using the photo manipulation software GIMP (http://www.gimp.org) (Figure 5B, 5C). The images were then fed back into ImageJ to analyze the intensity of the staining (Figure 5D, 5E, 7).

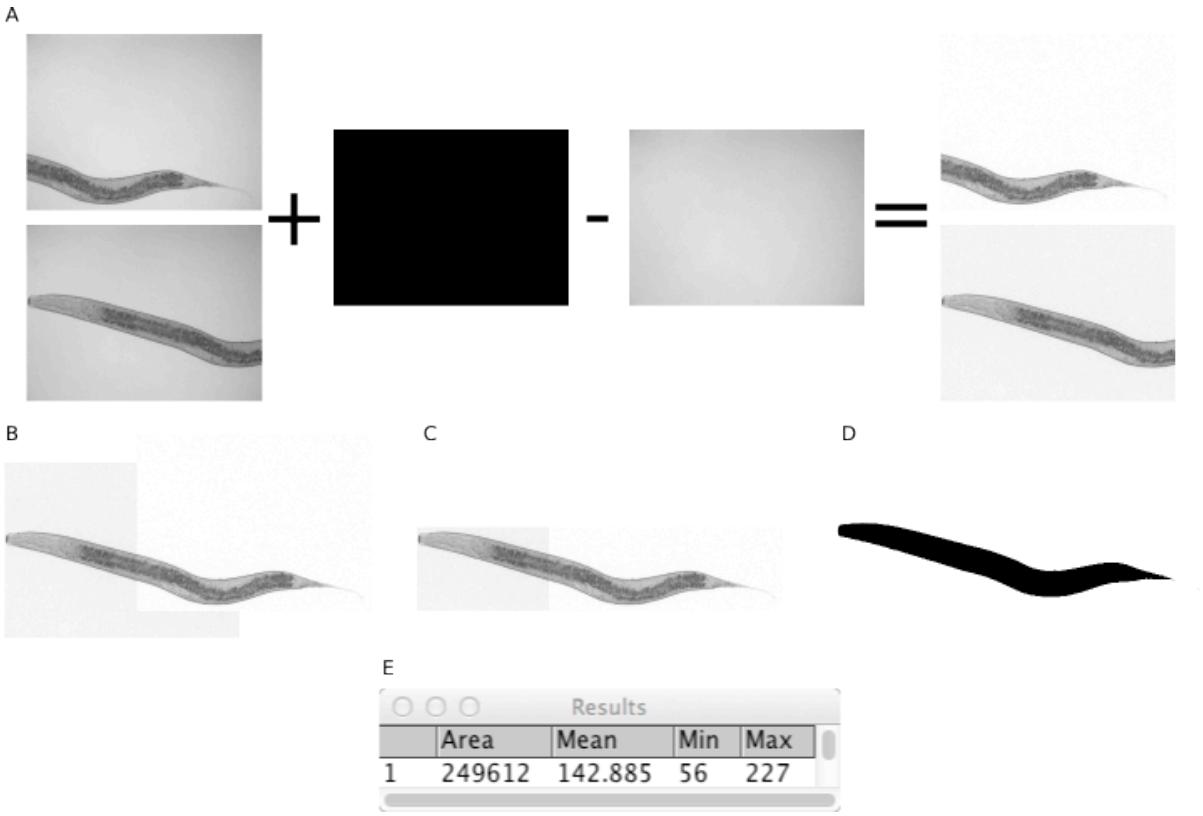


Figure 5. A combination of automatic and manual manipulations of image data was processed by ImageJ to determine staining intensity in *C. elegans*. (A) Image artifacts were removed from each picture to increase contrast. (B) Images were manually assembled. (C) Extra background was cropped out of the image. (D) A mask was automatically applied to the worm, and (E) image data from under the mask was extracted.

```
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory ");
list = getFileList(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
        showProgress(i+1, list.length);
        open("/Users/rhpollok/Desktop/Darkfield.tif");
        open("/Users/rhpollok/Desktop/Divisor.tif");
        open(dir1+list[i]);
        rename("Original");
        imageCalculator("Subtract create", "Original","Darkfield.tif");
        selectWindow("Result of Original");
        run("Calculator Plus", "i1=[Result of Original] i2=Divisor.tif operation
=[Divide: i2 = (i1/i2) x k1 + k2] k1=255 k2=0 create");
saveAs("Tiff", dir2+list[i]);
run("Close");
run("Close");
run("Close");
}
```

Figure 6. A macro designed to increase contrast and remove background noise in bright field microscope images using ImageJ and the Calculator Plus plugin.

```
dir1 = getDirectory("Choose Source Directory ");
list = getFileList(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
        showProgress(i+1, list.length);
        open(dir1+list[i]);
        run("8-bit");
        setAutoThreshold("RenyiEntropy");
        run("Analyze Particles...", "size=100000-Infinity       circularity=0.00
-1.00 show=Nothing display include");
}
```

Figure 7. A macro designed to analyze the intensity of Oil Red O staining in *C. elegans* using ImageJ.

Table 1: Primer List

PrimerA_T21B4_4	AAT GCA AAT AAC AAG AGG AAA AA
PrimerB3_T21B4_4_95_67	AGT CGA CCT GCA GGC ATG CAA GCT CCA TAG CCA ACC AAT TCC AC
PrimerC_95_67	AGC TTG CAT GCC TGC AGG TCG
PrimerD_95_67	AAG GGC CCG TAC GGC CGA CTA
PrimerA*_T21B4_4	TGT TCG AAT TCA ATG GTG GA
PrimerD*_95_67	GGA AAC AGT TAT GTT TGG TAT A
rig5promF	TCC ATG GAA CAC ACT TGC TG
rig5promR	CAA CCG GAG AAC TTG ATG TG
tyra2promF	GAA TGT GTG GGA GTA TGC AAG
tyra2promR	TTC CCC CAT ACC AAA AAT G
B1rig5F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT TGC AAA CTT GTG ATG TTT CTG G
B2rig5R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CCG AGA TAG TGC GAA GCA AGT
B1tyra2F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT TGC TCA GGT TTT TAT CTG TC
B2tyra2R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTT GAT GAC TCT GTT TTG AG
B4mCherryF	GGG GAC AAC TTT GTA TAG AAA AGT TGG GTG ATG GTG AGC AAG GGC GAG
B3mCherryR	GGG GAC AAC TTT GTA TAA TAA AGT TGT TTT ACT TGT ACA GCT CGT CCA T
flp21prom F	GGG ACG ATT CAA GTC GGT CT
flp21prom R	CGA GAA GAC ACG AAA GCA AG
flp2prom F	CTGTGTTCACTCTACCAGG
flp2promb R	TTGCGTGGTTTGCGACAATTGG
tyra2proma F	TGG GGG TGT CGT CTG AAA T
tyra2proma R	CCA TAC CAA AAA TGA AAT CAG AA
prab3_F	GAT CTT CAG ATG GGA GCA GTG G
prab3_R	TGT AGC GCC TTG AGG TTG TC
pflp21_gfp_stitch_F	TCA TCT TGC TTT CGT GTC TTC TCG GCT CCA AAG AAG AAG CGT AAG
pflp2_gfp_stitch_F	ACT TGC ATG GTT TGC GAC AAT TGG GCT CCA AAG AAG AAG CGT AAG
ptyra2_gfp_stitch_F	TTT CTG ATT TCA TTT TTG GTA TGG GCT CCA AAG AAG AAG CGT AAG
prig5_gfp_stitch_F	AAC CCA GAA ACA TCA CAA GTT TGC GCT CCA AAG AAG AAG CGT AAG
prab3_gfp_stitch_F	GGC GGA CAA CCT CAA GGC GCT ACA GCT CCA AAG AAG AAG CGT AAG
pPD9569_gfp_R	CAG ACA AGT TGG TAA TGG TAG C

Table 2: Plasmid List

Plasmid Name	Addgene ID #	Reference	Brief Description
pDONR 221	N/A	Nakagawa et al 2008	P1/P2 doner from Invitrogen
pGC188	19619	Voutev et al 2008	P4/P3 doner vector
pGC180	19616	Voutev et al 2008	R1/R2 destination vector
pGC247	19634	Voutev et al 2008	R1/R2 and R4/R3 destination vector
TU#710	15278	Zhang et al 2004	nzgfp
TU#711	15279	Zhang et al 2004	czgfp
TU#813	16082	Chelur et al 2007	caspase-3(p12)::nz
TU#814	16083	Chelur et al 2007	cz::caspase-3
pPD95_67	1490	Fire lab plasmid	empty GFP
pPD95_69	1491	Fire lab plasmid	empty GFP

CHAPTER THREE
Results

Introduction

C. elegans with a *gpb-2(ad541)* mutation are hypersensitive to starvation and experience excessive autophagy. An appropriate level of autophagy is vital and increases the survivability of the worm, but *gpb-2(ad541)* mutants essentially eat themselves alive when they are without food (Kang et al. 2007). The starvation-sensitive phenotype of *gpb-2(ad541)* is exacerbated by acetylcholine treatment, and *gpb-2(ad541)* worms die when they are grown in the presence of the muscarinic acetylcholine agonist arecoline (Avery 1993). Conversely, when *gpb-2(ad541)* worms are starved in the presence of the muscarinic acetylcholine antagonist atropine, their survival is increased compared to untreated worms (You et al. 2006) (Figure 8b, d). Similarly, when a muscarinic acetylcholine receptor *gar-3* loss-of-function mutation is introduced to the *gpb-2(ad541)* background, survival is increased (You et al. 2006) (Figure 9). Acetylcholine is an important neurotransmitter in *C. elegans* that is necessary to pass a starvation signal through the G_q pathway, which is regulated by *gpb-2* (Robatzek et al. 2001).

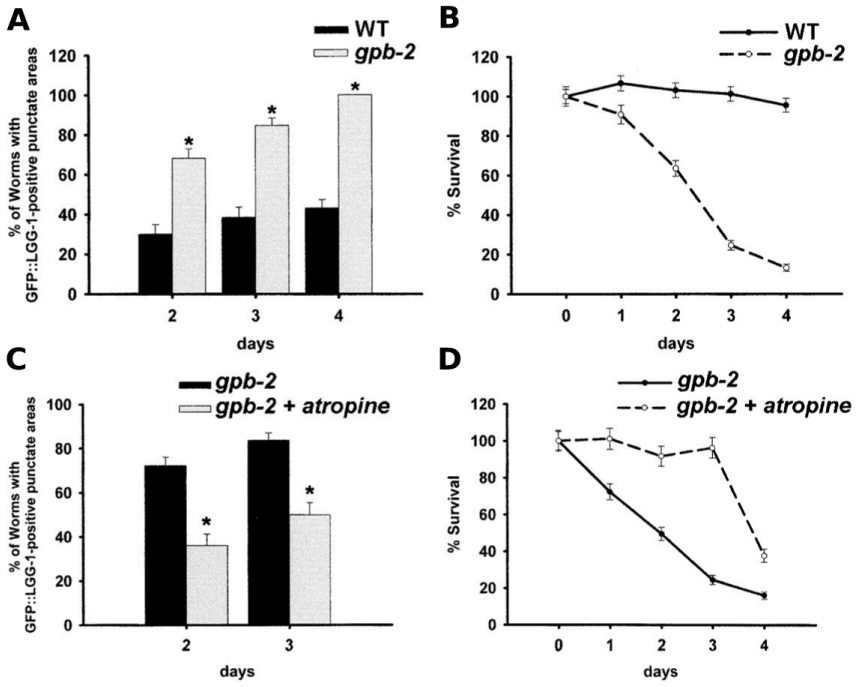


Figure 8. *gpb-2(ad541)* worms show increased GFP::LGG-1 punctate during starvation, indicating greater levels of autophagy. Excessive autophagy causes increased mortality in *C. elegans*. The cholinergic receptor antagonist atropine can inhibit the increased autophagy, and rescue starvation-induced lethality (Kang et al. 2007). (A) While some autophagy is normal during starvation, in *gpb-2(ad541)* worms there is much more autophagy. (B) *gpb-2(ad541)* worms experience starvation-induced lethality when starved at the L1 stage in M9 buffer. (C) Atropine reduces the autophagy seen in *gpb-2(ad541)* worms. (D) Atropine increases survival of *gpb-2(ad541)* worms during starvation. Reprinted with permission from CSHL Press: Genes & Development (Kang et al. 2007).

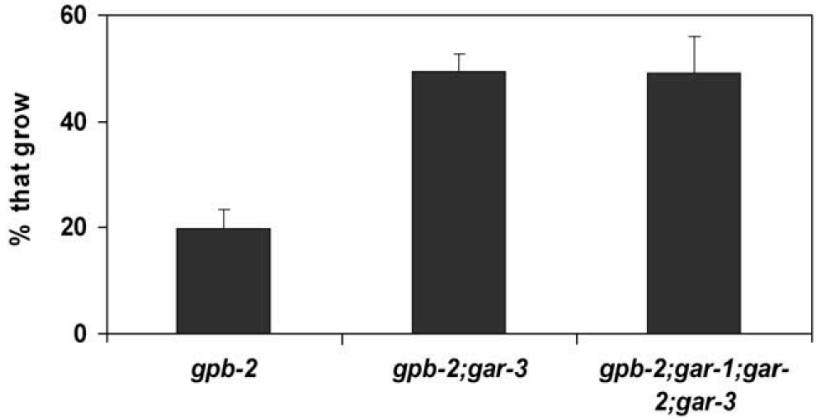


Figure 9. Muscarinic acetylcholine receptor *gar-3* can rescue some of the starvation-induced lethality seen in *gpb-2(ad541) C. elegans*. When *gpb-2* worms are starved for 3 days in buffer as L1s, approximately 20% grow. The double mutant *gpb-2; gar-3* has an increased survival rate, and this survival is not improved when additional *gar* mutations are introduced, suggesting *gar-3* signaling is regulated by *gpb-2*. Reprinted with permission from Elsevier: Cell Metabolism (You et al. 2006).

One mechanism by which *gpb-2(ad541) C. elegans* die is excessive autophagy in the pharynx during starvation (Kang et al. 2007). The worm's grinder is a specialized cuticle attached to muscles in the pharynx. When the grinder of the pharynx becomes damaged due to autophagy, the worm is incapable of efficiently extracting nutrients from bacteria, and is more likely to arrest and die (Figure 8). The pharynx contains a nervous system that is nearly autonomous from the remainder of the worm (Avery and Horvitz 1989) and, if acetylcholine is necessary for *gpb-2(ad541)* starvation-induced lethality, a source of that acetylcholine should be in the pharynx. There are 20 pharyngeal neurons, of which eight use acetylcholine as a neurotransmitter (Altun and Hall 2009). I hypothesize that by removing the cholinergic neurons involved in G_q signaling, excessive pharyngeal autophagy can be rescued, leading to a decrease in death caused by starvation of *gpb-2(ad541)* worms.

Of the cholinergic neurons in the pharynx of *C. elegans*, I strongly suspect that the MC neurons are responsible for G_q signaling within the pharynx. There are two MC neurons in the anterior bulb of the worm pharynx (Figure 1). The MC neurons are necessary for normal pumping of the pharynx, as the worm pumps much more slowly when the MC neurons have been destroyed by laser ablation (Avery and Horvitz 1989). EAT-2 encodes a nicotinic acetylcholine receptor postsynaptic to the MC neurons and, in an *eat-2(lf)* mutant, the pumping behavior is nearly identical to MC(-) worms (McKay et al. 2004). When our lab realized that acetylcholine promotes *gpb-2(ad541)* starvation-induced lethality, an experiment was performed in which numerous cholinergic neurons in the pharynx were ablated (Figure 10). The ablation experiment in *gpb-2(ad541)* worms suggests that ablation of the MC neurons improves survival, but the statistical significance was quite low. To more confidently test whether MC ablation can rescue *gpb-2(ad541)* starvation sensitivity, I designed new experiments to validate this hypothesis.

A) Starved 3 Days on M9 Agar Plates

Neuron(s) Ablated	Fertile to Infertile/Dead *C. elegans*
Mock Ablation	9/17*
I1 (L/R)	3/7
M1	6/6
M2 (L/R)	3/4
M4	1/4
MC (L/R)	4/5*

*p ≥ 0.05

B) Starved 4 Days on M9 Agarose Plates

Neuron(s) Ablated	Fertile to Infertile/Dead *C. elegans*
Mock Ablation	0/7
M1	1/11
MC (L/R)	3/5
M1 + MC (L/R)	4/6
Combined Mock + M1	1/18*
Combined MC (L/R) + [M1 + MC(L/R)]	7/11*

*p < 0.005

C) Starved 4 Days on M9 Agarose Plates

Neuron(s) Ablated	Fertile to Infertile/Dead *C. elegans*
Mock Ablation	6/17
MC	10/15

p ≥ 0.05

Figure 10. Laser ablation assay on pharyngeal cholinergic neurons. Ablation of cholinergic neurons that participate in G_q signaling may rescue starvation-induced lethality in *gpb-2(ad541) C. elegans.* (A) When no neuron is ablated and *C. elegans* are starved on agar plates, 9/17 grew into mature adults. Worm survival was most improved when M1 or MC neurons were ablated. (B) Starving worms on agarose plates for four days increases the stringency. In this condition, survival is most improved when MC and MC + M1 neurons are ablated. All results where the MC neurons were not ablated were grouped together in "Combined Mock + M1". All results in which the MC neurons were ablated were grouped together in "Combined MC (L/R) + [M1 + MC(L/R)]". Survival was significantly increased in worms with ablated MC neurons compared to those without MC ablated. (C) MC ablation improves survival compared to mock ablation, but not significantly.

Laser ablation in *C. elegans*

Using laser ablation to identify the G_q signaling cholinergic neurons in the pharynx of *C. elegans* has the advantage of precision. If one set of neurons does not confirm the hypothesis, it is theoretically simple to repeat the experiment on a new group of targets. There is no need to spend weeks or months designing transgenic constructs; one simply

needs to know the anatomy of the pharynx. A caveat to these types of laser ablation studies is in worm recovery. Normally, in an L1 starvation assay, worms are hatched and starved in M9 buffer in the absence of food. After the requisite time has passed, worms are aliquoted onto a food source, and worm recovery is recorded.

In typical laser ablation studies, *C. elegans* are recovered on NGM plates covered in bacterial food. My experimental design differs in that worms must be starved immediately following neuron ablation. Unfortunately, when worms lack bacteria, they begin to forage for new sources of food, meaning that worms can crawl to the edge and off of an agar plate, effectively removing them from the experiment. The stringency of starvation can also be more difficult to control when the worms are recovered on plates. Even without additives, there are some nutrients present in agar that can increase worm survival during starvation compared to starvation in buffer (Figure 10, Figure 8b). Agarose plates provide a more nutrient-free media for starving worms, and are a more stringent starvation condition. Originally, I attempted to optimize the starvation assay using agarose plates (Figure 11). While it is possible, even accounting for worm loss due to foraging, I was not convinced by the results. It is difficult to transfer worms without using bacteria on the end of a worm pick, and some bacteria will adhere to the worm during transfer. Any bacteria that are transferred with the worm can confound the starvation results. Mineral oil can replace the bacteria on a pick, but worms will sometimes swallow oil and become sick. To address these problems, I redesigned the experiment to use M9 buffer and 96-well round bottom plates. This eliminates the solid media, preventing the worms from foraging, and it allows the transfer of worms using pipetors instead of worm picks.

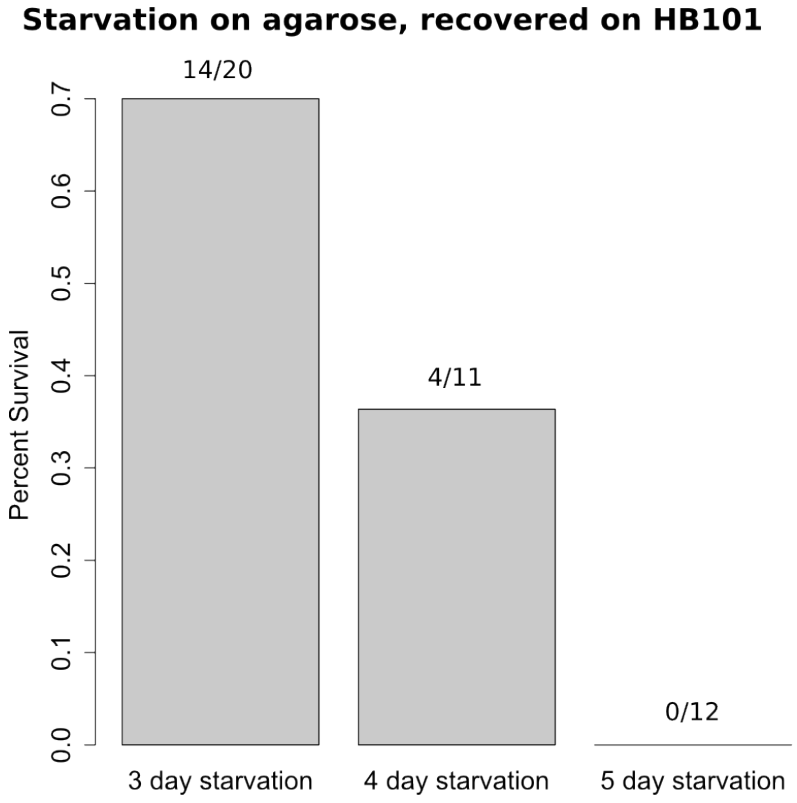


Figure 11. *gpb-2(ad541)* mutants that were hatched on food and transferred to agarose plates. After 3, 4, or 5 days of starvation, the worms were transferred to HB101 *E. coli.*

When *C. elegans* are removed from a slide after laser operation, most of the remaining bacteria are pulled off with a cover slip. When the worms are suspended in a drop of M9 buffer and aspirated into a 96-well plate, the majority of worms are recovered. A small number of worms are lost in the transfer from slide to well, or then later from well to plate, but there are far fewer worms damaged or partially fed using this adjusted technique. Starving worms in M9 buffer makes for a much more consistent starvation condition and reduces the variables contributing to starvation.

An ideal dynamic range for starvation-induced lethality allows most, but not all, specimens to die in the control condition. When the starvation is not completely lethal, it is possible to detect differences such as quality of laser ablation. If the laser is not perfectly focused on a MC neuron, the lumen of the pharynx can be punctured, killing the worm. It is important to know if a worm survives or dies due to the controlled variables, and not through outside factors such as error during ablation. Most, but not all, *gpb-2(ad541)* worms should not survive starvation. With this in mind, I decided that a third of the experimental worms should survive during a mock ablation, where no actual laser surgery occurs.

In our lab, when *gpb-2(ad541) C. elegans* have been starved for three days in M9 buffer and recovered on HB101 *E. coli*, roughly 25% survive (Figure 8b). My experimental design allows the worms to hatch in food instead of buffer, and become no more than two hours old before laser ablation. The worms are also starved in M9 buffer that is not aerated. As a result of these two modifications, 62.5% of *gpb-2(ad541) C. elegans* survive starvation when prepared for laser ablation and recovered on HB101 after three days of starvation. To increase the lethality of starvation, I changed the recovery bacteria from *E. coli* to DA1877 *Comamonas*. Pharyngeal autophagy can impair the ability of *gpb-2(ad541)* worms to extract nutrients, and *Comamonas* is a more stringent recovery condition (Figure 12). Unfortunately, when worms are recovered on *Comamonas,* the condition was complete lethality. When starved for three days, neither *E. coli* nor *Comamonas* allowed for the targeted dynamic range.

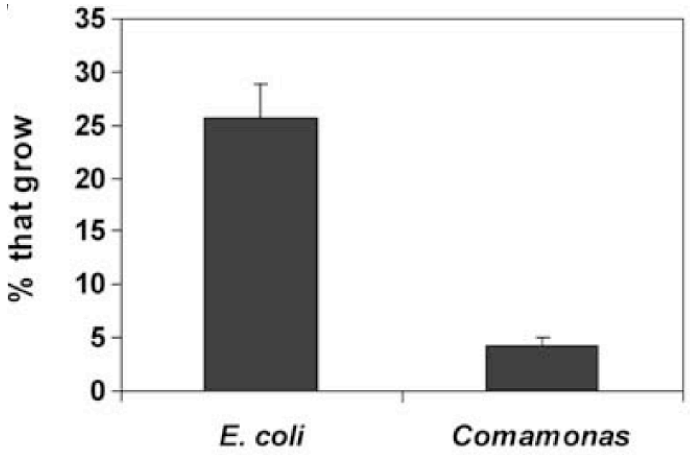


Figure 12. *gpb-2(ad541) C. elegans* starved for three days and then recovered on HB101 *E. coli* or *Comamonas*. When worms are recovered on *Comamonas*, fewer survive than on HB101. We believe this is because the grinder of the worm is damaged, and the animals are incapable of efficiently extracting nutrients from the bacteria. *Comamonas* is physically smaller than *E. coli*, and we believe this makes it more difficult for the worm to grind. Reprinted with permission from Elsevier: Cell Metabolism (You et al. 2006).

Recovering *C. elegans* on DA1877 *Comamonas* after three days of starvation is a more stringent starvation condition than desired, but it can still test the hypothesis that the MC cholinergic neurons are responsible for the starvation response in the *gpb-2(ad541)* worms. If no *gpb-2(ad541)* worms can survive a mock ablation with a three day starvation, it is necessary to compare the ratio of wild-type worms that survive a mock ablation versus MC neuron ablation. Some number of wild-type worms will die from laser ablation due to operator error, and it can be assumed that error rate is consistent among all experimental conditions. If many control worms survive the MC ablation, it is reasonable to assume that successful neuron kills occur in the experimental worms as well.

Laser ablation results

The first experimental condition tested with laser ablation involved *gpb-2(ad541) C. elegans*. The worms were MC- or mock-ablated, and were then starved for one day. After one day of starvation, the worms were recovered on *Comamonas.* All worms that were mock-ablated survived, and 78% of the MC-ablated worms survived (Figure 144). The worms that were operated on also pumped slowly, confirming successful ablation of the MC neurons. The next condition tested was to extend the starvation to three days. In this situation, all of the mock-ablated worms died, and all of the MC-ablated worms died (Figure 13). This set of experiments failed to support the hypothesis that ablation of MC neurons can rescue *gpb-2(ad541)* from starvation-induced lethality.

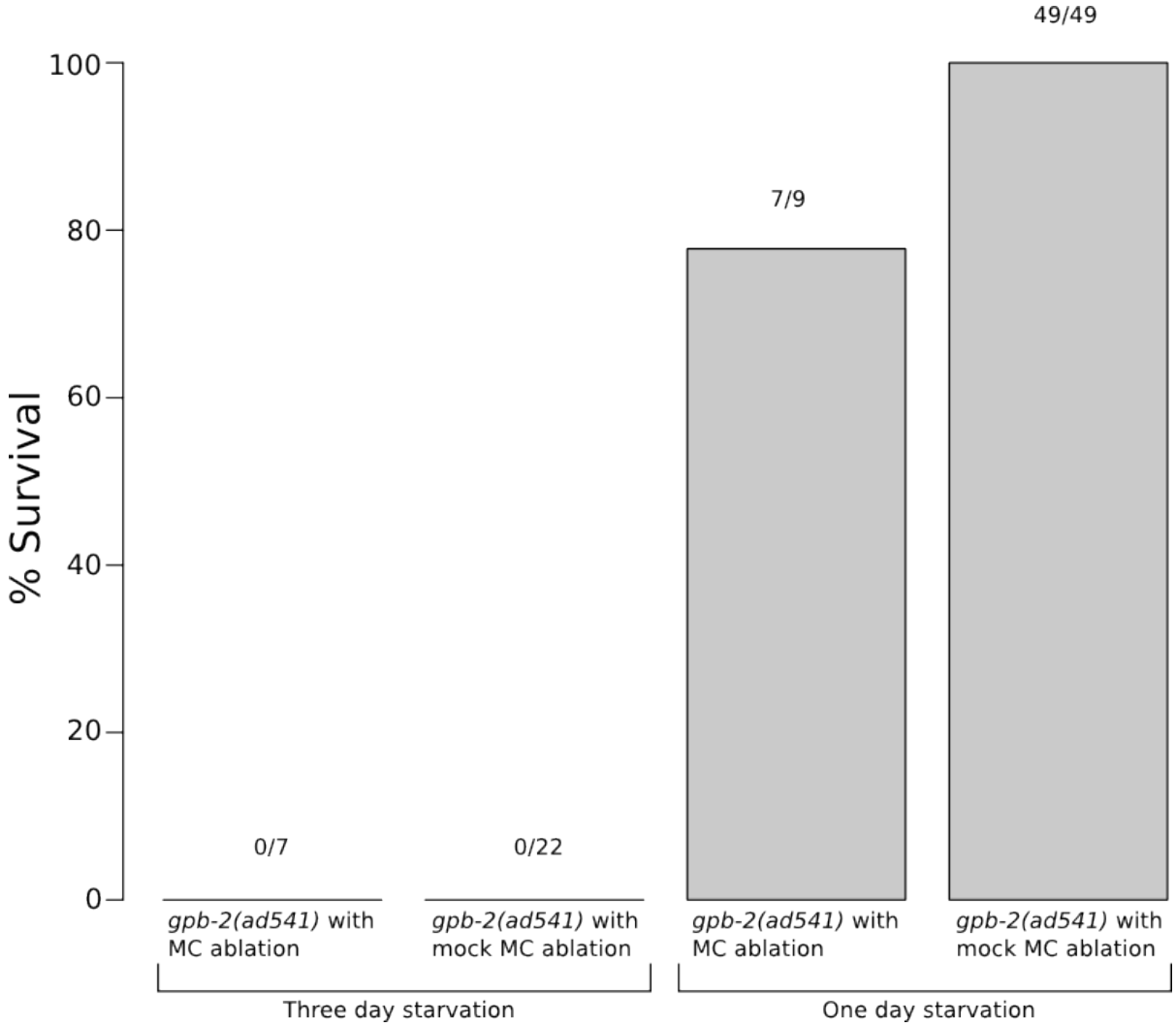


Figure 13. *gpb-2(ad541) C. elegans* after MC laser ablation or mock laser ablation. Worms were starved after ablation for either one or three days.

Optimization of starvation conditions

Despite the failure to rescue starvation-induced lethality with MC neuron laser ablation, I aimed to lessen the stringency of starvation. The MC neurons could still have a role in starvation response, but it may be a subtler role that is masked under harsh starvation conditions. If starvation is reduced to two days with recovery on *Comamonas*, 45% of the

worms survive. When the duration of starvation remains at three days, but the bacteria used for recovery is switched to HB101 *E. coli*, 62% of the worms survive. It is possible to increase the stringency of starvation by transferring worms to unseeded NGM plates, and then adding live bacteria culture. This does not provide as much food as an already seeded plate, but additional bacteria grows up over the course of a few days. When *gpb-2(ad541)* worms are starved for three days and transferred to freshly applied HB101 *E. coli*, 9% of the worms survive.

Laser ablation is a powerful technique that can dissect neuron function in *C. elegans*, but it is not perfectly suited for starvation assays. Without the ability to visually validate successful kills after shooting, it is necessary to perform a large number of operations quickly. Optimizing and developing the technique required many months of work with relatively little output. With these caveats in mind, I concurrently worked to develop an alternative method to supplement the laser ablation studies.

Genetic manipulation for MC neuron death

It is possible to subvert the apoptotic machinery to induce death in targeted cells or tissues. In *C. elegans*, expression of EGL-1 or an activated caspase causes a cell to undergo apoptosis. In order to produce these proteins in the correct location, a promoter that specifically expresses in that region must exist. Unfortunately, when I started exploring methods to genetically kill the MC neurons, there were no known pharynx-specific promoters that expressed only in MC.

While searching for a *C. elegans* MC-specific promoter, a colleague sent an image and asked our lab to identify the neurons in which a GFP construct was expressed (Figure 14). To our surprise, the GFP construct appeared to fluoresce in two pharyngeal neurons approximately where the MC neurons are located. The construct contained the promoter for *ador-1* driving expression of GFP. To validate that *ador-1* expresses in the MC neurons, I used a stitching reaction to fuse the *ador-1* promoter region of genomic DNA to GFP. Using genomic DNA from N2 worms, I PCR-amplified the 4-kb region immediately upstream from *ador-1*. I then PCR-amplified GFP from the pPD95.67 plasmid. Using PCR, I added a complimentary linking region to the 3' *ador-1* promoter region and the 5' GFP coding region. After fusing the generated fragments together, I injected the linear DNA into N2 worms. The transgenic worms expressed GFP in the pharynx, but the expression was in the I2 neurons, not the MC neurons.

Figure 14. The promoter from *ador-1* drives GFP expression in the pharynx. We hypothesized that the two neurons visible in the anterior pharynx were the MC neurons, but we later identified them as I2(L/R) (Han personal communication).

Combinatorial expression

Without an available single promoter to drive expression in the MC neurons, I aimed to develop methods using promoter combinations to generate my desired specificity. I searched through reported expression profiles, looking for known promoters that express in the MC neurons early in worm development. I then compared all of the expression profiles, looking for combinations of two promoters that both express in MC, but do not overlap elsewhere in the worm. The predicted combinations to test were: *pflp-21* + *prig-6*, *pflp-2* + *prig-6*, and *ptyra-2* + *prig-5* (Figure 15).

Candidate promoters to target MC neurons

Promoter	Expression	Protein
flp-21 + rig-6	MC	RecCasp, RecGFP
flp-2 + rig-6	MC	RecCasp, RecGFP
tyra-2 + rig-5	MC	RecCasp, RecGFP
sra-7	ASK	RecCasp, RecGFP
srg-8	ASK	RecCasp, RecGFP
flp-21	ADL, ASI, ASH, ASE, FLP, URA, MC, M4, M2	RecGFP
flp-2	AIA, RID, PVW, I5, MC	RecGFP
rig-6	AVA, AVB, AVE, PVC, AIB, AUA, AVG, RIB, RIC, SAA, SIA, SIB, RIF, RIM, RMD, RME, SMD, DA, DB, VA, VB, M5, NSM, MC, I3, MI	RecGFP
rig-5	AVD, AIN?, AUA?, RIC?, RIF? RMD, SMDV, I2, MC, M4, M3?, M2?	RecGFP
tyra-2	MC, NSM, ASE	RecGFP

Figure 15. Expression profiles of *C. elegans* genes taken from wormbase.org. The combinations of *pflp-21 + prig-6, pflp-2 + prig-6,* and *ptyra-2 + prig-5* are predicted to co-express in the MC neurons. *psra-7* and *psrg-8* only express in ASK and were included as controls.

I explored two options to promote apoptosis in MC neurons through a combination of promoters. The first option was a system developed by the Hubbard lab (Voutev and Hubbard 2008). The Hubbard system uses one promoter to drive expression of FLP, and the second promoter to express the protein of interest. The protein of interest has a stop sequence between the promoter and itself, preventing its translation. The stop sequence is flanked with FRT sites. When the first promoter, which drives FLP, is co-expressed with the second

promoter, which drives the protein of interest, the stop sites are excised allowing transcription to take place. For my purposes, I attempted to drive the expression of EGL-1 to induce apoptosis (Figure 16). Unfortunately, I was never able to get the Hubbard expression system to work in my hands. One problem may have been my inexperience with the Gateway method of plasmid construction, which was particularly complicated by the multiple insertions necessary into a single plasmid. Regardless of the reason, I did not successfully create any transgenic worms.

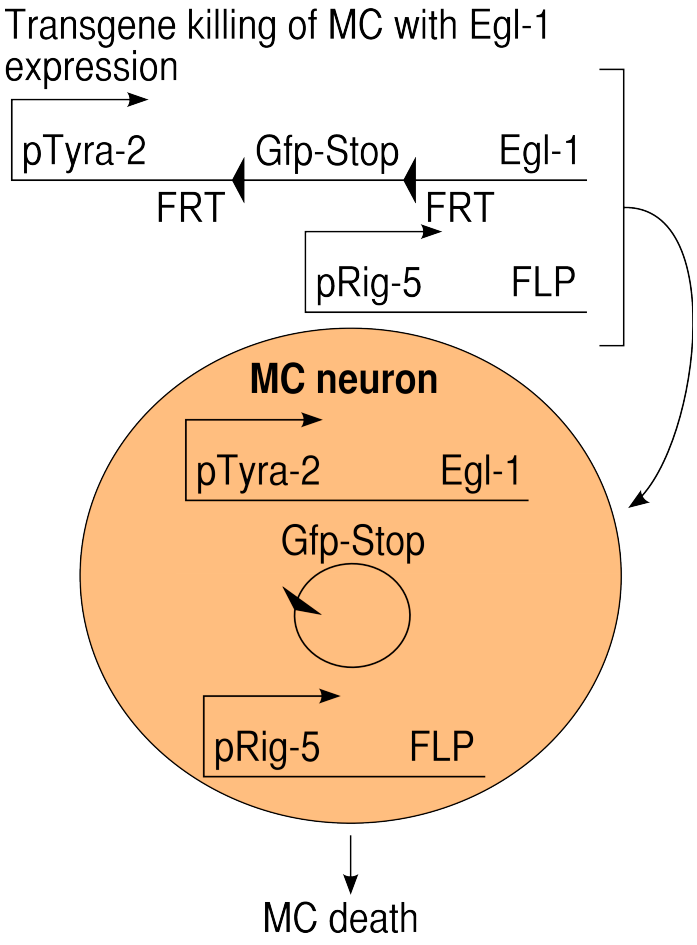


Figure 16. Two constructs are introduced into *C. elegans* and, in this example, they are regulated by *ptyra-2* and *prig-5*. When they are expressed separately nothing happens but, when they are co-expressed in MC, the GFP-Stop is excised, allowing expression of EGL-1. When EGL-1 is expressed, the MC neuron undergoes apoptosis.

The Chalfie lab developed the second combinatorial expression system I explored for MC killing in *C. elegans* (Zhang et al. 2004; Chelur and Chalfie 2007). Apoptosis is executed by an inactive caspase that is cleaved, leading to dimerization of the subunits into the active conformation (Cohen 1997). The Chalfie lab developed a system in which each half of the dimer is fused to a leucine zipper. When these two subunits are co-expressed, the leucine zipper brings together both halves of the caspase, which then becomes an active recombinant caspase (RecCaspase) (Figure 17). The Chalfie lab used this same idea to generate recombinant GFP (RecGFP). The RecGFP provided a useful control to validate expression before attempting to kill the MC neurons. Many of the RecGFP constructs were built and prepared for injection into wild-type worms. Due to other priorities, this experiment is still ongoing, but many of the constructs have been built and are ready to be injected.

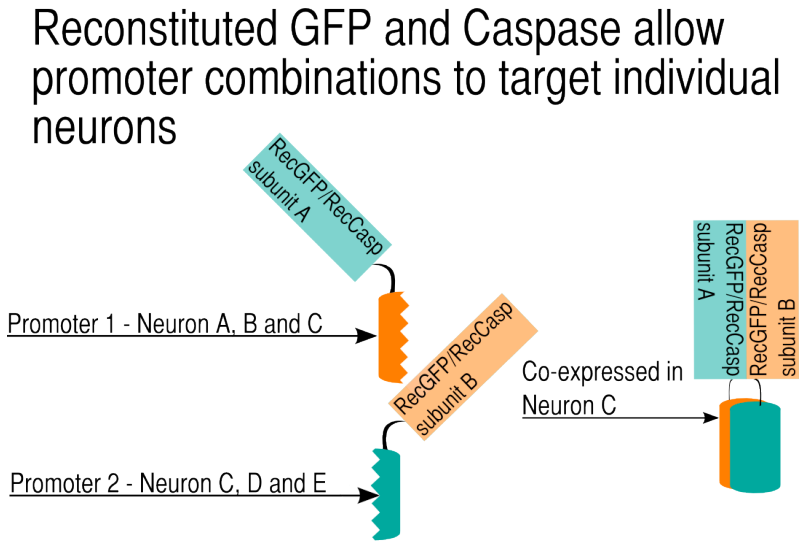


Figure 17. Inactive GFP or caspase subunits can become active when their individual subunits are brought together. Each component of the whole protein is attached to a leucine zipper that, when expressed with its match, associates. When the two subunits associate, activity is restored to either GFP or the constitutively-active caspase. In the case of the caspase, this initiates apoptosis.

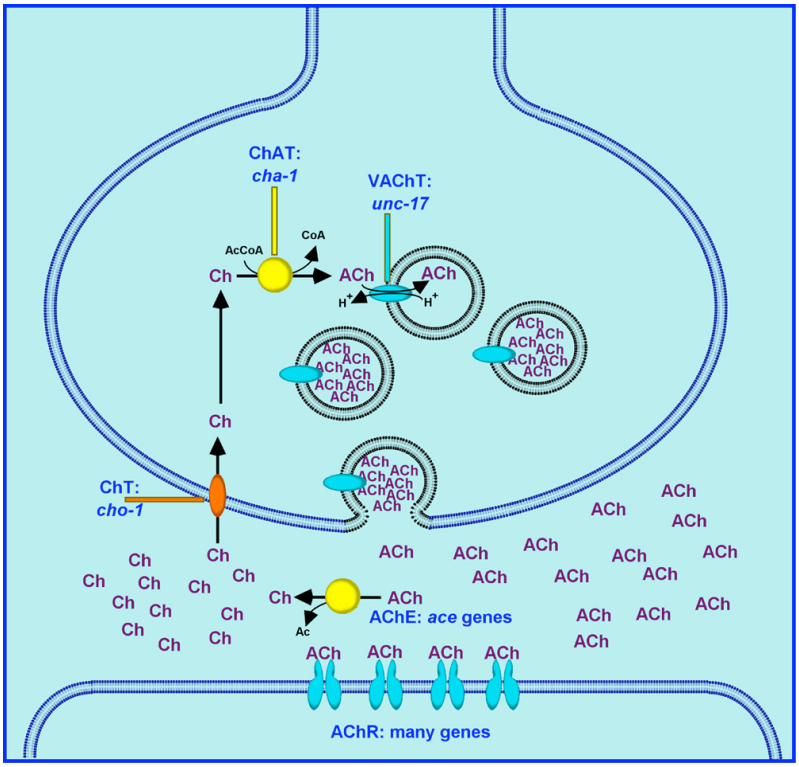


Figure 18. Acetylcholine processing, release, and uptake in *C. elegans*. *cha-1(p1152)* and *unc-17(e245)* are partial loss-of-function mutants, with reduced ability to prepare acetylcholine for release in cholinergic neurons. Reprinted under Creative Commons from Wormbook.org (Rand 2007).

Acetylcholine defective *C. elegans* mutants to test starvation-sensitivity

While killing the cholinergic MC neurons may rescue starvation-induced lethality of *gpb-2(ad541) C. elegans,* an alternative approach is to reduce the amount of acetylcholine released. There are viable worms that are deficient in loading acetylcholine into secretory vesicles. If a broad decrease in acetylcholine signaling renders *gpb-2(ad541)* worms more viable during starvation, then selectively rescuing acetylcholine release may also identify cells necessary for propagating a starvation signal.

In *C. elegans, cha-1* acetylates choline, and *unc-17* loads acetylcholine into synaptic vesicles (Figure 18) (Rand and Russell 1984). The *cha-1(p1152)* and *unc-17(e245)* alleles are loss of function mutations that are viable, but their movement is somewhat uncoordinated. I generated two worm strains with a *gpb-2(ad541)* background, one strain with *cha-1(p1152)* and the other with *unc-17(e245)*. I expected to see an increase in survival after starvation in each strain, because inhibiting release of acetylcholine should also inhibit starvation sensitivity in *gpb-2(ad541)* mutants. Results showed no appreciable increase in survival post starvation for either double mutant and revealed that *cha-1(p1152)* is starvation-sensitive on its own (Figure 19). While these results do not disprove that the MC neurons are involved in starvation signaling in the pharynx, they suggest a more complicated mechanism by which acetylcholine functions in starvation signaling.

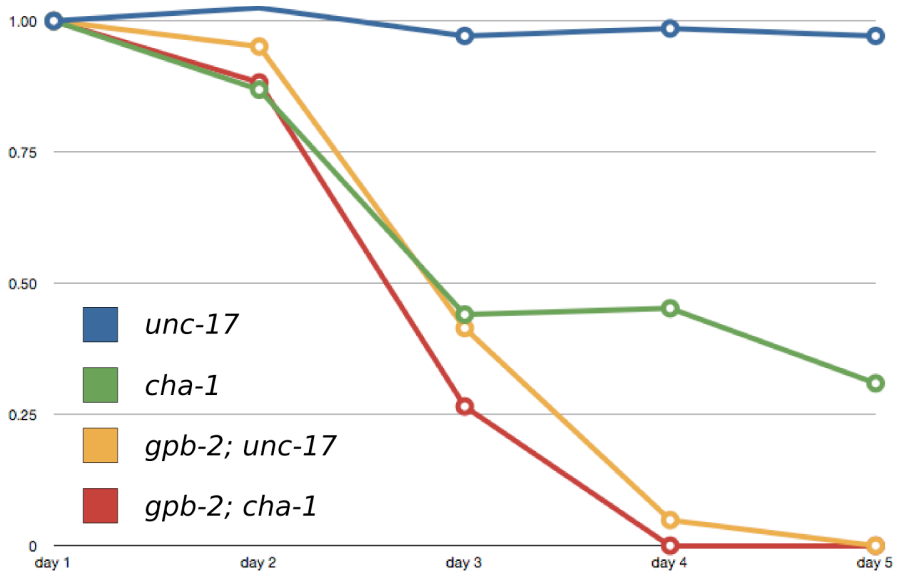


Figure 19. Mutant *C. elegans* deficient in acetylcholine release are still susceptible to starvation-induced lethality when paired with *gpb-2*. Atropine can rescue starvation-induced lethality in *gpb-2* worms. Since an acetylcholine receptor antagonist inhibits starvation-induced lethality, I hypothesized that reduced acetylcholine release would also rescue starvation-induced lethality. Not only do the double mutants fail to rescue, but *cha-1* itself is sensitive to starvation.

Oil Red O studies

Previous studies by our lab focused on blocking starvation-induced lethality in the *gpb-2(ad541) C. elegans* background. Various nutrients, such as leucine, inhibit this lethality (Figure 3) (Kang and Avery 2009), and some loss-of-function mutations, such as *flp-20* and *mgl-2*, rescue *gpb-2(ad541)* mutants. Introducing a *gcy-28(tm2411)* loss-of-function mutation reverses the rescue seen in *gpb-2; flp-20* and *gpb-2 mgl-2* worms. When *gcy-28(tm2411)* worms were observed during starvation, even without *gpb-2(ad541)*, the worms appeared much paler in color (Figure 20). Using Oil Red O to stain for fat, our lab observed that starved *gcy-28(tm2411)* worms lose fat much more quickly than starved N2 worms. We thought that *gcy-28*, a receptor type guanylate cyclase, might be responsible for interpreting a starvation signal in the gut and changing how the worm metabolizes fat. I took charge of this project, hoping to validate our ideas, by exploring the mechanism by which *gcy-28* is involved in fat metabolism.

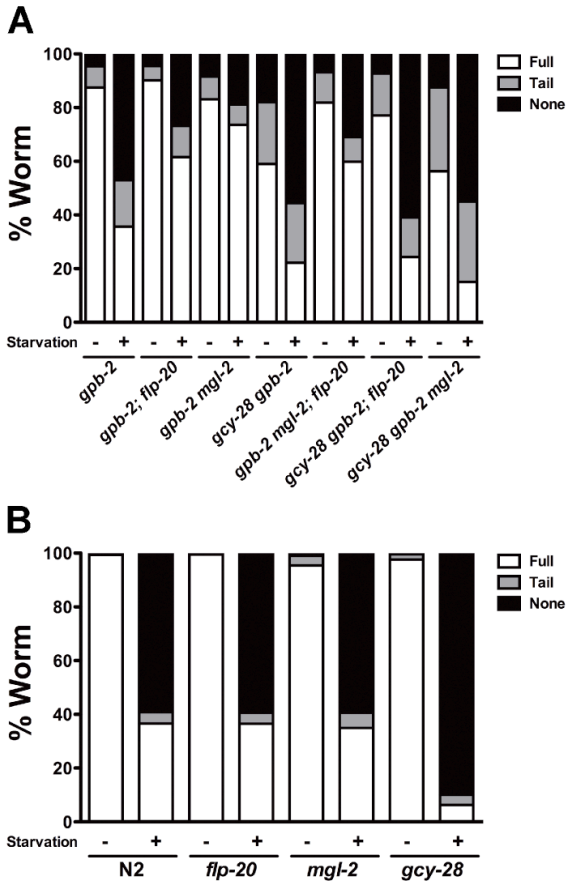


Figure 20. Starvation sensitivity correlates with fat staining using Oil Red O. Oil Red O identifies hydrophobic fat in *C. elegans*. A "full" worm is stained from the posterior pharynx to the tail, "tail" is stained predominately in the back fifth of the worm, and "none" has no significant staining. (a) When L4-young adult worms are starved for four hours, much of the fat staining is not seen in *gpb-2* worms. In *gpb-2; flp-20* and *gpb-2 mgl-2* worms, the staining is more consistent with the wild-type N2 phenotype. When *gcy-28* is added into the background, fat staining is the least pronounced. (b) When worms are starved for 24 hours in the single mutant backgrounds of *flp-20, mgl-2,* and *gcy-28*, only *gcy-28* fat staining differs from wild-type worms (Kang personal communication).

One of the criticisms of our past work with Oil Red O staining concerned the quality of pictures. My goal was to quantify how much fat was in the worm and, to do this, I needed to capture much higher resolution pictures. While fat quantification can be done biochemically, I felt there was relevant information in the location of stored fat and the

visible pattern of fat utilization at various stages of the worm life cycle. Also, I planned to do rescue experiments in which *gcy-28* is restored only in certain regions of the gut, giving a mosaic rescue in that tissue. To address past criticisms in Oil Red O imaging and to test the role of *gcy-28* in fat utilization in the intestines, my first step was in standardizing the preparation process for Oil Red O staining in *C. elegans*.

Before Oil Red O staining in *C. elegans* can be carried out, the conditions of starvation must be determined. Two important variables in starvation are the amount of time the worm is grown on bacteria, and the amount of time the worm is starved. *C. elegans* have different energy requirements at different stages of their lives, and the amount of time that the worm is grown determines what stage of life they will be in when an experiment is performed. When the worms enter a late adult stage, they become egg-laying factories. Eggs are typically enriched in fat, and adult worms dedicate their resources to pouring their fat into the eggs. Thus, eggs will stain bright red with Oil Red O. Worms filled with eggs can make the visual determination of fat storage more difficult, so I preferred to eliminate that variable if possible. There are also certain stages in which a worm will arrest or go into a dauer state if there is no food present, and I wanted to avoid these arrest states for my analysis. With these caveats in mind, I decided to focus on worms late in the fourth larval stage (L4) and the early adult stage of their life.

Optimizing and standardizing conditions to repeat Oil Red O staining

C. elegans are synchronized at the first larval stage (L1), and then allowed to grow on food. Normal conditions in the literature typically involve worms grown at 20°C and on

OP50 *E. coli* (Figure 21) (Byerly et al. 1976). Under normal conditions, L1 worms require approximately 33 hours to enter the L4 stage, and will lay their first egg 21 hours later. Higher quality food, such as HB101 *E. coli*, and increased temperature can alter the worm's metabolism and speed up maturity. Different mutations can also cause worms to grow at a different rate. While it is possible to normalize growth between worm strains with developmental checkpoints, the experimental design would be greatly simplified if differences are minimal within the chosen time points. Since I am interested in the rate at which fat is utilized, it is useful to use worms with an initial large amount of stored lipids. To identify when worms have relatively abundant fat and are roughly synchronized, I tested various growth conditions with different food sources and feeding times.

Development at Different Temperatures

	16°C	20°C	25°C
Egg laid	0 hr	0 hr	0 hr
Egg hatches	16 - 18 hr	10 - 12 hr	8 - 9 hr
First-molt lethargus	36.5 hr	26 hr	18.0 hr
Second-molt lethargus	48 hr	34.5 hr	25.5 hr
Third-molt lethargus	60.0 hr	43.5 hr	31 hr
Fourth-molt lethargus	75 hr	56 hr	39 hr
Egg-laying begins	~90 hr	~65 hr	~47 hr
Egg-laying maximal	~140 hr	~96 hr	~62 hr
Egg-laying ends	~180 hr	~128 hr	~88 hr

Figure 21. When worms are grown on OP50 *E. coli*, their developmental rate is largely determined by the temperature at which they are grown (Byerly et al. 1976).

Initially, I attempted to replicate a prior experiment using N2 and *gcy-28(ky713) C. elegans*. I chose the *gcy-28(ky713)* point mutation as opposed to the deletion allele *tm2411* because there were transgenic rescue animals already available. I grew the worms for 44 hours, and then starved them for 12 hours. Initially, I was testing which bacteria would allow worms to develop excess fat and remain synchronized with each other for these assays. Some bacteria cause N2 worms to store more fat (Brooks et al. 2009), while other strains can alter the growth rate(Shtonda and Avery 2006). I grew the worms on HB101 or DA837 *E.coli*. When N2 worms are grown on DA837 and then starved, they lose all fat, and results are not consistent with earlier results (Figure 22). When *gcy-28(ky713)* are grown on DA837, they develop less quickly and are noticeably smaller. Growing N2 and *gcy-28(ky713)* on HB101 appeared to keep the worms roughly synchronized, and the starvation staining was more consistent with earlier data. I repeated this experiment with a 50-hour feeding, followed by a 12-hour starvation (Figure 23), but the worms developed too quickly into adulthood, and both N2 wild type and *gcy-28(ky713)* worms matured and began egg development. When the worms were older, the N2 worms typically did not stain fully. Growing worms for 44 hours on HB101 at 20°C appeared to be the optimum condition to replicate our previous results.

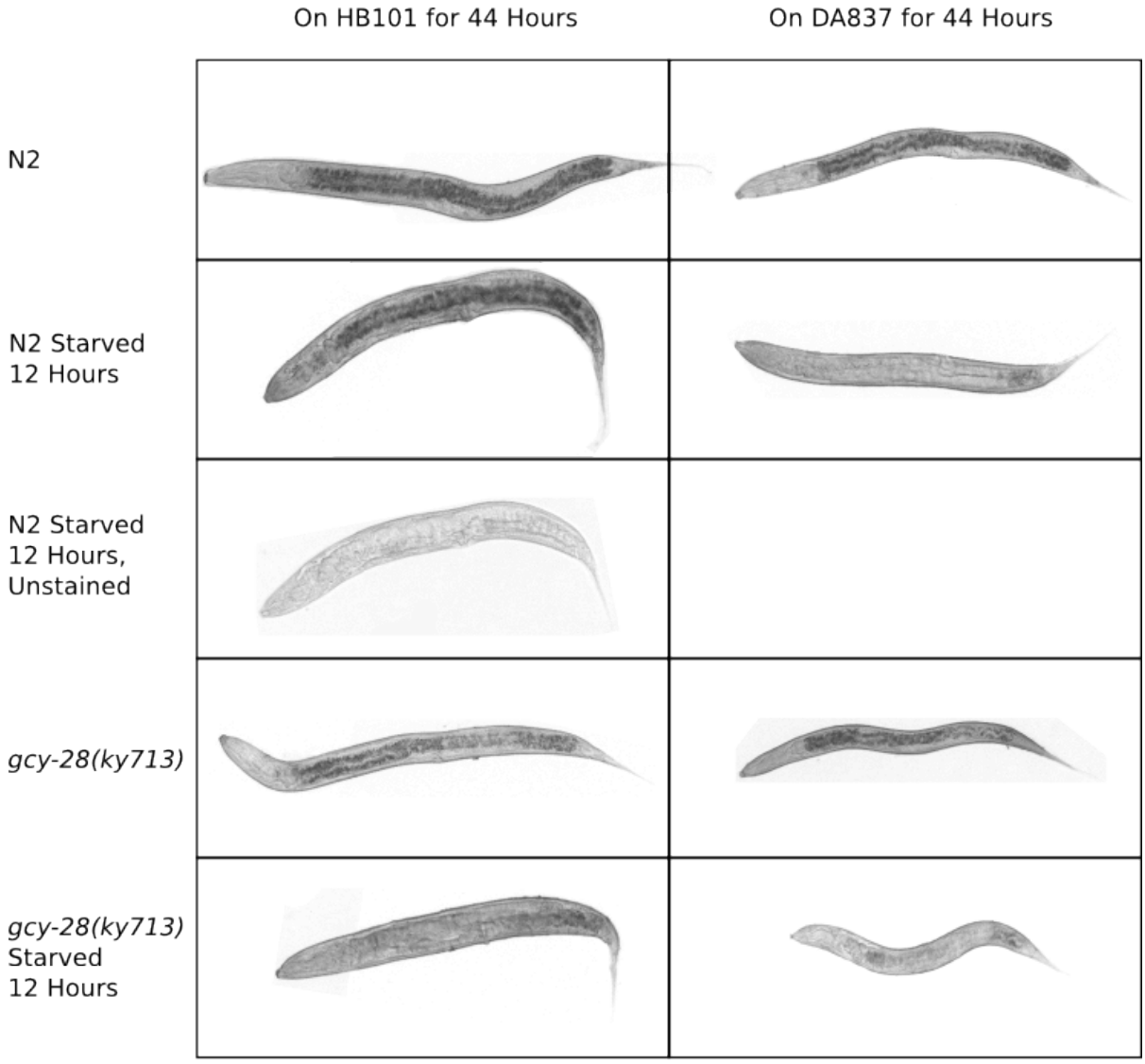


Figure 22. N2 and *gcy-28(ky713)* worms were grown for 44 hours and either starved for 12 hours and stained with Oil Red O or immediately stained without starvation.

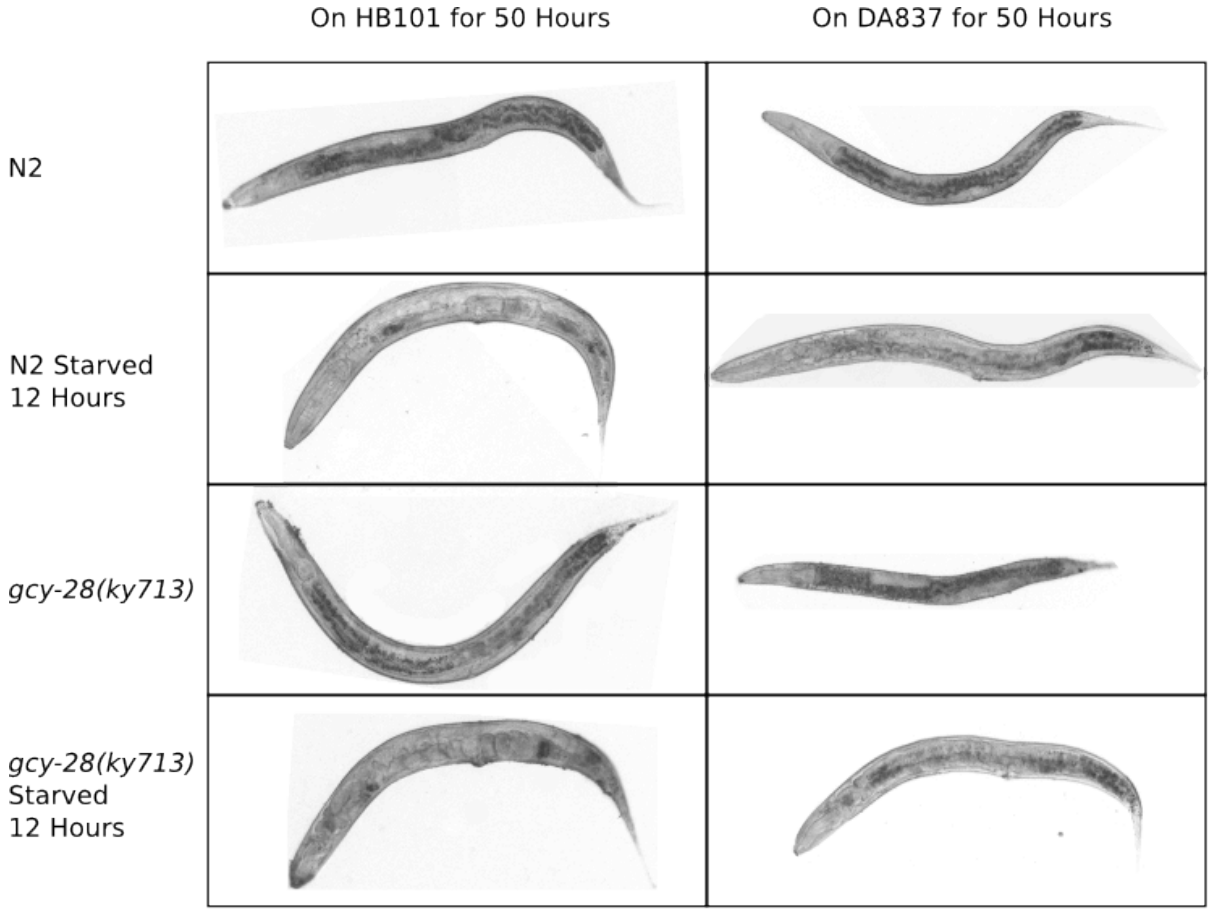


Figure 23. N2 and *gcy-28(ky713)* worms were grown for 50 hours and either starved for 12 hours and stained with Oil Red O or immediately stained without starvation.

Despite finding growth conditions that allowed the N2 and *gcy-28(ky713) C. elegans* to approach a similar staining pattern as that seen previously, the mutant starved condition exhibited unexpected staining. Instead of a clear tail staining phenotype, there was a very light splotchy staining throughout the gut in *gcy-28(ky713)* worms after starvation. An inconsistency in the experimental procedure was recognized: previous results utilized the *tm2411* deletion allele. I repeated the 44 hours of feeding, followed by 12 hours of starvation, in mutant worms containing the *gcy-28(tm2411)* allele (Figure 24), as done previously. Using

gcy-28(tm2411) resulted in fat staining patterns that closely matched previous work, supporting the hypothesis that *gcy-28* is a downstream receptor for starvation in the gut.

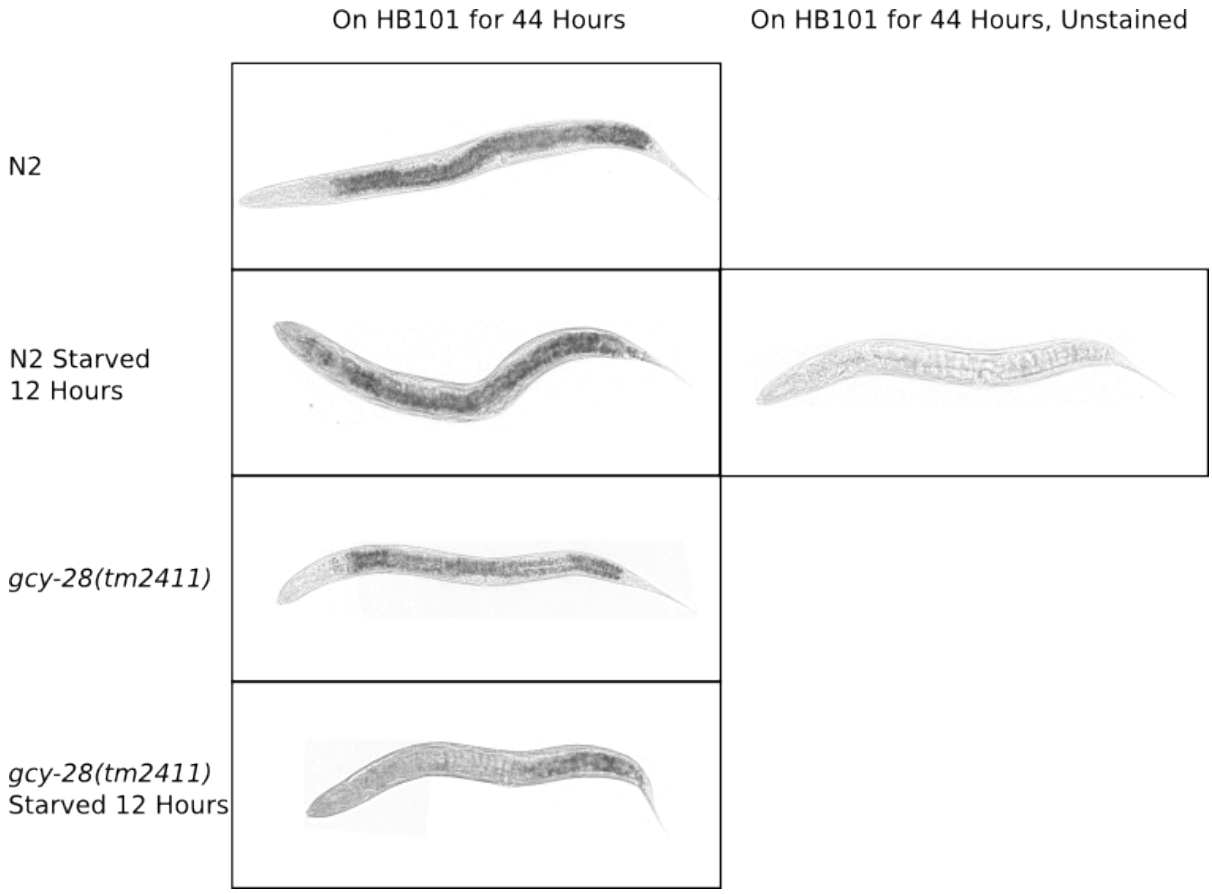


Figure 24. N2 and *gcy-28(tm2411)* worms were grown for 44 hours and either starved for 12 hours and stained with Oil Red O or immediately stained without starvation. These worms were prepared in Dallas, TX.

Validation of results

After the lab moved from Dallas to Richmond, I aimed to validate the conditions of feeding, starving, and staining *C. elegans* that I had established, and then attempt a gut-specific rescue of *gcy-28* to restore fat staining in a tissue-specific manner. Unfortunately,

my staining results using the same conditions were vastly different in Richmond (Figure 25) than in Dallas (Figure 24). The contrast in the pictures is slightly different because of the new microscope and camera setup, but it is clear that the starved N2 and *gcy-28(tm2411)* worms are stained much differently. Instead of a continuous stain from the pharynx to the tail in N2 worms, there is a wide unstained section in the midbody. Egg development is also quite clear in both N2 and *gcy-28(tm2411)* worms. I then aimed to identify what had changed in my experiment between Dallas and Richmond.

Figure 25. N2 and *gcy-28(tm2411)* worms were grown for 44 hours and either starved for 12 hours and stained with Oil Red O or immediately stained without starvation. These worms were prepared in Richmond, VA.

The results of my Oil Red O staining were different between Richmond and Dallas; thus, my first thought was to test my Oil Red O staining solution concentrations on mixed stage worms. Using mixed stage worms that had never been starved would help identify if

the problem was with the worms or with the Oil Red O. For this experiment, I used worm plates that were convenient and ready for staining. There was no starvation component so the exact genotype should not matter. The fixing condition was identical in each case, and the only variable manipulated was the concentration of Oil Red O stock solution mixed with water. Normally, a 60% Oil Red O stock solution diluted with PBS is used. When a 60% Oil Red O solution was compared to a 50% or 40% concentration, it was clear that the 60% solution produced the most consistent stain. When the concentration of stock solution was 50% or 40%, the stain appeared to come out of solution and appear as crystals that would coat the worm. At 60% stock solution, the portions of the worm that managed to stain looked similar to earlier results (Figure 26). Also, in the mixed stage, it was clear that L3, L4, and adult worms stained similarly to earlier experiments in the well-fed condition. The difference in staining did not appear to be due to buffers or staining solution changes.

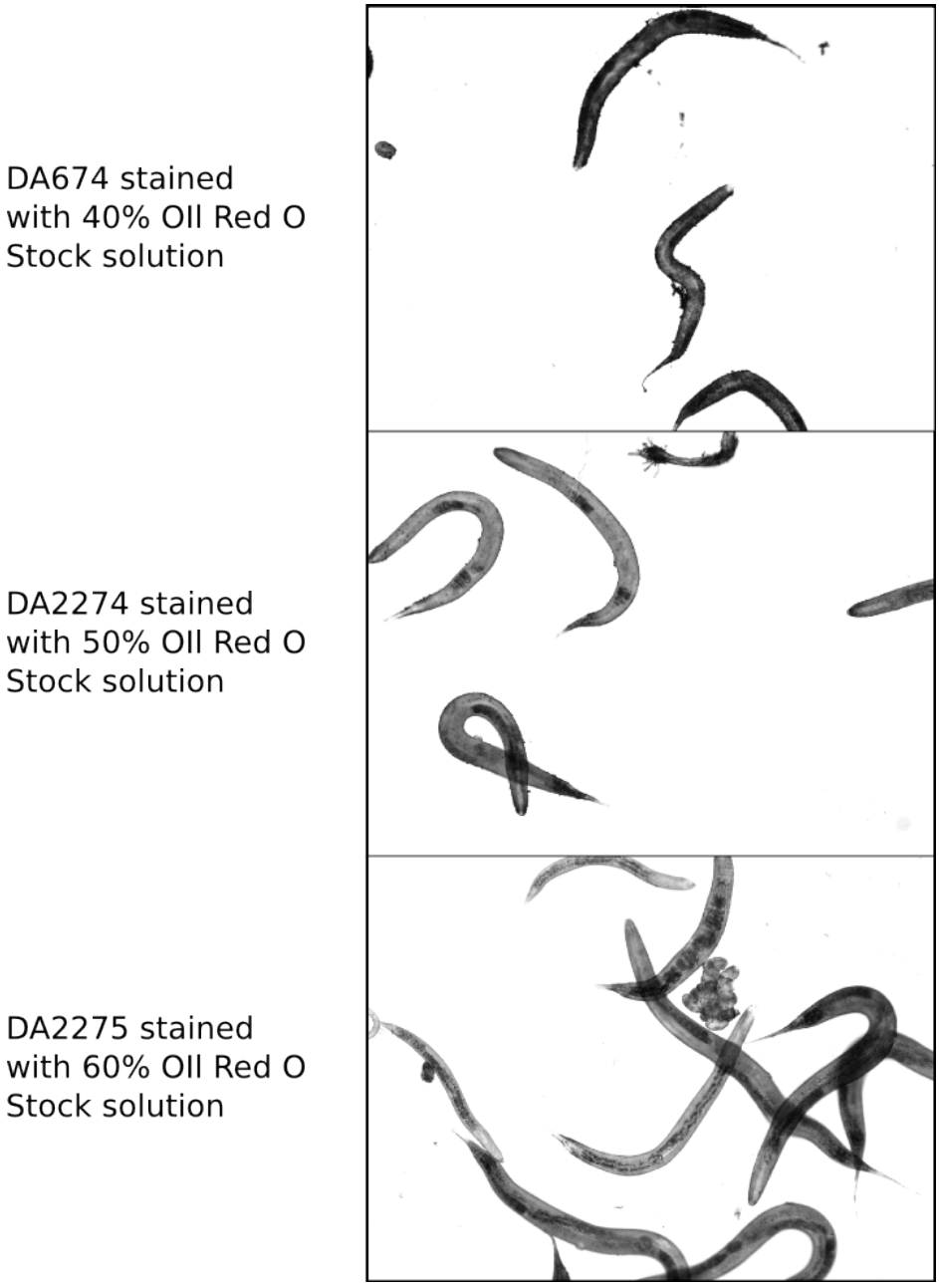


Figure 26. Mixed stage worms were stained with different concentrations of Oil Red O stock solution mixed with PBS. Normal Oil Red O working solution is a 60% Oil Red O stock solution, which produced the best results. Different worm strains were used because they were conveniently available.

Since the Oil Red O staining buffers stain fat in well-fed worms, I re-examined the *C. elegans* growth and starvation conditions. In Dallas, all incubations were done in a temperature-controlled room. In Richmond, worms were grown and fed in an incubator but starved on my bench. Typically, it is a few degrees warmer on the lab bench, and a 12-hour starvation at room temperature accelerates the growth and metabolism of the worms. I repeated my 44 hours of feeding followed by 12 hours of starvation in both N2 and *gcy-28(tm2411)* worms (Figure 27). Before and after starvation, the N2 worms were fully stained from pharynx to tail. The *gcy-28* worms did not stain as consistently as the N2 worms during the fed condition. The staining was much lighter in the *gcy-28(tm2411)* fed condition compared to the N2 worms, and was different from the identical experiment in Dallas in which both fed N2 and *gcy-28(tm2411)* looked identical (Figure 24). I believe there was a slight difference in temperature between the Dallas incubator and the Richmond incubator. The growth rates of N2 and *gcy-28(tm2411)* worms are not exactly the same, and I needed to find temporal conditions that keep the initial staining in each condition as similar as possible.

To determine the feeding time that gives the most consistent results between N2 and *gcy-28(tm2411) C. elegans*, I compared the size and staining patterns of each strain when grown for 40–44 hours on HB101 (Figure 28). N2 worms grow and stain quite consistently throughout the 40–44 hour time period, but there is quite a bit of variability in the *gcy-28(tm2411)* worms. *gcy-28(tm2411)* worms appear smaller during the first few hours of starvation, and do not stain consistently. From 42 hours and beyond of feeding, *gcy-28(tm2411)* worms exhibit increased staining and approach the size of N2 worms. I did not see much difference in worms older than 42 hours but, when worms are grown for 44 hours,

they begin developing eggs after a 12-hour starvation. To minimize the time spent developing eggs, I set 42 hours of feeding as the standard for future experiments.

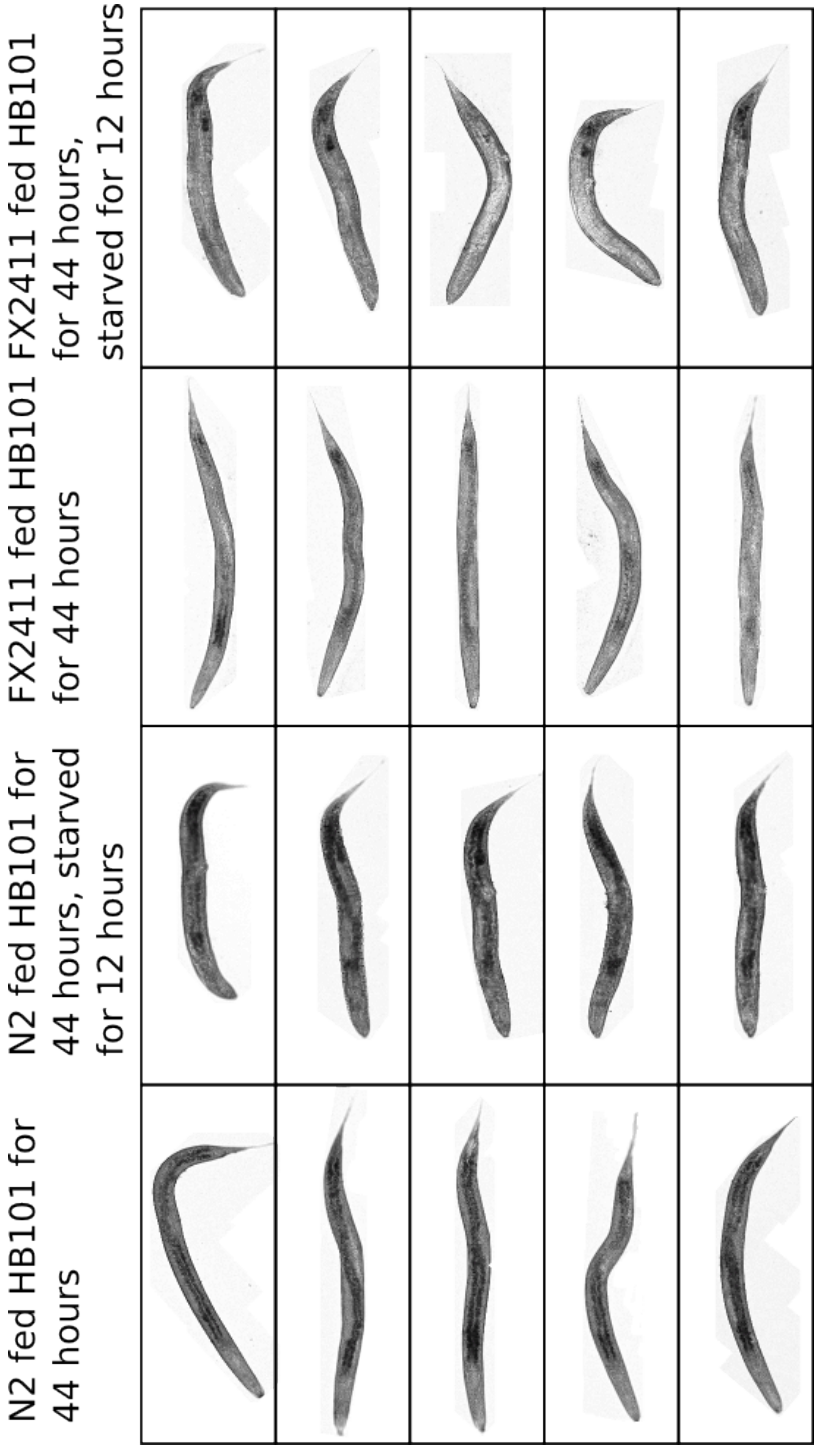


Figure 27. N2 and *gcy-28(tm2411)* worms were grown for 44 hours and starved for 12 hours, and then stained with Oil Red O. While the N2 worms yield consistent results with those from Dallas, the fed *gcy-28(tm2411)* worms are much more lightly stained, indicating less stored fat.

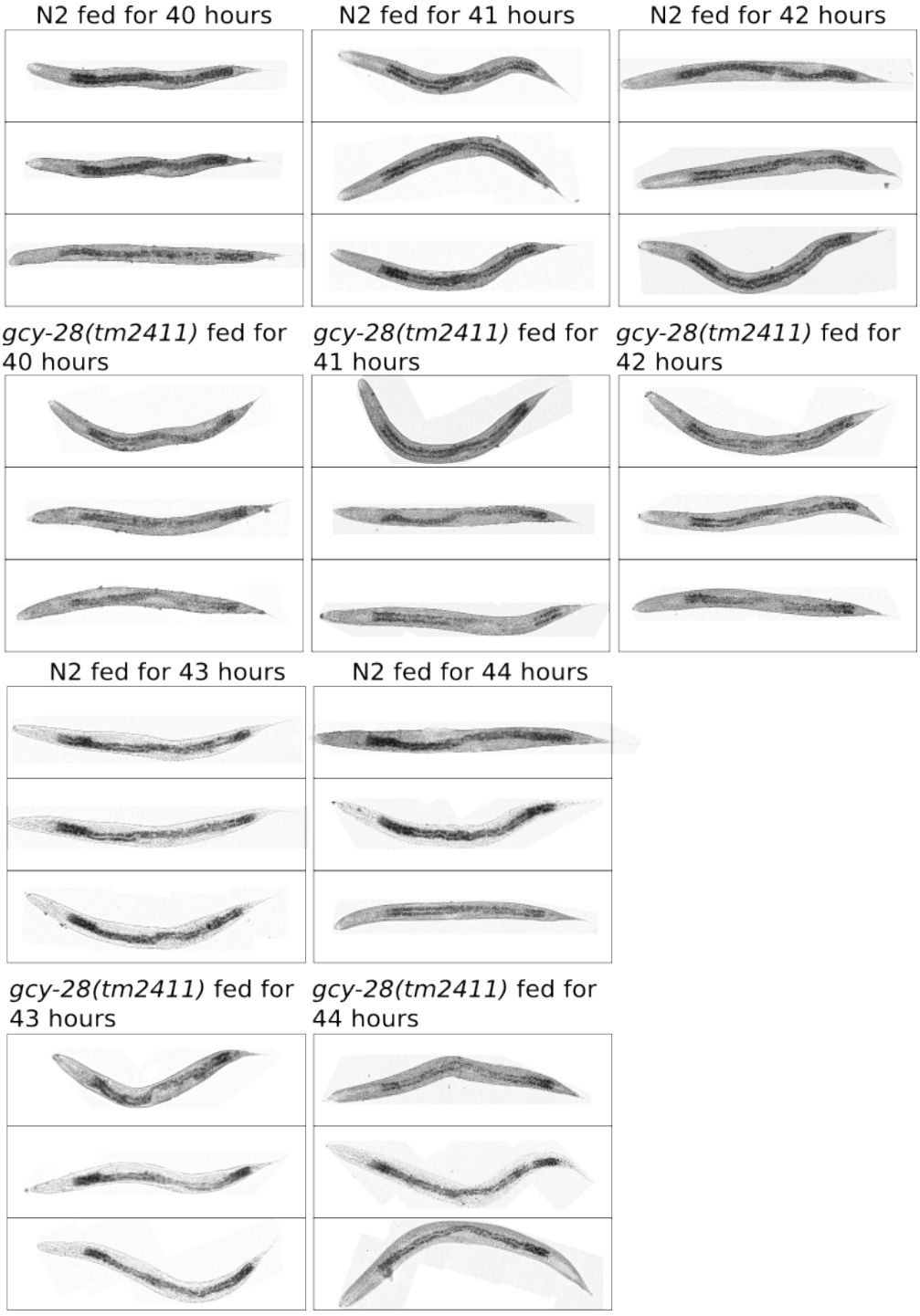


Figure 28. N2 and *gcy-28(tm2411)* worms were synchronized and fed HB101 for 40–44 hours. Worm size and Oil Red O fat staining was compared, and the most similar morphology between N2 and *gcy-28* appears to be at 42 hours.

Oil Red O stains inconsistently

With a 42-hour feeding time identified as an ideal condition for both N2 and *gcy-28(tm2411) C. elegans,* I repeated the full experiment where worms are fed for 42 hours and then starved for 12 hours (Figure 29). The fed N2 and *gcy-28(tm2411)* worms looked similar, as expected, but the starved worms still did not match the results I obtained in Dallas. Starved N2 worms did not have a solid staining throughout the gut. Instead, there was usually a clearing in the midbody of the worm, with fat staining anterior and posterior of the vulva. While more lightly stained, the *gcy-28(tm2411)* worms looked quite similar to the N2 worms. There is almost no detectable staining when starvation is extended to 24 hours in either N2 or *gcy-28(tm2411)* worms, and the worms look nearly identical (Figure 30). In previous work, when N2 worms were well fed and then starved for 24 hours (Figure 20b), a third of the worms exhibited a full fat staining phenotype. Despite my best efforts, I cannot get consistent staining results between my own work in Dallas and Richmond, or with previous data generated by others in the lab.

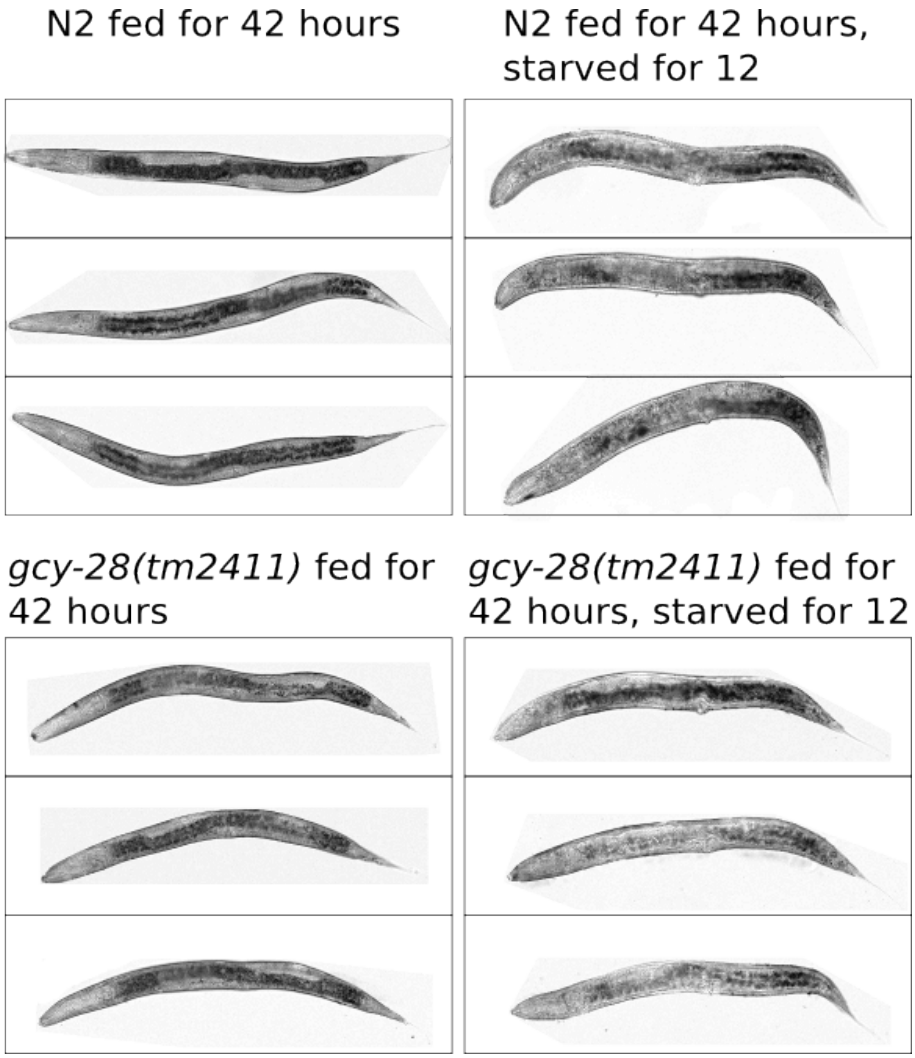


Figure 29. N2 and *gcy-28(tm2411)* worms were grown for 42 hours and either starved for 12 hours and stained with Oil Red O or immediately stained without starvation. Both N2 and *gcy-28* worms stain similarly after feeding. During starvation, the expected pattern of full staining was observed in the N2 worms, but a localized posterior staining in *gcy-28(tm2411)* worms was not seen. In both N2 and *gcy-28(tm2411)* worms, there seems to be a clearing of fat from the midbody, but significant amounts of fat staining on the posterior and anterior sides.

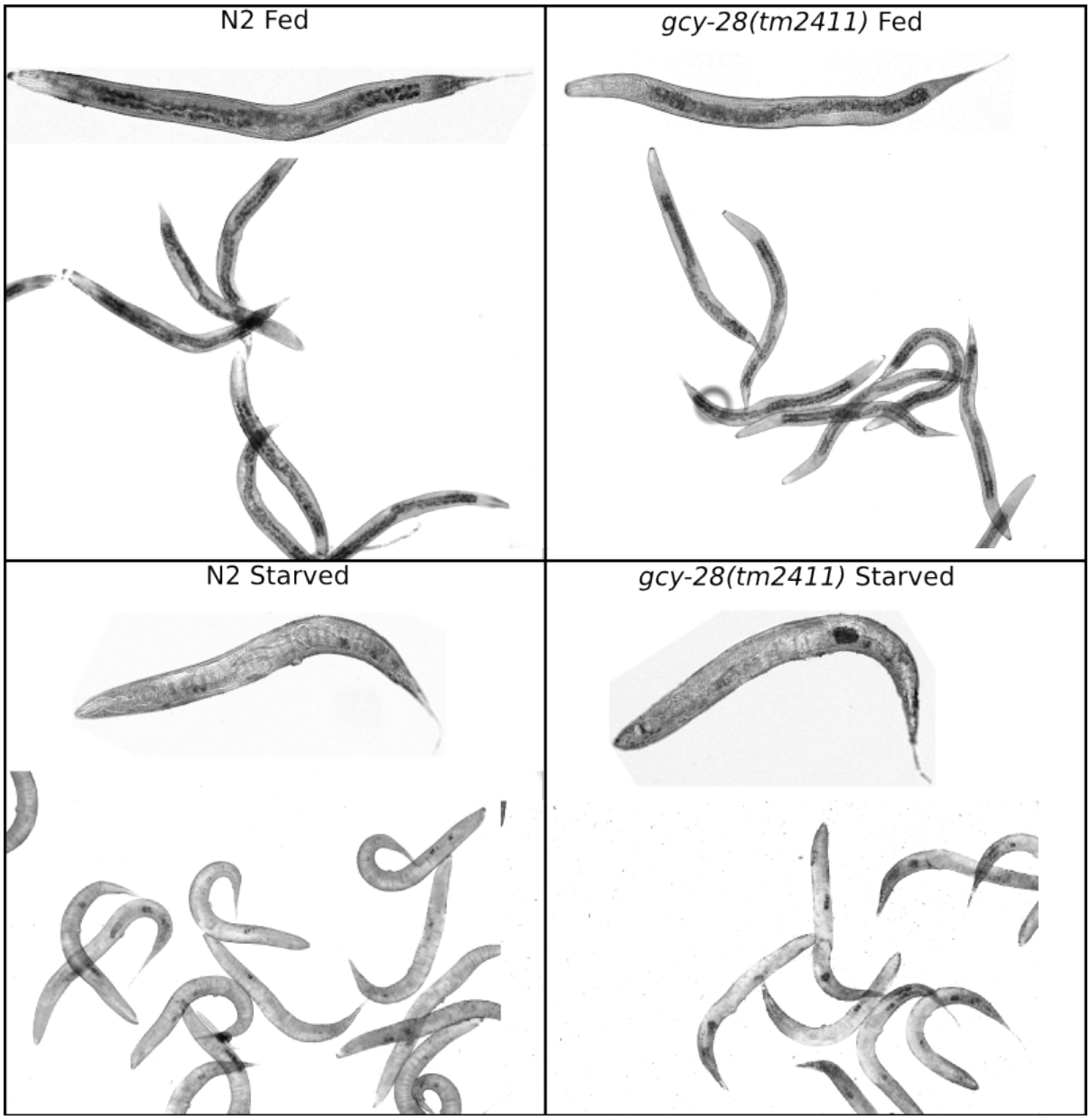


Figure 30. N2 and *gcy-28(tm2411) C. elegans* grown for 42 hours and then either starved for 24 hours and stained with Oil Red O or immediately stained without starvation. N2 and *gcy-28(tm2411)* worms stain similarly after a 42-hour growth on HB101 at 20°C. While roughly a third of the N2 worms are expected to retain full fat staining after a 24-hour starvation, N2 worms have almost no fat staining after this starvation. Likewise, the lack of fat staining in *gcy-28(tm2411)* worms is almost identical with the 24-hour starved N2 worms.

Reexamining how fat stains in *C. elegans*

Failing to identify consistent Oil Red O staining conditions in the *C. elegans gcy-28(tm2411)* deletion mutant, I stained a mixed-stage preparation of the *gcy-28(ky713)* point mutation. I wanted to get an idea of how the worms would stain at all stages in development without starvation, and I wanted to explore fat staining in both *gcy-28(tm2411)* and *gcy-28(ky713)* genotypes. The worms were grown at 20°C on HB101 bacteria. The stain consistently labels fat throughout the entire worm in adults (Figure 31A). In L4 and young adult worms, the staining appears to extend from the pharynx to the tail, but there are still heterogeneous staining and splotchy sections that are not consistent with results from Dallas (Figure 31B). Worms that are at the L3 stage or younger do not have a consistent pattern of fat staining (Figure 31C, D).

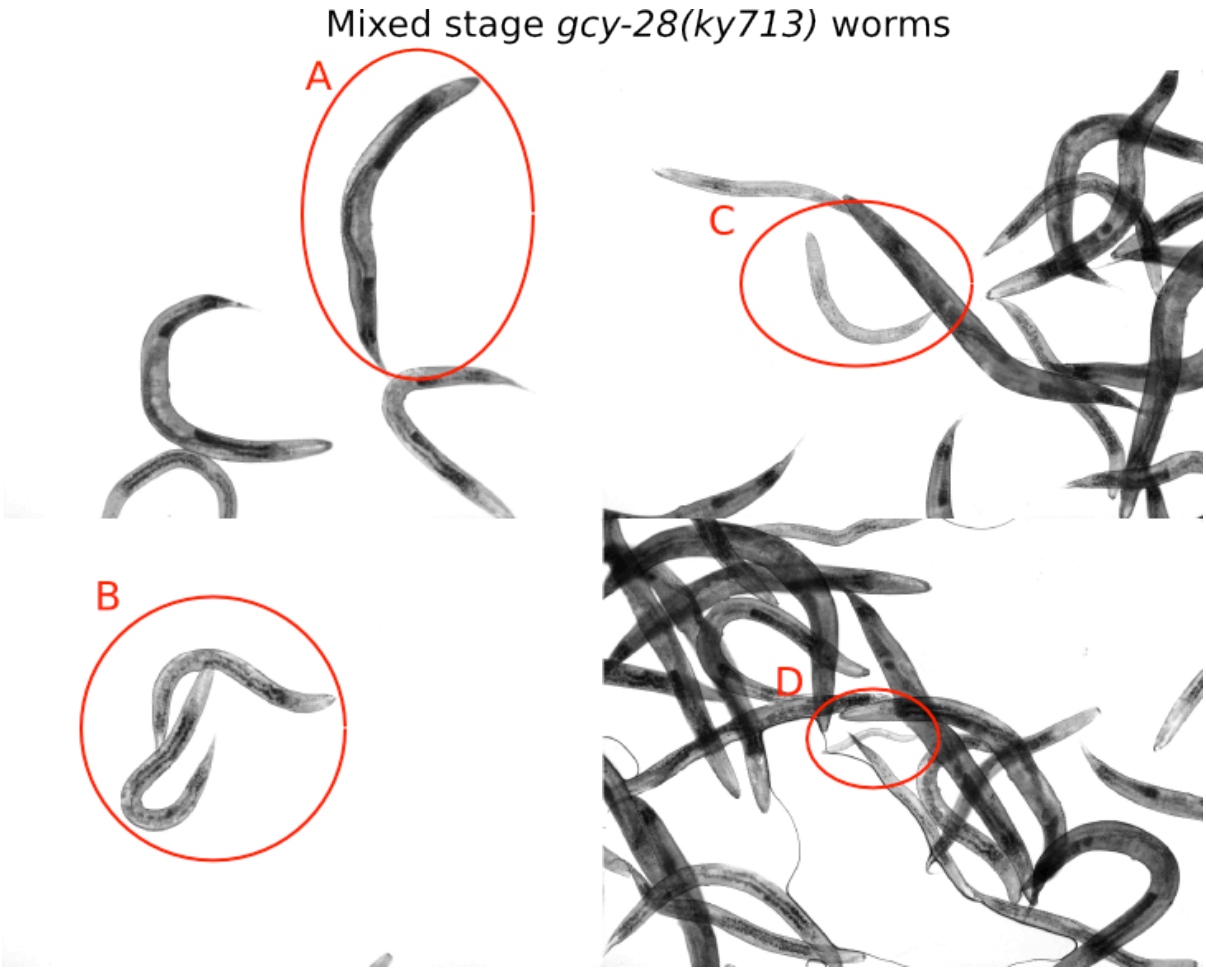


Figure 31. Mixed stage *gcy-28(ky713) C. elegans* stained with Oil Red O. Images of numerous worms at various life stages show how well worms that are homozygous for the point mutation *gcy-28(713)* stain when grown on HB101 *E. coli* at 20°C. (A) Adult worm, (B) L4 worms, (C) L3 worm, and (D) L2 worm.

With an optimized feeding time to get the most consistent Oil Red O fat staining in *C. elegans*, I decided to more closely observe how the pattern of fat staining changes with starvation over time. When a worm is at the late L4 or early adult stage and has not been starved, I typically see a dark Oil Red O stain from the pharynx to the anus. As fat is utilized in the worm during starvation, the staining decreases on the anterior side first, with the fat staining gradually fading in an anterior to posterior pattern. Since incubation temperatures

may be higher in Richmond, VA compared to those in Dallas, TX, the rate of fat utilization in worms may also be correspondingly increased. When N2 worms are starved for 12 or 24 hours, the pattern of fat utilization may have progressed completely to the point of no fat staining visible. By looking at worms earlier during starvation, I hoped to identify a pattern of fat utilization from the anterior to the posterior. I grew N2 worms for 42 hours on HB101 *E. coli* at 20°C, and then starved them for 0, 3, 6, 12, or 24 hours (Figure 32). At 0 and 3 hours of starvation, N2 worms maintain their fat throughout their body. At 6 hours, the fat staining is nearly abolished, with some inconsistent staining in the tail. At 12 and 24 hours of starvation, there are no appreciable levels of fat in the worm. One interesting feature to note is the molted cuticle seen at 3 hours of starvation. Three hour starved worms had been plated 45 hours earlier, and the fourth-molt lethargus occurs 44–46 hours after egg hatching (Figure 21). The shedding of the cuticle suggests that the development rate is consistent with the literature. One of my concerns was that the worm was directing its stored fat into egg production, but at 6 hours of starvation there is very little fat staining and no evidence of developing eggs. At 12 and 24 hours starvation there are some visible oocytes, but the oocytes develop after most fat has gone away. The discrepancy between results I obtained in Richmond and in Dallas does not seem to be caused by developmental timing or staining effectiveness.

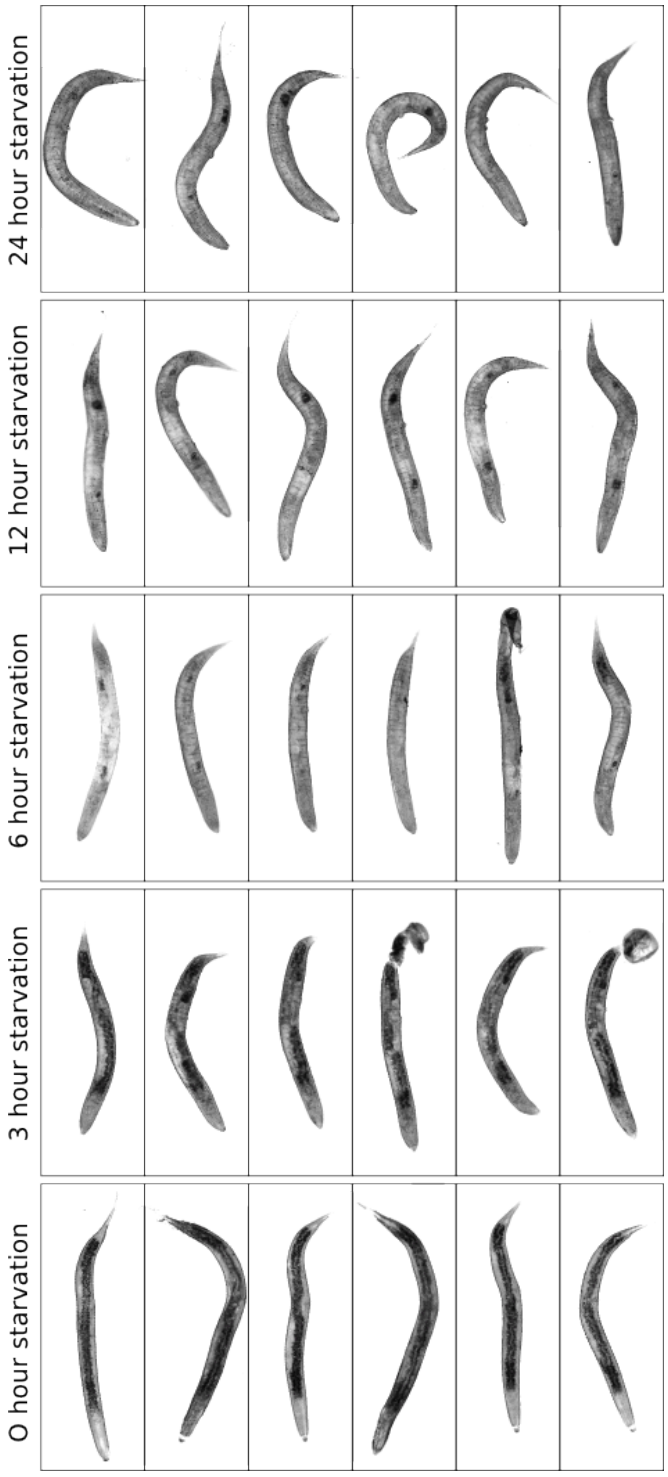


Figure 32. N2 *C. elegans* fed for 42 hours on HB101 *E. coli* at 20°C, and then starved for 0, 3, 6, 12, or 24 hours before staining with Oil Red O. Worms maintain their fat staining when starved for 0 or 3 hours, but quickly and completely lose their stored fat afterwards.

Testing differences in protocols on Oil Red O staining pattern

Our lab originally performed these experiments using a Sudan Black staining protocol (Wang et al. 2008) with Oil Red O that broke the *C. elegans* cuticle with a freeze crack. Unaware of differences between protocols, I used a more recent Oil Red O staining protocol published by the same lab (O'Rourke et al. 2009), and this is the protocol I followed when staining worms. Colleagues expressed concern that the altered protocol may be causing the differences that had been seen. There was also some concern that I was growing the worms for too long. Previous researchers in the lab did not grow the worms for over 40 hours. I repeated the experiment using a freeze-crack protocol and a 37-hour feeding (Figure 33). N2 and *gcy-28(tm2411)* worms were grown for 37 hours on HB101 *E. coli*, and then starved for 12 hours. Worms were fixed either with or without a freeze-crack step. Worms that were treated with a freeze-crack appeared identical to worms that were not exposed to a freeze-crack, suggesting that this additional step was not responsible for past results.

I had not previously tested feeding times of less than 40 hours (Figure 28), and I wanted to systematically observe how well *C. elegans* could store and retain fat with this shorter growth time. N2 and *gcy-28(tm2411)* worms were grown for 37 hours on HB101 *E. coli* and then starved for 0, 2, 4, 6, 8, or 10 hours at 20°C (Figure 34). Without any starvation, *gcy-28(tm2411)* worms failed to stain with Oil Red O as well as N2 worms, either indicating that they do not store fat as quickly, or that they grow more slowly than N2 worms. In both N2 and *gcy-28(tm2411)* worms, there was very little fat staining after just two hours of starvation.

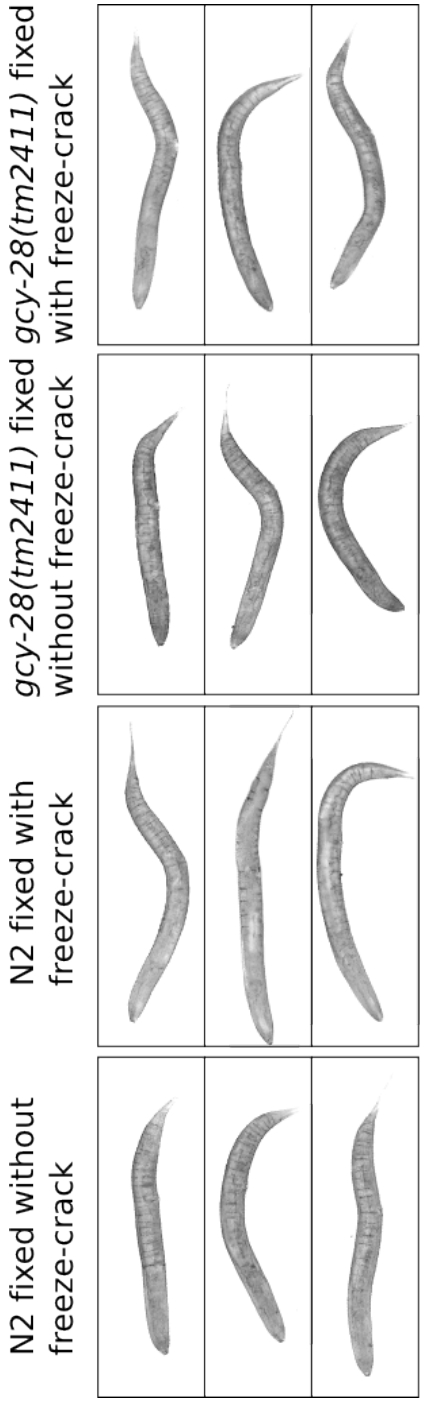


Figure 33. N2 and *gcy-28(tm2411)* worms grown 37 hours on HB101 and starved for 12 hours, then fixed with or without a freeze-crack step before Oil Red O staining. The original protocol followed by Chanhee Kang used a freeze-crack step, and this step might cause a difference in Oil Red O staining quality. There does not seem to be any difference in staining between worms treated with or without a freeze-crack step.

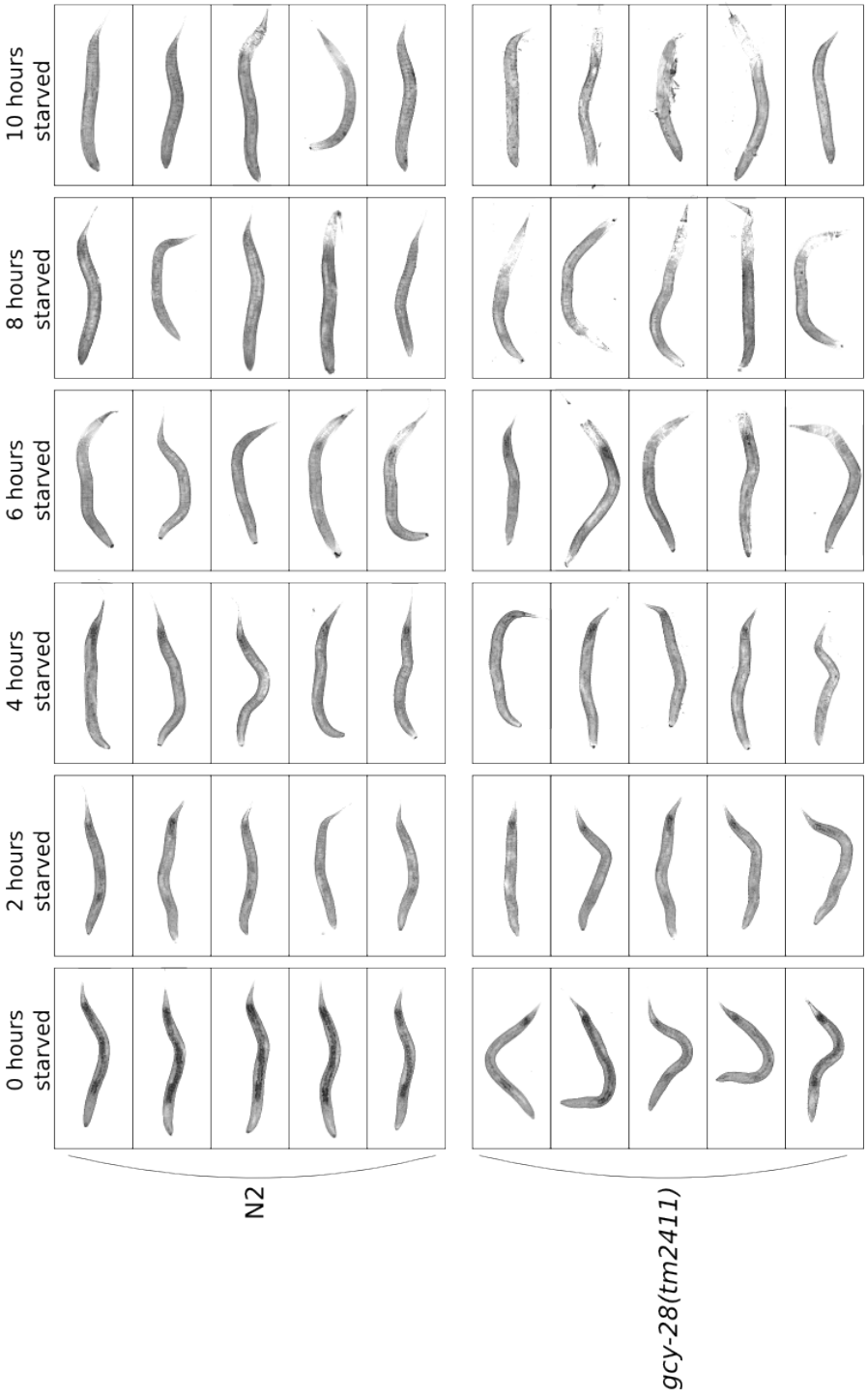


Figure 34. N2 and *gcy-28(tm2411) C. elegans* grown for 37 hours on HB101, starved for 0, 2, 4, 6, 8, or 10 hours, and then stained with Oil Red O. Staining at 0 hours in *gcy-28* worms was inconsistent in younger worms, showing that 37 hours of growth is not adequate. In N2 and *gcy-28* worms, there is little Oil Red O staining seen after 2 hours of starvation.

Uncontrolled temperature effects

Temperature can be difficult to regulate, and temperature has a significant effect on the growth rate of *C. elegans*. Even in our lab incubators, I have noticed the temperature is usually slightly cooler on the bottom of our 20°C incubator than the top or middle. In Dallas, worms were grown in a temperature-controlled room at 19°C. The difference between 19° and 20°C is small, but it is possibly responsible for the radically different results. A 1°C difference in temperature is difficult to reliably reproduce, so I wanted to test another standard worm incubation temperature, 15°C. The tested feeding times of 48 and 55 hours on HB101 were followed by a 15-hour starvation in N2 or *gpb-2(ad541)* worms (Figure 35). I used *gpb-2* as a negative control that should not stain well after starvation. Neither set of feeding/starving conditions produced worms with significant fat staining, leading me to conclude that fat retention after starvation is too difficult a phenotype to pursue further.

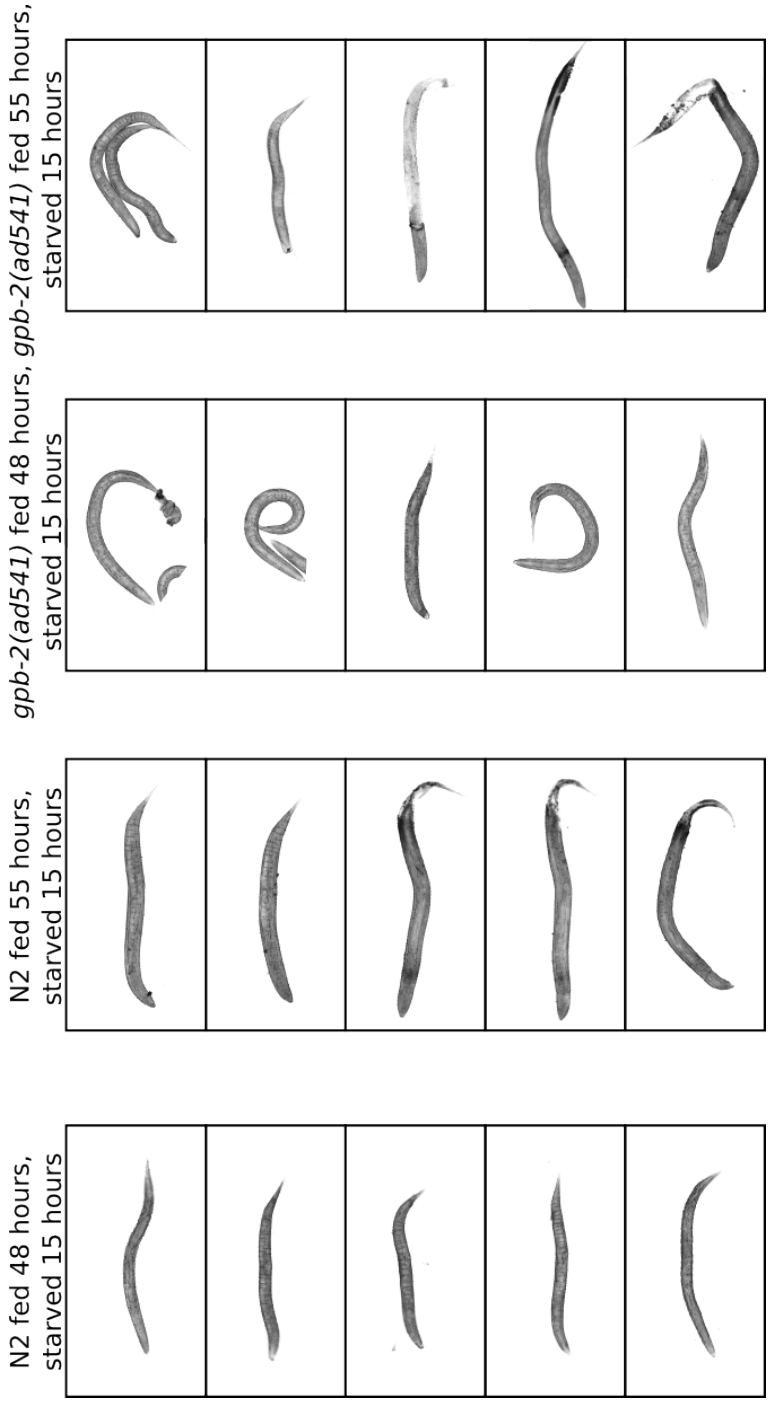


Figure 35. N2 and *gpb-2(ad541)* worms were grown at 15°C for 48 or 55 hours. Worms were then starved for 15 hours and stained with Oil Red O. Earlier staining discrepancies may have been temperature dependent and I hoped to find conditions that achieved a more consistent staining at 15°C. N2 worms did not retain significant fat when grown at a lower temperature.

CHAPTER FOUR
Discussion

Exploring starvation using *gpb-2*

Much of the work done with the *gpb-2(ad541) C. elegans* mutant background has been to identify the biochemical and physiological changes that take place during a starvation response. The work of Dr. Chanhee Kang began taking a closer look at how the starvation signal is propagated throughout the worm. Dr. Kang identified the AIB and AIY neurons as important for amplifying or repressing a starvation response (Kang and Avery 2009). The aim of the work presented here was to further identify cells and tissues in the worm that initiate and react to a starvation response. My initial goals were to validate the role of the MC neurons in propagating a starvation signal and to determine if the receptor type guanylate cyclase, GCY-28, is responsible for fat utilization in the gut of the worm.

Laser ablation of the MC neurons

Starvation in *gpb-2(ad541) C. elegans* can present a wide variety of phenotypes, but the L1 death coupled with excessive autophagy in the pharynx makes that organ convenient to dissect the signaling pathway. Because the pharynx is somewhat isolated neurologically from the rest of the worm, the physiological components between starvation and death are vastly reduced. *gpb-2(ad541)* worms are acetylcholine-sensitive, and it is likely that if acetylcholine is initiating autophagy in the pharynx, the source of acetylcholine is the pharynx. If the source of acetylcholine can be located, the next aim is to identify the mechanism by which acetylcholine release is regulated. The two problems needing to be

addressed were which neuron(s) are initiating autophagy in the pharynx and how the neuron(s) can be reliably manipulated to test for other signaling components.

One of the most obvious ways to manipulate a cell is to simply kill it, and then observe what happens to the organism. This is a strength of *C. elegans* because of its invariant cell lineage, and was the first course of action that I considered. The two methods for killing cells in the worm that I explored were using a laser ablation technique and genetically killing with a transgenic pro-apoptotic construct.

The main advantage of a laser ablation study is that it is possible to kill a variety of neurons in *C. elegans* relatively quickly, and results can be validated with a high level of confidence. The caveat with laser ablation is that surgery is necessary to test future hypotheses following the original discovery. A screen for signaling molecules that activate the cholinergic neuron of interest would need an additional series of laser ablation operations for each candidate tested. Ultimately, an inordinate amount of time could be spent with laser ablations and, while that is not necessarily a bad thing, a faster and easier method needs to be developed.

The benefits and caveats of killing cells in *C. elegans* genetically are opposite those of laser ablation. Cell killing is accomplished by inducing apoptosis by a promoter-driven transgenic construct. Making an apoptotic transgene can take weeks or months, and it is impossible to know if the hypothetical construct will be functional until it has been injected into the worm. Even if the construct works as expected and the target cells are ablated, the process must be repeated on a new set of neurons if the candidates fail to rescue the phenotype. There is a large initial investment of time with genetic ablation, but the great

advantage is the time saved in future studies. Under ideal conditions and not counting time for validation or preparation of media, approximately 10 worms can be operated on with laser ablation every 30 minutes. With a transgenic construct, thousands of worms can be ready with even less preparation, making large screens and more intricate experiments more practical.

The pilot study, which suggested that ablation of the MC neurons could rescue *gpb-2(ad541)* starvation sensitivity in L1 *C. elegans*, produced inconsistent results. In the first set of experiments, where worms were starved for three days and then recovered on agar plates, there was increased survival in MC-ablated worms compared to the wild-type worms (Figure 10). Unfortunately, the sample size was quite low, and more than half of the mock ablation control worms survived. When the conditions were tweaked so that recovery took place on agarose plates, the mock ablation control worms did not survive consistently. When the mock ablation experiment was repeated on agarose plates, the ratio of worms surviving was quite different from those of the pilot study (Figure 11). When comparing starvation on agarose to starvation in buffer (Figure 13), there were even more differences. Ultimately, I concluded that starvation sensitivity in *gpb-2(ad541)* was much more consistent when worms were starved in buffer, and proceeded based on this conclusion.

The conditions for recovery of *C. elegans* after laser ablation are just as important as the precise conditions of starvation. Mutant *gpb-2(ad541) C. elegans* that have been starved have variable levels of autophagy in the pharynx that correspond to the stringency of starvation. Pharyngeal autophagy limits the ability of worms to extract nutrients from bacteria, and some bacteria are easier for *gpb-2(ad541)* worms to eat after starvation.

Comamonas is more difficult for starved *gpb-2(ad541)* worms to recover on than HB101 *E. coli* (Figure 12). Past experiments in which mock-ablated *gpb-2(ad541)* worms were starved on agarose and then recovered on HB101 had 0/7 and 6/17 worms survive after 4 days starvation (Figure 10). In my hands, 4/11 *gpb-2(ad541)* worms survived a 4-day starvation on agarose when recovered on HB101 (Figure 11). I hoped to shorten the starvation time and make survival of mock-ablated *gpb-2(ad541)* worms more consistent by switching to the more stringent recovery bacteria *Comamonas.*

I believe the most appropriate balance between stringent and permissive starvation conditions is to err on the side of less sensitivity. If all mock-ablated *C. elegans* die, then any worms that can manage to survive will become a much more significant result. I could have tested a combination of both stringent and permissive starvation, but over the course of many months, my rate of successful ablations was quite low with little improvement. As a result, I focused my efforts on a more stringent 3-day starvation followed by recovery on *Comamonas*. I also used 1-day starvation as a control. No *gpb-2(ad541)* worms should die after a 1-day starvation, so any that fail to grow should be from error in laser ablation.

The most simple conclusion from my laser ablation study is that killing MC neurons does not rescue *gpb-2(ad541)*-induced starvation sensitivity in *C. elegans*. Since most of the worms survived MC ablation after a 1-day starvation, the laser operation itself was not killing a majority of the worms. Laser ablation of MC did not promote survival in *gpb-2(ad541)* worms after a 3-day starvation. The small number of worms operated on could be part of the problem, but the difficulty of the technique prevents quickly testing a large sample

of worms. The technical complexities and the lack of controls make it difficult to develop concrete conclusions from a laser ablation followed by starvation in *C. elegans.*

The main difference between my laser ablation experiments and those done earlier was the starvation step. Typically when *C. elegans* have their pharyngeal neurons ablated, they are immediately returned to food and allowed to recover for up to one day. This makes it possible to observe the health of the worm, and allows it time to grow larger. The larger the worm, the easier it is to visualize cell nuclei and determine if the targeted neurons were killed. Introducing a starvation step immediately after laser ablation introduced numerous complications, which I spent a great deal of time trying to address during optimization of my assay. While there were complications with the stringency and length of starvation, I believe the most problematic variable was validation of the killed neurons.

It is possible to mount *C. elegans* onto a slide after 24 hours of starvation following laser ablation for validation of cell death but, without food, the worms will remain in an L1 arrested stage. When the worms are at the L1 stage, they are incredibly delicate and are more prone to physical damage while being physically manipulated. It is also difficult to immediately see the MC neurons, and the worms cannot remain on the slide for more than 15 minutes or the sodium azide anesthesia could start introducing organism-wide nerve damage. It is not practical to confirm cell death after the starved worms are allowed to recover, because many *gpb-2(ad541)* mutants will have died even without laser ablation. I am not satisfied with the present method, in which mock MC-ablated survival rates are applied to laser MC-ablated survival rates. If the laser MC-ablated survival rate is higher than the mock, I assume the laser killing successfully rescues *gpb-2(ad541)* starvation sensitivity. There is

no direct observation of MC laser ablation. With an experiment that is already so complicated and lacking definitive controls to confirm successful kills, it is impossible to build decisive conclusions based on the data collected.

Genetic ablation of MC neurons

Genetic ablation of cells has been used to kill targeted cells in our lab previously. EGL-1 initiates a signaling cascade that ultimately activates the caspase CED-3. A transgenic construct consisting of a promoter and EGL-1 can be injected into *C. elegans,* and the chosen promoter directs the location at which EGL-1 is expressed. Unfortunately, there are no known promoters that express specifically in the MC neurons.

The most straightforward approach to genetically ablate the MC neurons is with a single promoter. While there are no such promoters in the literature, a personal communication suggested that there might be a suitable candidate. When GFP is attached to the *ador-1* promoter, there are clearly two neurons that exhibit GFP fluorescence in the anterior bulb of the worm (Figure 14). The neurons are in the proper location to conceivably be the MC neurons. To validate this, I generated my own *pador-1::GFP* construct using a stitching PCR technique. The transgenic worm that I generated had very localized expression in two pharyngeal neurons but, unfortunately, they were not the MC neurons. I managed to develop a worm that expresses GFP in the I2 neurons and in the nerve ring.

While there was no known single MC-specific promoter, I discovered a handful of expression profiles that, when combined, should produce the desired MC expression pattern. The *C. elegans* community has an extensive website, wormbase.org, that details the

expression pattern of thousands of genes at all life stages of the worm. Examining these records, searching for known expression profiles that include MC, I generated a list of combinations that should be specific at an early stage of development (Figure 15). Expression that happens early in development is important because neuronal corpses can still be active for several hours. I found the promoter combinations of *flp-21* + *rig-6, flp-2* + *rig-6,* and *tyra-2* + *rig-5* that should specifically express in the MC neurons.

Originally, I tried to utilize a complicated *C. elegans* expression system that used FLP/FRT (Voutev and Hubbard 2008) to achieve the MC-specific expression of EGL-1. One promoter would drive expression of EGL-1, but a FRT flanked GFP-Stop sequence is immediately upstream. The second promoter would drive expression of FLP and, in cells in which both promoters drive expression, the GFP-Stop would FLP out. The design of these constructs is advantageous in the fact that the GFP-Stop sequence will fluoresce and help validate the promoter expression. The component of this technique that was most attractive was its use of Invitrogen's "Gateway" system. Once the donor plasmids were created, it should have been a quick and simple job to generate the handful of plasmids necessary for injection. Unfortunately, after many months of troubleshooting, I could never get the Gateway system to work and, ultimately, gave up on this intriguing setup for a more traditional cloning strategy.

A reconstituted GFP or caspase system allows for a combination of promoters to either fluoresce or kill cells when each promoter is co-expressed. Both reconstituted GFP and apoptosis-inducing caspase are dimers, and these dimers are non-functional when they are separated. When the separate dimers are co-expressed, they still will not become functional

on their own. When each component of the GFP or caspase dimer is joined to a leucine-zipper domain, the leucine-zipper will bring the two components of the dimers together to form a functional protein (Figure 17).

Designing RecGFP and RecCaspase constructs does not give functional templates that can be reused, such as the entry clones that are made using the Gateway method. Once appropriate primers and PCR conditions have been established, however, it is relatively simple to produce the required end product. The time it took me to build RecGFP constructs ready for injection was considerably less than the time spent on the FLP/FRT scheme. While the FLP/FRT system was more elegant and potentially useful for other projects, the time and cost savings through the use of the RecGFP system were more appropriate.

Injections of various RecGFP constructs began but never progressed very far. This project was dropped, not because of any inherent difficulty, but because my priorities were focused elsewhere.

Genetic ablation and laser ablation of the MC neurons in *C. elegans* either has not supported the hypothesis that MC is the cholinergic neuron propagating starvation sensitivity in the pharynx, or the necessary studies have not been completed. I did pursue one other idea to address the role of MC, with respect to acetylcholine release and starvation sensitivity in *gpb-2(ad541)* worms.

Manipulation of acetylcholine release in *C. elegans*

Atropine, a muscarinic acetylcholine receptor antagonist, can rescue the starvation sensitivity of *gpb-2(ad541)*. The role of atropine is the basis for my hypothesis that

cholinergic neurons in the pharynx relay a starvation signal. Ablating neurons is one way to impair cholinergic signaling, but it should also be possible to restore cholinergic signaling to a worm that is deficient. If mutant worms impaired in the release of acetylcholine can better survive *gpb-2(ad541)*-starvation sensitivity, then selectively rescuing acetylcholine release could identify where GPB-2 is the most important.

I crossed *gpb-2(ad541)* into two different acetylcholine mutants, *unc-17* and *cha-1*, that are involved with loading acetylcholine into synaptic vesicles (Figure 18). CHA-1 catalyzes acetylcholine synthesis, and UNC-17 shuttles acetylcholine into vesicles for release. The *cha-1(p1152)* and *unc-17(e245)* alleles are both loss-of-function mutations that make the worm more uncoordinated. If my hypothesis is correct, *gpb-2; unc-17* and *gpb-2; cha-1* should be less sensitive to starvation than *gpb-2(ad541)*. Both *gpb-2(ad541); unc17(e245)* and *gpb-2(ad541); cha-1(p1152)* are starvation-sensitive (Figure 19). What especially concerned me was the fact that without *gpb-2(ad541)* in the background, *cha-1(p1152)* is sensitive to starvation by itself. While this result is interesting, it complicates the hypothesis that acetylcholine is necessary to propagate a starvation response in the worm.

gcy-28 and Oil Red O staining

Fat staining of *gcy-28(tm2411)* worms with Oil Red O was supposed to be the end of a story about AIB neuron secretion of the FLP-20 neuropeptide in *C. elegans*. We hypothesized that the receptor type guanylyl cyclase GCY-28 is a component in transducing the FLP-20 signal through the gut to regulate fat utilization. Previous work indicated that fat staining in the gut was a phenotype that could be treated like starvation sensitivity. Initially,

we found that when *gpb-2(ad541)* worms are starved, there is less fat staining than that seen in wild-type N2 animals. The *flp-20(pk1596)* deletion allele can rescue *gpb-2(ad541)* starvation sensitivity and fat utilization, but *gcy-28(tm2411)* suppresses the *flp-20(pk1596)* rescue (Figure 36, 20). *gcy-28(tm2411)* is expressed in various head neurons and throughout the gut, and has been shown to act in olfactory food sensing and foraging behaviors(Tsunozaki et al. 2008). I was hoping to determine the role of the gut specific *gcy-28,* and I intended to create both gut specific and gut mosaic rescue of *gcy-28* to firmly establish a function in that tissue.

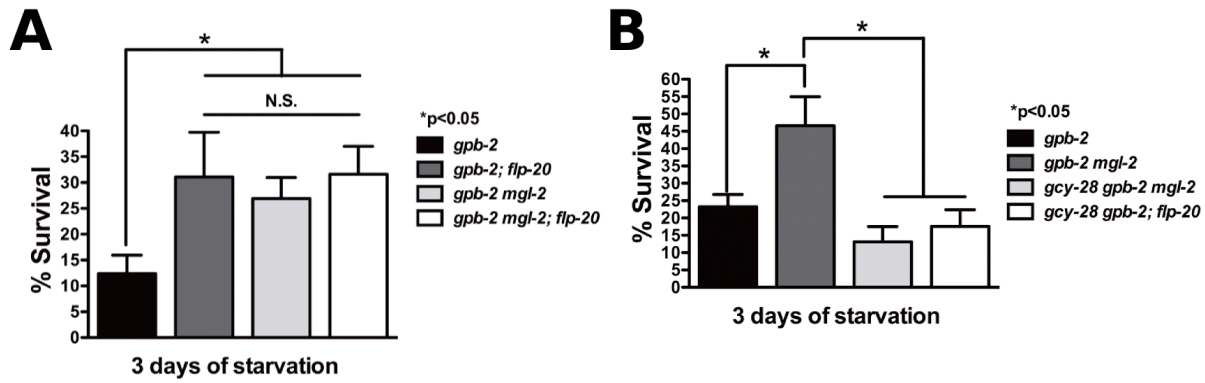


Figure 36. *flp-20* and *mgl-2* can inhibit starvation-induced lethality due to *gpb-2*, but this sensitivity is restored with the addition of a *gcy-28* mutation. (A) After three days of starvation, about 12% of *gpb-2* mutant *C. elegans* survive. Loss of function in either *flp-20* or *mgl-2* can rescue some survival. (B) When *gcy-28* is included in the background of either *gpb-2 mgl-2* or *gpb-2; flp-20*, the starvation sensitive phenotype is restored (Kang personal communication).

Previous results showed that there is a clear difference between how late stage/adult N2 *C. elegans* that are starved can retain fat compared to equivalent *gcy-28(tm2411)* worms (Figure 20). I was able to repeat the experiments that showed *gcy-28(tm2411)* worms stored less fat in the gut than N2 worms after starvation (Figure 24), but when my lab moved from Dallas, TX to Richmond, VA, I was unable to obtain similar results (Figure 25). The short-

term goals for my project shifted from determining the role of *gcy-28* in fat metabolism to finding conditions that affected fat metabolism in late stage N2 worms.

Fat staining experiments performed previously in the laboratory did not have explicit conditions in regard to feeding time, starvation conditions, and staining protocols. This was due to results being reproducible regardless of deviations in protocol. When I took over the fat staining project, I aimed to better control these variables. Once I encountered serious problems in replicating past results, I was forced to spend an inordinate amount of time trouble shooting all aspects of the project.

At the 2012 *C. elegans* metabolism meeting, many colleagues discussed problems they had in different starvation assays. Anecdotally, I heard about students insisting their worms grow on select shelves of an incubator because of temperature differences between the top and bottom shelves influencing their results. At UT Southwestern, our worms were housed in a temperature-controlled room that was large enough to limit temperature fluctuations. The incubator room was also slightly below the standard worm growth temperature of 20°C. I did not think such small differences in temperature could influence the Oil Red O staining experiment, but I reconsidered that idea after the meeting.

When I tested how *C. elegans* retain fat after starvation at lower temperatures, I decided to focus on 15°C. 15°C is a standard temperature in our lab incubators, and incubators set to this temperature are always available. I was not interested in doing an exhaustive experiment where I attempted to alter the temperature in smaller increments. I decided that if a phenotype is so transient, and that only incredibly specific growth conditions could reveal that phenotype, then I could not be confident my conclusions were reproducible.

When N2 worms, with *gpb-2(ad541)* worms as a negative control, were grown and starved at 15°C, I did not see the fat retention phenotype I needed to continue the *gcy-28(tm2411)* studies.

Recent developments for new alternatives

A recent study done by O'Rourke and Ruvkun (O'Rourke and Ruvkun 2013) touched on themes similar to my work, in that nutrient availability inhibited fat utilization in *C. elegans*, and the absence of nutrients promoted fat utilization. My hypothesis was that the GCY-28 in the gut was necessary to inhibit excessive fat utilization, and that FLP-20 was a starvation signal in the same regulatory pathway as GCY-28. O'Rourke found that MXL-3 is a transcriptional repressor that inhibits fat utilization when nutrients are present, and that HLH-30 is a transcriptional enhancer that promotes fat utilization when nutrients are absent. The transcription level regulation described fits nicely into the ideas I explored, but I believe it also provides a way to more accurately test the role of *gcy-28.*

The excessive autophagy during starvation of *gpb-2(ad541) C. elegans* can cause death, but autophagy is also an important process in fat utilization. When worms are starved, *hlh-30* promotes fat utilization through lysosomal lipase and autophagy genes. If the excessive autophagy in *gpb-2(ad541)* worms is caused by overexpression of *hlh-30*, then crossing an *hlh-30(lf)* mutant into a *gpb-2(ad541)* background could possibly rescue both the lethality due to excessive autophagy and the increased fat utilization. *flp-20* could either promote expression of *hlh-30* or inhibit expression of the repressor *mxl-3.* The way *gcy-28* restores starvation sensitivity to a *gpb-2; flp-20* mutant would be consistent with the

hypothesis that *gcy-28* promotes repression of autophagy through *mxl-3*. There are numerous experiments that can be done with both *hlh-30* and *mxl-3* in the context of my research. If *hlh-30*/*mxl-3* are the transcriptional regulators at the end of a signaling pathway initiated by acetylcholine, my ideas could potentially be tested in a manner less dependent on starvation/recovery conditions.

APPENDIX A
Transcription factor *zfp-1* enhances cell death defects in *C. elegans*

Of the 1090 somatic cells generated during development of *C. elegans* hermaphrodites, 131 undergo programmed cell death. Transcription factors that regulate cell-specific apoptosis in *C. elegans* have been associated with cancer (Reviewed in Potts and Cameron 2011). We aimed to identify novel transcription factors that regulate programmed cell death in *C. elegans* by performing an RNAi screen against transcription factors in a sensitized cell death defective background. A homolog of human leukemia associated AF10, *zfp-1,* was identified as a hit, as it was found to promote cell death in *C. elegans*. Our initial characterization of the programmed cell death defects of *zfp-1* mutants is described here.

We performed an RNAi screen of 387 transcription factors and 263 chromatin remodeling factors using *ced-3(n2427); P_{lin-11}gfp* worms to identify genes that regulate cell death in the ventral nerve cord. The *P_{lin-11}gfp* reporter expresses GFP in the VC motor neurons of the ventral nerve cord, and provides a quick and reliable assay for identifying regulators of cell death (Reddien et al. 2001). In *P_{lin-11}gfp* (otherwise genetically wild-type) worms (Figure 1A), four cells in the ventral nerve cord are clearly visible under a dissecting fluorescence microscope; two of the six VC neurons are obscured by fluorescence in vulval cells, which also express the reporter. In worms with mutations that result in a strong cell death defect, *P_{lin-11}gfp* is expressed in five additional

cells in the ventral nerve cord. These additional cells are the lineal equivalents of ventral neurons in the anterior and posterior ventral nerve cord, which undergo programmed cell death in wild type animals. In mutants with partial defects in programmed cell death, an intermediate number of additional cells survive and express $P_{lin\text{-}11}gfp$. Worms defective in engulfment of cell corpses (*ced-1(e1735)*) or with a partial loss of function mutation in the caspase (*ced-3(n2427)*) have an intermediate number of additional cells that survive and express $P_{lin\text{-}11}gfp$ (Figure 1B, 1C). The sensitized *ced-1(e1735)* and *ced-3(n2427)* genetic backgrounds can enhance detection of weak cell death defects.

The strongest cell survival phenotype observed, both in the *ced-3(n2427)* background and in a secondary screen using *ced-1(e1735),* was in animals fed a *zfp-1(RNAi)* construct (data not shown). The *zfp-1(ok554)* deletion allele removes the leucine-zipper domain, rendering the protein non-functional (Cui et al. 2006). When *zfp-1(ok554)* is crossed into the $p_{lin\text{-}11}gfp$ reporter, there is a small, but significant, increase in the number of surviving ventral nerve cells (Figure 1D). The weak effect of *zfp-1(ok554)* in an otherwise wild type reporter background is enhanced in *ced-1* and *ced-3* mutants with partial defects in programmed cell death (Figure 1E, 1F). Other transcription factors, such as *lin-35,* can enhance a weak cell death defect (Reddien et al. 2007), and *zfp-1* regulates a similar subset of genes (Grishok et al. 2008). It is possible that *zfp-1* transcriptionally regulates cell death in a manner similar to that of *lin-35*. Thus, knockdown of *zfp-1* might elicit a cell death phenotype as a result of its activity in transcriptional regulation.

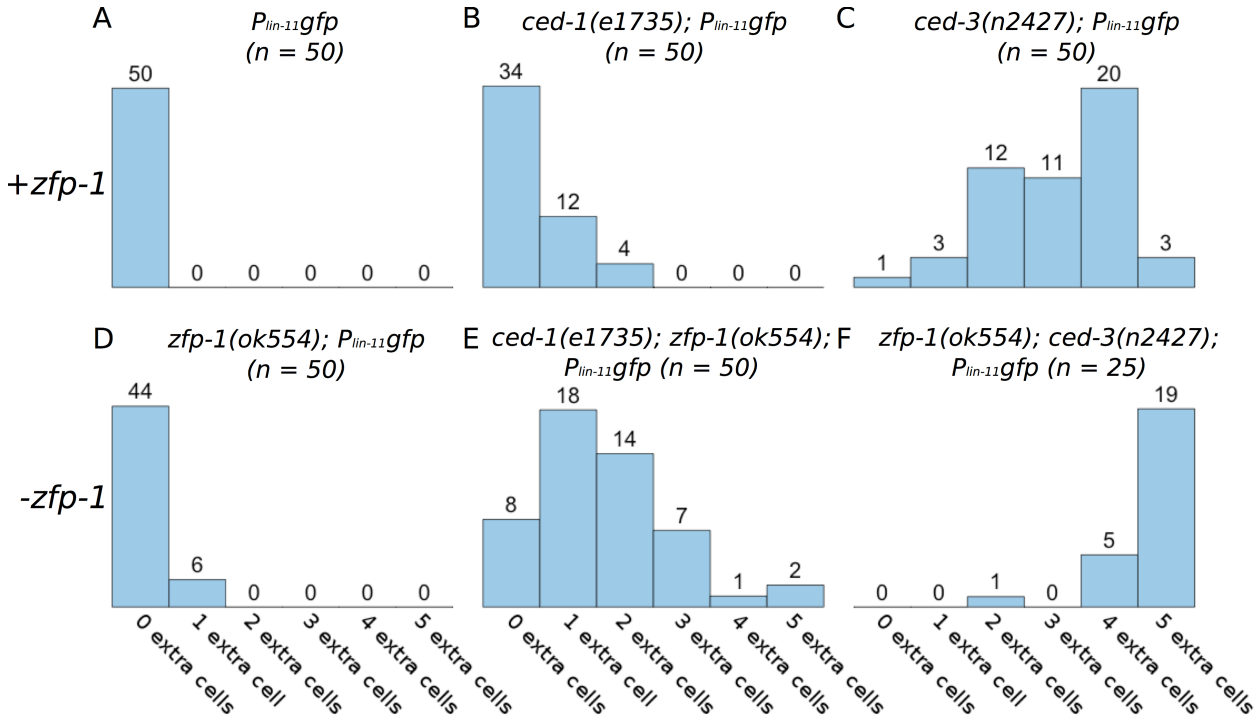


Figure 37: *Plin-11gfp* causes ventral neurons to fluoresce, and defects in apoptosis causes more cells to survive and fluoresce (A, B, C). A loss of *zfp-1* increases the number of surviving ventral neurons (D, E, F).

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