Characterization of the Spinster Gene Ortholog *C13C4.5* in *Caenorhabditis elegans*

By

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Nicholas Quintyne, and has been approved by the members of her/his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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The dauer larva is an alternate larval stage which allows the nematode *C. elegans* to survive environmental stress during development. Dauer formation requires autophagy, a cellular process responsible for degrading and recycling cytoplasmic components. I investigated the role of a *spinster* ortholog, *C13C4.5*, by examining the effects of *C13C4.5* loss-of-function and by generating a transgenic strain which expressed a C13C4.5::GFP fusion protein. Under normal conditions *C13C4.5*::GFP is expressed diffusely in the intestine, but under autophagy-promoting conditions the expression pattern becomes more punctate. This is consistent with localization of *C13C4.5* to autophagolysosomes during autophagy, as has been shown for *spinster* in *D. melanogaster*. Loss of *C13C4.5* function in a dauer constitutive mutant resulted in a reduction in the proportion of animals entering into the dauer stage. Together these data suggest that *C13C4.5* is involved in dauer formation and the autophagy pathway.

TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Autophagy

In order to maintain homeostasis, cells employ processes such as the ubiquitin-proteasome pathway and the autophagy pathways. While the ubiquitin-proteasome pathway is useful in selectively degrading and recycling smaller amounts of protein, autophagy can be useful in the relatively non-specific degradation of large proteins, organelles, or large quantities of proteins (Suzuki and Ohsumi, 2007). Proteins and organelles are generally targeted for degradation when they have accumulated too much damage or have become too old. There are three major types of autophagy: chaperone-mediated autophagy, microautophagy, and macroautophagy. Each type involves delivery of sequestered cellular cargo to the lysosome for degradation (Melendez et al., 2009). For this project autophagy will refer only to macroautophagy.

Macroautophagy is the pathway through which a cell targets either long-lived proteins or cytoplasm-localized organelles for degradation. The process has many stages, but these can be condensed into four main processes: vesicle nucleation, vesicle elongation, docking and fusion, and finally cargo degradation (Melendez et al., 2009). The process begins when a cell receives signals from the environment indicating a reduction in nutrient availability which turns on pathways for adapting to starvation conditions. These starvation signals proceed to inhibit the target of rapamycin (TOR) complex. The TOR signaling pathway is a well conserved nutrient sensing pathway and a major regulator of autophagy (Watanabe et al., 2011). In addition to TOR, regulation of autophagy is controlled by three other major proteins: Class I and Class III Pi3 Kinases

(PI3K), the insulin-like receptor, and MEK1/2. TOR and the Class I PI3Ks negatively regulate autophagy, while Class III PI3K and MEK1/2 positively regulate autophagy (Melendez et al., 2009).

During periods of normal growth and high nutrient levels the TOR complex inhibits the autophagy pathway. The inhibition of the TOR complex by reduced nutrient availability results in the autophagy pathway being turned on, and leads to the nucleation of a vesicle. This vesicle is initially termed an isolation membrane but is renamed as an autophagosome upon completion of the vesicle (Suzuki and Ohsumi, 2007). The autophagosome is next filled with cargo to be degraded. Once filled it then docks and fuses with a lysosome, forming an autophagolysosome. The cargo is now exposed to lysosomal hydrolases which degrade the components inside the autophagolysosome. After being degraded, the components are re-distributed to the cell to be reused (Melendez et al., 2009).

Autophagy and Dauer Formation in the Nematode C. elegans

In the nematode worm *C. elegans*, autophagy can be turned on in response to a need for altering its morphology, controlling its lifespan, regulating necrotic cell death, removing protein aggregates, and conducting apoptosis (Kovacs et al., 2010). Autophagy has also been shown to be essential for both entry into the alternate dauer larva and survival during this diapause stage (Kovacs et al., 2010; Melendez et al., 2003).

The dauer larval stage is an alternate developmental stage which *C. elegans* can enter in the face of unfavorable environmental conditions and allows the animal to

survive until appropriate growth conditions return (Hu, 2007). In the presence of high food, low temperatures, and low population density *C. elegans* undergoes normal development from an egg to an adult through four larval stages. The unfavorable environmental conditions which promote dauer arrest are low food supply, high population density, and relatively high temperatures. The low food supply signals are also able to upregulate autophagy. If these conditions are reversed the worm can exit the dauer stage and normal development can recommence. The dauer larva is physically characterized by its constricted, elongated appearance, altered cuticle, closed mouth and anus, constricted pharynx, and darker coloring. Using the altered body shape and darker coloring it is easy to distinguish adult worms from worms which have arrested in the dauer larval stage.

There are four major pathways which control entry into the dauer stage: the guanylyl cyclase pathway, the TGFβ-like pathway, the insulin-like pathway, and the steroid hormone pathway. Genetic analysis of dauer formation has identified a number of genes that are critical to this process and mutations which can lead to either dauer constitutive phenotypes (*daf-c*) or dauer defective (*daf-d)*. The dauer constitutive phenotype is characterized by the worm improperly arresting in the alternate dauer stage in the presence of food and low population densities. Dauer defective mutants are the opposite: they do not arrest in the dauer larval stage in response to low food level and high population densities. Many of the *daf-c* mutations are temperature sensitive which causes the worm to inappropriately arrest at the dauer stage if grown at the restrictive temperature, while allowing it to develop normally at the permissive temperature (Hu,

2007).

Using a dauer constitutive mutant it was shown that knockdown of genes required for autophagy, via RNA interference (RNAi), prevents worms from arresting in the dauer stage. Additionally, using the *LGG-1*::GFP marker for autophagy, it was shown that knockdown of *bec-1* via RNAi also leads to aberrant formation of autophagolysosomes (Melendez et al., 2003). These data show that autophagy is required for proper formation of the dauer larva. Therefore any genes which are required for autophagy should also be required for proper dauer formation.

The Spinster Protein

The re-distribution of autophagic cargo to the cell is a critical feature of autophagy. In *D. melanogaster* spinster proteins have been implicated in programmed cell death, neural degeneration, autophagic lysosome reformation, and mTOR activation (Rong 2011 et al.; Nakano et al., 2001). Dermaut and coworkers (2005) found that mutations in the *spinster* gene lead to carbohydrate accumulation in autophagolysosomes based on carbohydrate staining. Rong and coworkers (2011) subsequently showed that without an active spinster protein autophagolysosomes become abnormally enlarged, suggesting that the accumulating carbohydrates are no longer being redistributed to the cell for reuse. In addition, they showed that the spinster protein in *D. melanogaster* is essential for re-activation of the mTOR complex upon completion of autophagy, implicating it as an essential component in the feedback regulation of autophagy. Following autophagy the spinster protein is also essential for reformation of lysosomes.

The spinster protein is part of the permease superfamily, suggesting that it may act as a sugar transporter although no strong biochemical data exist to support this observation.

Spinster orthologs exist in humans (HSpin1) and zebrafish (*not really started*, *nrs-1*). The *spinster* ortholog in humans has been implicated a type of apoptosis which is not dependent upon caspase release, but instead relies on the autophagy pathway (Yanagisawa et al., 2003). In zebrafish, the *spinster* ortholog has been implicated in encoding a membrane-bound protein expressed in the yolk during embryogenesis. Without this protein, the embryo is not viable (Young et al., 2002). Nakano and coworkers (2001) reported *spinster* orthologs in the nematode *C. elegans* based on sequence similarity but to date there have been no reports on their function. The worm orthologs do maintain a conserved region as being critical for the ability of the *spinster* gene to act as a sugar transporter, so it is possible they have similar functions (Rong et al., 2011).

Spinster Genes in C. elegans

Since autophagy is required for dauer formation and *spinster* is required for completion of autophagy in *D. melanogaster* it can be hypothesized that disruption of *C. elegans spinster* genes should prevent dauer formation. Based on this hypothesis, knockdown of candidate *spinster* orthologs in a dauer constitutive mutant may lead to a decrease in the ability of the mutant animal to arrest in the dauer larval state. This hypothesis was tested with the following four objectives:

1) Identify *spinster* orthologs in *C. elegans* using sequence homology to *D. melanogaster* spinster protein.
2) Examine the effect of loss of function of candidate *spinster* orthologs in a dauer constitutive mutant using RNAi.
3) Examine expression pattern of candidate *spinster* gene under autophagy-promoting conditions.
4) Examine the dauer formation phenotypes of a candidate *spinster* gene deletion in a dauer constitutive mutant.

METHODS

C. elegans Strains

The following strains were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota: N2 (wild type), DR1572[*daf-2(e1368)III*], and RB1678[*C13C4.5(ok2087)V*].

Basic C. elegans Solutions

Nematode Growth Media (NGM) - 1 L (H_2O), 3 g NaCl, 17 g agar and 2.5 g peptone. This solution was autoclaved at 121°C for 50 minutes and then cooled to 57°C. After cooling 1 mL 1 M $CaCl_2$, 1 mL 5mg/ml cholesterol in ethanol, 1 mL 1 M $MgSO_4$, and 25 mL 1 M KPO_4 were added. If the plates were to be used for RNAi procedures then 2 mL IPTG at 1 M and 1 mL carbenicillin at 25ng/mL was added. 10 mL NGM solution was added to each 5 cm petri dish.

LB Broth, Miller (Novagen, Germany) – 25 g LB Broth, Miller was added to 1 L H_2O and autoclaved.

M9 - 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4 \cdot 7H_2O$, and 985 mL H_2O were combined and autoclaved.

Routine C. elegans Maintenance

The bacterial strain *E. coli* OP50 was used as a food source for routine experiments. OP50 bacteria were grown by inoculating a single colony into Luria Broth and incubating overnight at 37°C. After incubating overnight 300 µL of the suspended

bacteria were aliquoted onto each NGM petri dish. This bacterial lawn was allowed to dry overnight before use.

The nematode worm *C. elegans* was grown at 20° C on 5cm petri dishes filled with NGM and spotted with an *E. coli* lawn. *C. elegans* matures to adulthood in three to four days and then begin to lay progeny. Due to their ability to lay 300 worms in the following days worms were transferred as necessary to ensure populations stayed fed (Stiernagle, 2006).

Dauer Assay on RNAi Bacterial Lawns

RNAi knockdown of the *spinster* gene orthologs was performed by feeding worms bacterial strains which expressed double stranded RNA specific to the target sequence.

RNAi plates were made as stated in the Basic *C. elegans* Solution section. As a food source the HT115 bacteria strain containing the RNAi vector L4440 was inoculated into LB broth containing ampicillin, grown overnight, and spotted in 300 µL aliquots onto RNAi NGM plates. These plates were used within a week of the RNAi bacterial lawn drying to minimize light-induced breakdown of IPTG, which could reduce the ability of IPTG to induce double stranded RNA. The RNAi strains corresponding to *C13C4.5, C39E9.10,* or *F09A5.1* were obtained from the MRC RNAi Library (Geneservice, CA) and were sequenced prior to RNAi experiments. An empty L4440 RNAi vector was used as a negative control.

Dauer assays were conducted using the DR1572[*daf-2(e1368)III*] worm strain.

This strain is a temperature sensitive dauer constitutive mutant. At 20°C it develops normally while at 25°C the worms arrest in the dauer larval stage.

The assay was carried out by growing a population of about 20 DR1572[*daf-2(e1368)III*] worms on each of the RNAi strains at 20°C for the first generation. 100 eggs were picked from the first generation's progeny, and then grown at 25°C. Two generations of RNAi exposure were used to maximize the effects of the RNAi. After allowing the second generation to grow for three days at the restrictive temperature, the populations were scored for the number of dauer worms versus non-dauer worms based on the visible phenotypes. Dauer worms are skinny, elongated, and dark while their adult counter parts are large, fat, and less darkly colored.

Generation of Male C. elegans

Male *C. elegans* occur naturally at a frequency of 1 in 500 under normal growth conditions, since their existence relies on non-disjunction of the X chromosome during meiosis in the germ line (Lints et al., 2009). To cause this event to occur more frequently the worms are heat shocked. Each population to be heat shocked consisted of 5 L4 hermaphrodites. The heat shock was carried out at 30°C for six hours. The worms were then returned to 20°C and allowed to lay the F1 generation. This generation was screened for males to be used in genetic crosses. If the number of males was inadequate for a genetic cross (~20 individuals) then they were mated with hermaphrodites of the same genotype to produce additional males. 50% of the progeny from a cross between a male and a hermaphrodite will be male.

Backcrossing the C13C4.5 Deletion Mutant

Backcrossing mutant strains into the wild type strain is necessary to remove additional mutations that may have been introduced during the mutagenesis procedure. The mutants are only screened for the desired mutation, so backcrossing into the wild type is done to remove any unwanted mutations that may have occurred alongside the desired one.

The typical backcross involves crossing a single wild type worm with 2 to 3 homozygous mutant hermaphrodites to generate heterozygous, hermaphrodite worms. These hermaphrodites are then allowed to self-fertilize and the worms which are homozygous for the deletion are crossed once again with a male wild type worm. This type of cross takes two months to complete the five backcrosses.

The alternate backcross strategy is outlined in Figure 1 and allows for completion in three weeks and begins by initially mating 10 wild type males with 3 mutant hermaphrodite worms, producing a F1 population in which all the males and hermaphrodites are heterozygous for the *C13C4.5* deletion. This is considered to be the first backcross. 10 of these heterozygous F1 males were then mated with 3 wild type hermaphrodites. The F2 progeny of this cross results in both males and hermaphrodites which are either wild type or heterozygous for the *C13C4.5* deletion. 20 of these F2 males were chosen at random and put onto individual NGM plates and mated with 2 wild type hermaphrodites to generate the F3 progeny. Once the F3 progeny has been laid the 20 F2 males were individually PCR genotyped to determine which were heterozygous for

the *C13C4.5* deletion. Male F3 progeny from a heterozygous F2 male were then selected to continue in the backcross. These F3 progeny were now considered to be backcrossed 2 times. This process was repeated three more times to complete the five backcross events. After the fifth backcross instead of choosing males from the progeny population, hermaphrodites were chosen. These hermaphrodites were either homozygous wild type or heterozygous for the *C13C4.5* deletion, so 40 were chosen at random, put onto individual NGM plates, and allowed to self-fertilize. Once their progeny was laid, the hermaphrodite parents were PCR genotyped (see Molecular Biology Methods section) and any population which came from a heterozygous parent contained hermaphrodite worms that were either wild type, heterozygous for the deletion, or homozygous mutant. 40 L4 hermaphrodites were chosen at random from a heterozygous parent and allowed to self-fertilize before genotyping the 40 parents for being homozygous for the deletion. The homozygous parent progeny was kept and considered to be the completed backcross strain.

Generation of a daf-2; C13C4.5 Double Mutant

The cross began by mating male MGL69 worms with hermaphrodites which were homozygous for the *daf-2(e1368)* point mutation. The F1 progeny from this mating had both males and hermaphrodites that were all heterozygous for both the *daf-2* and *C13C4.5* deletion. 20 F1 hermaphrodites were selected and plated onto individual plates and allowed to self-fertilize. The F2 progeny from each animal contained worms which

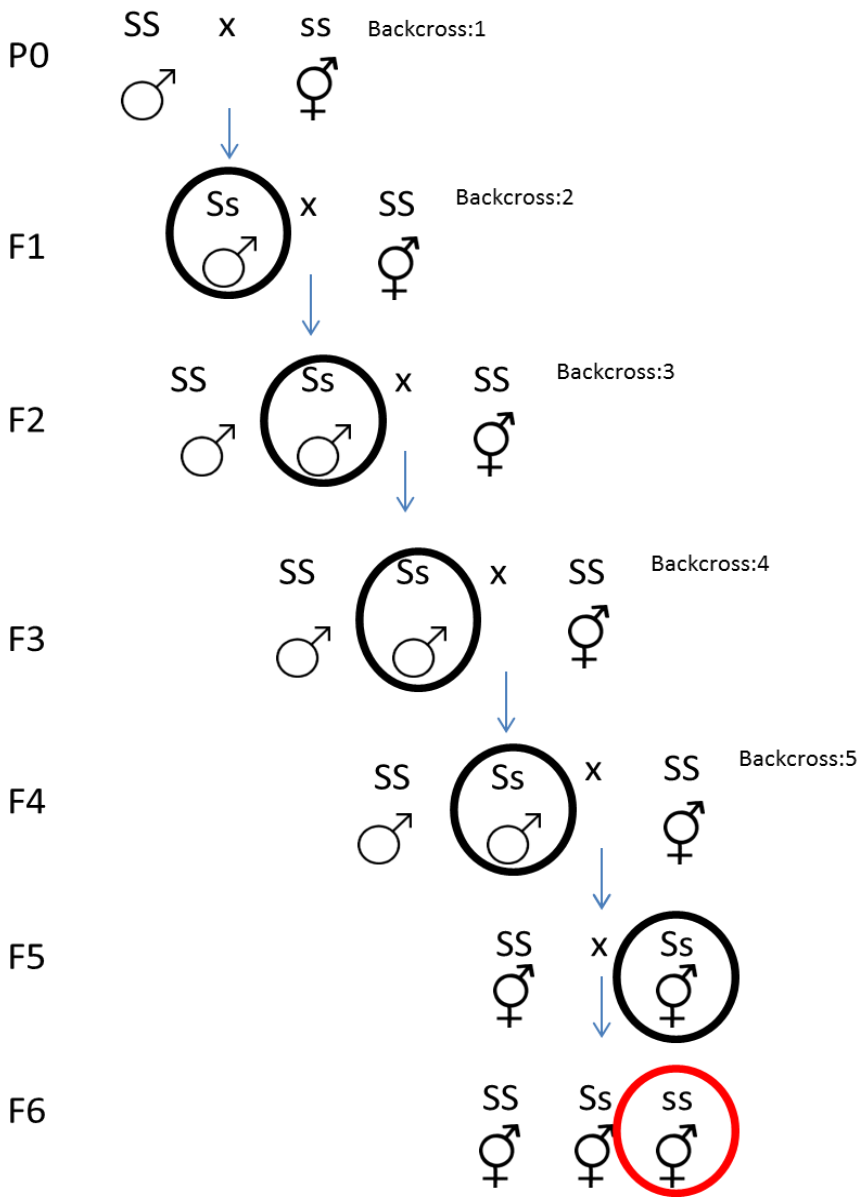
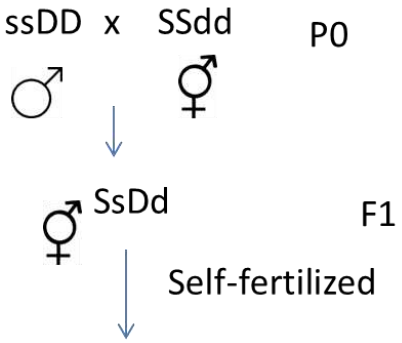


Figure 1: Strategy for Backcrossing C13C4.5 into the Wild Type (N2) Strain. "S" denotes a wild type version of the *C13C4.5* gene while "s" denotes the version with the deletion. Animals which are ss are homozygous for the deletion, Ss animals have one copy of the wild type and deletion, and SS have both wild type copies. Circled genotypes are those which were used to continue the cross. The red circled genotype in the F6 population is the final backcrossed animal.

segregated typically for a Mendelian two-factor cross. To identify the animals which were homozygous for the *daf-2* mutation and the *C13C4.5* deletion the animals were PCR genotyped for the *C13C4.5* deletion. Any worms carrying this were then sequenced by Operon using the daf2seqintF and daf2seqIntR primers for the *daf-2* mutation. A strain containing both mutations was termed MGL97 (Figure 2).

Dauer Assay with MGL97[daf-2(e1368)III; C13C4.5(ok2087)V]

The *C. elegans* strain MGL97[*daf-2(e1368)III; C13C4.5(ok2087)V]* was used for a dauer assay. 20 adult nematode worms were used to lay eggs onto standard NGM plates for 2 hours on day 1. After laying their progeny the adults were removed from the plate and ~100 eggs were put onto a new NGM plate and allowed to grow at 25°C for three days. The worms are then scored for proportion of dauer vs. non-dauer worms.



	SD	Sd	sD	sd
SD	SSDD	SSDd	SsDD	SsDd
Sd	SSDd	SSdd	SsDd	Ssdd
sD	SsDD	SSDd	ssDd	ssDd
sd	SsDd	Ssdd	ssDd	ssdd

F2

Figure 2: Summary of the crosses used to generate the daf-2;C13C4.5 mutant.

The chart shows the possible genetic outcomes from the self-cross of the F1 worms which are heterozygous for both mutations. The red and purple outcomes were the results screened for by the PCR genotyping, and the final desired genotype in purple was identified by both PCR genotyping for the *C13C4.5* deletion and sequencing of the *daf-2* mutation.

Molecular Biology Methods

Primers

The following oligonucleotide primers were synthesized by Operon. These primers arrived at a stock concentration of 100 µM and were diluted to a working concentration of 2 µM. They were stored in a freezer at -20°C. The primer names and their sequences are listed below.

C13C4.5proF1: cacatgtccagctgcctattggacc

C13C4.5proF2: ctgcctattggaccttctgttttc

C13C4.5gDNAR/GFPtail: gaaaagttcttctcctttactcatcatatgttgaacttcggaaccag

C13diF: gcatcttatgcaattatctcc

C13diR: cagacaagagcatctgcacgaac

C13dR: catatgttgaacttcggaaccag

daf2seqintF: cttgtgcttatgatcgtcttct

daf2seqIntR: gcgtcaaatccgtcgttgaatcg

GFPN-F: atgagtaaaggagaagaacttttcac

GFPD: aagggcccgtacggccgacta

GFPD*: ggaaacagttatgtttggtata

Gel Electrophoresis

1% gels were made by mixing 0.3g of agarose (Invitrogen, WI) into 30mL of ultrapure H_2O and then heating and stirring until the agarose was dissolved. Once dissolved, 1.5 µL of ethidium bromide was added to the solution. The gel was then cast in

a horizontal gel tank containing combs to form the wells. Once set, the gel was covered in a 1x TAE buffer, which was diluted from a 50x TAE buffer stock solution (121 g Tris-base, 28.6 mL acetic acid, 50 mL 0.5 M EDTA pH8.0, 750 mL H_2O). The outer most wells were loaded with 5 µL of a standard DNA ladder solution (Bioline's Hyperladder I (Bioline, Taunton, MA)). Into the remaining wells 1 µL sample DNA combined with 5 µL 6x loading buffer (Nalgene, NY) was added. The gel was then run at 100V for 30 minutes.

DNA was visualized by UV transillumination (Alpha Innotech: Red, Proteinsimple, CA).

Plasmid DNA Purification from Overnight Bacteria Cultures

Plasmid DNA purification was performed using the QIAprep® Spin Miniprep Kit (QIAGEN, CA) according to manufacturer's instructions.

DNA Purification from PCR Reactions

PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN) according to manufacturer's instructions. 30 µL of the EB buffer was used for elution.

DNA Extraction and Purification from Agarose Gel

After gel electrophoresis the DNA fragment of interest was visualized under UV light and excised from the gel using a clean scalpel blade. The gel section was transferred

to a pre-weighed microcentrifuge tube and the DNA was extracted and purified using the QIAquick® Gel Extraction Kit (QIAGEN). The procedure was carried out according to manufacturer's instructions.

DNA Sequencing

Purified DNA was sequenced by a commercial company (Operon, AL) and prepared according to their guidelines (http://www.operon.com/). Results were analyzed using Chromas (Technelysium, MN) and SeqBuilder (DNASTAR, WI) computer software.

Quantification of DNA

DNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, DE), or by comparison of DNA run on a gel to standards run alongside it. The standards were Bioline's DNA Hyperladder I and the process of comparison follows their guidelines.

Preparation of Genomic DNA from C. elegans

In order to obtain genomic DNA for use as a template for PCR amplification worms were lysed in Worm Lysis Buffer. Worm Lysis Buffer contains 1.5 µL proteinase K and 250 µL of Single Worm Lysis buffer (50 mM KCL, 10 mM Tris-base pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40 (IGEPAL), 0.45% Tween-20, 0.01% Gelatin). This solution was aliquoted into PCR tubes in 5 µL volumes. Into each aliquot the appropriate number

of worms was added. The tubes were then incubated at 60°C for 60 minutes and then at 95°C for 15 minutes in a GeneAmp PCR System 9700 (Invitrogen).

Polymerase Chain Reaction (PCR) Amplification of Genomic DNA

PCR amplification of genomic DNA was performed using the Expand High Fidelity PCR System (Roche, IN) for DNA fragments up to 5 kb in length, Expand Long Template PCR System (Roche) for fragments over 5 kb, and the GoTaq Flexi PCR kits (Promega, WI). For any general PCR reaction in which the correct sequence was not critical, the GoTaq Flexi PCR system was used with the standard procedure ratios of polymerase, primers, $MgCl_2$, and dNTPs. Each reaction contained about 5 ng of DNA. Typical cycling conditions were 94°C for 5 min for 1 cycle, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min/kb for 35 cycles, and 72°C for 4 min for 1 cycle. If the DNA was going to be used for injections, sequencing, or otherwise required a high level of accuracy the Expand High Fidelity (Roche) or Expand Long Template (Roche) kits were used. Their procedures were followed as defined in their protocol manual (Roche, 2005; Roche, 2005).

Transformation of C. elegans

Transformation of *C. elegans* was carried out by Dr. Neale Harrison of the Gill Lab at The Scripps Research Institute -Scripps Florida.

1.) Generation of DNA Mixture for Injection

The DNA mixture to be injected was obtained by combining the purified PCR fusion product with a purified plasmid containing the co-injection marker p*unc-25*::mRFP .The PCR fusion product was at a concentration of 5ng/µL and the co-injection marker at a concentration of 18ng/µL. The DNA preparation was centrifuged at 14,000rpm to pellet any contaminants.

2.) Mounting C. elegans for Injection

Young, first day adult hermaphrodite worms are used for injections. 20-40 µL of a 2% agarose solution was heated in a microwave until molten and applied to a glass coverslip. This was then flattened with a second cover slip until very thin. Once the pad solidified, the second slide was removed by sliding it off of the first slide. These pads were then dried overnight. Before adding worms onto the pad a small drop of Halocarbon Oil 700 (Sigma, MO) was added. Worms were placed into this drop to immobilize them for injection.

3.) Creation of Needles for Microinjection

To inject the DNA mixture microinjection needles were used. To create these borosilicate glass capillary tubes 1.0mm O.D x 0.78 I.D were pulled using a model P-10 needle puller (Narishige, Japan). The DNA mixture was loaded using capillary action from a pipette.

4.) Injection Procedure

The needle loaded with the DNA mixture was placed into a three dimensional micromanipulator system mounted on an AXIO Observer.A1 inverted microscope (Zeiss, Germany). Under 40x magnification mounted first day adult *C. elegans* were

manipulated into place until the distal arm of the gonad of the worm was at a 30° angle with the needle. The needle was then positioned into the distal arm of the gonad and the DNA mixture injected using the IM 300 Microinjector (Narishige) via-pressure from a nitrogen cylinder. Each injected worm was floated out of the oil using M9 and placed onto a standard NGM plate. A total number of 11 worms were injected with the *C13C4.5* fusion PCR product.

Identification and Maintenance of the pC13C4.5::C13C4.5::GFP strain

Each plate containing a single injected worm was given 48-96 hrs to lay progeny. This progeny (the F1 generation) was examined under a Stereo Discovery.V8 microscope (Zeiss, Germany). Transgenic F1s were identified by the *unc-25::*mRFP co-injection marker being visible when using a RFP filter. All positive F1s were transferred to individual plates and allowed to generate the F2 generation. The F2 generation was screened in the same way as the F1. Any F2 transgenic *C. elegans* were put onto their own plate and labeled as transgenic lines. Transgenic F2 lines obtained from a different F1 parent were considered independent since each will contain a different number of extrachromosomal arrays and therefore may act different. The extrachromosomal arrays present in the transgenic animals do not exhibit 100% transmission frequency. To maintain the transgenic lines, progeny expressing the co-injection marker were transferred away from their non-transgenic siblings.

Induction of Autophagy in the pC13C4.5::C13C4.5::GFP Transgenic Strain

In order to examine the expression of *pC13C4.5::C13C4.5::*GFP under normal and autophagy-promoting conditions the transgenic worms were exposed to either the L4440 control RNAi or to *let-363* RNAi. The *let-363* RNAi was a kind gift from Dr. Kailiang Ja at Florida Atlantic University. Its sequence was confirmed by sequencing PCR amplified plasmid DNA and Operon stock M13 primers.

RNAi plates were made as described earlier. Transgenic animals were grown on bacteria containing the RNAi for two generations to maximize the effects of the RNAi before being mounted and imaged to examine their expression pattern.

Mounting C. elegans for Imaging

C. elegans were mounted on 5% agar pads. To make these pads four glass slides were needed. Two of the slides have a strip of autoclave tape across them. One clean slide was put between the two with the autoclave tape. The 5% agar solution was then heated until molten (~20 seconds in the microwave). 50 µL of molten agar was pipetted onto the clean slide. A fourth glass slide was put across the one clean and two with the tape. It was then used to flatten then agar pad while it cools, applying pressure onto where the two strips of tape are to ensure the pad was of adequate thickness. 10 µL of 100 mM sodium azide was placed onto a glass cover slip. Into this droplet the worms to be imaged were added. This droplet side of the cover slip was then placed onto the agar pad.

Imaging C. elegans

C. elegans were visualized using an AXIO Observer.A1 microscope (Zeiss) and imaged using an AXIOcam ICm1 camera (Zeiss).The software used to take and store the images was Axiovision Rel.4.8 9 (Zeiss). For imaging L1 larvae a 20x lens was used. A 10x lens was used for L4 larvae and 4x for adults.

RESULTS

Identification of C. elegans Spinster Gene Orthologs

To verify the known *spinster* gene orthologs in *C. elegans* the sequence of the *D. melanogaster* Spinster type I protein was used in a BLAST/BLAT search of the *C. elegans* genome to find the homologous protein sequences (http://www.wormbase.org/tools/blast_blat). The results of the search confirm the known *C13C4.5 and C39E9.10* spinster orthologs (Nakano et al., 2001) and identify two new possible orthologs: *Y111B2A.19* and *F09A5.1* (Figure 3).

Identification of Spinster RNAi Constructs

Two RNAi feeding libraries were available in the Gill Lab: the MRC RNAi library (Geneservice), and the ORF RNAi library (Geneservice). The MRC library contained the following clones: V-8K11 - *C13C4.5* gene, IV-6P22 - *C39E9.10*, and X-6M09 - *F09A5.1.* The strains from the ORF library were 11070-D12 (*C13C4.5)*, 11067-A2 (*Y111B2A.19)*, 11050-F1 (*C39E9.10)*, and 11078-E1 (*F09A5.1)*. Plasmid DNA was purified from each strain and the RNAi insert was sequenced using M13 primers. Their sequences were then aligned to the genes there were supposed to target for knockdown. It was determined that none of the RNAi strains from the ORF RNAi library matched their target sequences and therefore were not used. In contrast, all of the strains from the MRC library showed exact homology to their target genes. Therefore the RNAi strains used to knockdown the possible *spinster* orthologs were V-8K11, IV-6P22, and X-6M09. Unfortunately the MRC library did not have a strain which targeted *Y11B2A.19* and thus

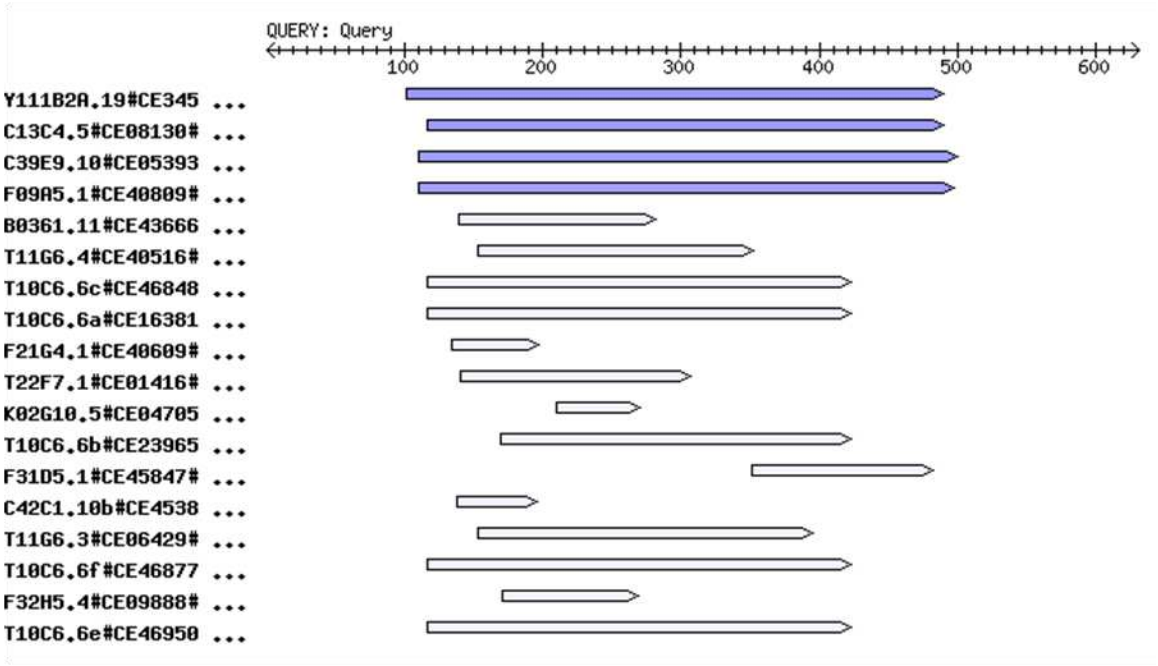


Figure 3: Identification of C. elegans Spinster Gene Orthologs. Two *C. elegans spinster* orthologs, *C13C4.5* and *C39E9.10*, have already been identified by sequence homology to the *D. melanogaster* spinster protein. Using the *Drosophila* sequence and the BLAST/BLAT search engine, two additional orthologs were identified: *Y111B2A.19* and *F09A5.1*. The top four proteins are all at least 60% homologous to the *D. melanogaster* protein, with the top result being 62%.

it was not possible to examine this gene by RNAi.

Dauer Assay of DR1572[daf-2(e1368)III] Strain on Individual Spinster RNAi

To identify which of the three possible *spinster* orthologs being studied was the strongest candidate, a dauer assay was performed using the DR1572[*daf-2(e1368)III*] strain. The number of dauer vs. non-dauer animals after the second generation of RNAi exposure is shown in Figure 4. The graph shows an average of three biological replicates. Each of these biological replicates consisted of two technical replicates of about 100 animals. The data shows a marked increase in the proportion of adults for the animals which have been exposed to *C13C4.5* RNAi compared to control RNAi. Subsequent experiments were focused on *C13C4.5* since it showed the strongest effect on dauer formation. Although the effect of the knockdown of *F09A5.1* was not as strong as seen with *C13C4.5* the observed effect may warrant further investigation in the future.

Generation of Transgenic C. elegans Expressing C13C4.5 fused to GFP

The ability of *C13C4.5* RNAi to inhibit entry into the dauer form in a *daf-2* mutant suggested that this gene could be involved in autophagy. To provide supporting evidence for such a role a transgenic worm expressing a *pC13C4.5::C13C4.5*::*GFP* fusion protein under the control of the endogenous *C13C4.5* promoter was generated by first creating a *pC13C4.5::C13C4.5*::*GFP* PCR fusion construct and then injecting this construct into the gonad of an adult animal.

The general strategy for generation of the construct is shown in Figure 5 and

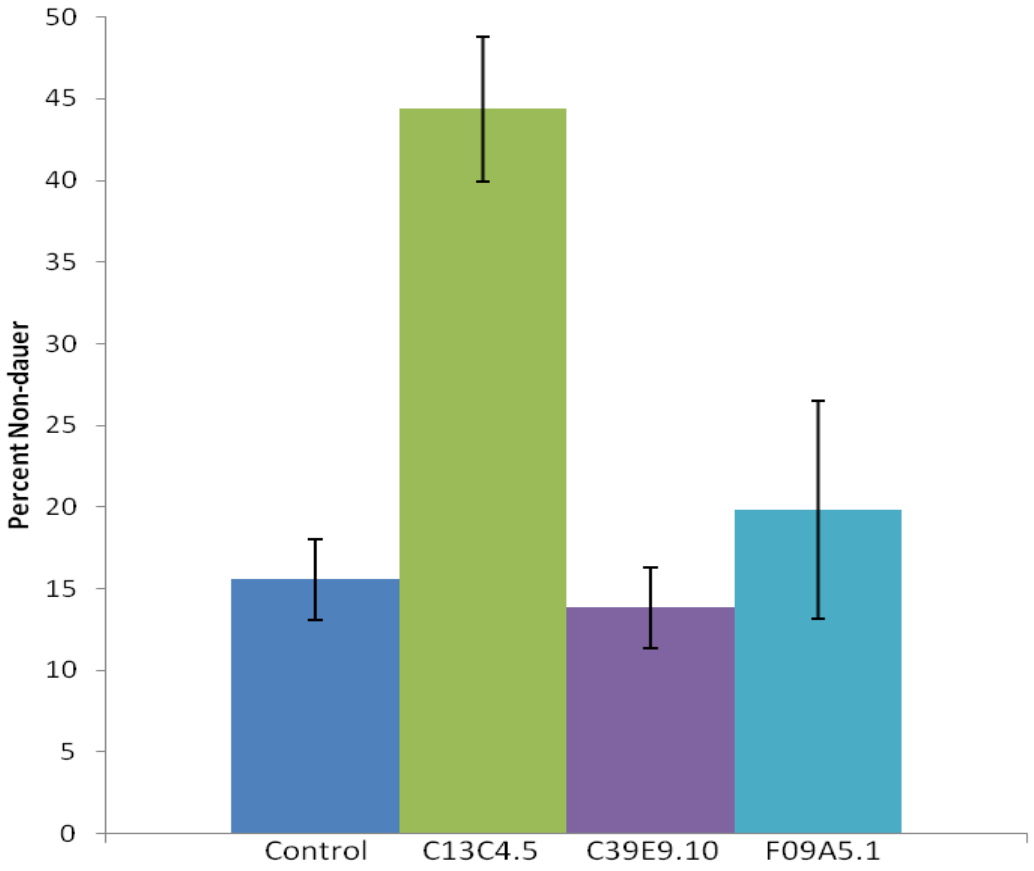


Figure 4: RNAi of Spinster Orthologs in the Dauer Constitutive Strain daf-2(e1368). RNAi was used to knockdown three of the candidate *spinster* orthologs in a temperature sensitive dauer constitutive mutant. The data shows that loss of function of *C13C4.5* results in a marked increase in non-dauer animals compared with control RNAi.

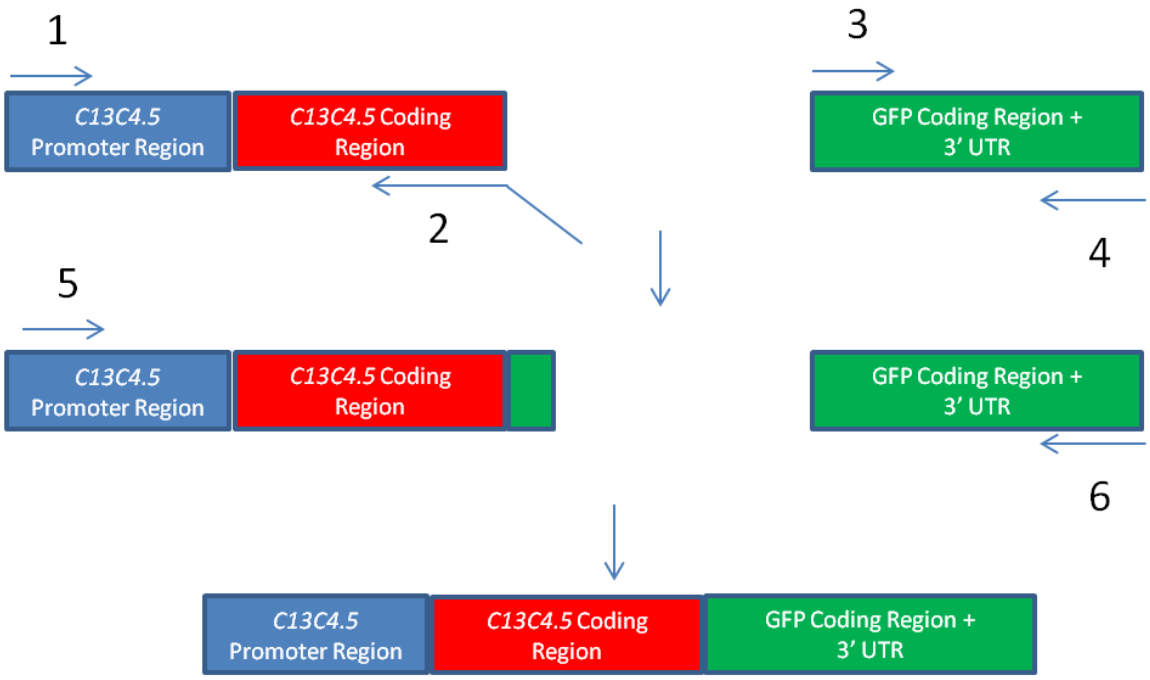


Figure 5: Strategy for Creating a GFP Fusion Construct From GFP DNA and C13C4.5 Genomic DNA. The *pC13C4.5::C13C4.5::GFP* fusion construct was made by first amplifying the 6kb *pC13C4.5::C13C4.5* genomic region using primer 1 (C13C4.5proF1) and primer 2 (C13C4.5gDNAR/GFPtail). In addition to amplifying the *C13C4.5* DNA primer 2 also has a tail hanging off which is complementary to the *GFP* DNA. This region is added to the *pC13C4.5::C13C4.5* DNA and allows for the fusion step to proceed. Next the 1.7kb *GFP* region was amplified by primer 3 (GFPN-F) and primer 4 (GFPD), and then the two regions were fused together to form a 7.7kb *pC13C4.5::C13C4.5::GFP* construct using primer 5 (C13C4.5proF2) and primer 6 (GFPD*).

involved amplifying the target promoter and coding sequence from genomic DNA, amplifying GFP from a plasmid, and then fusing the two sequences using fusion PCR.

First, genomic DNA was obtained by lysing worms. Using this genomic DNA as a template Expand Long Template PCR was performed using the forward primer C13C4.5proF1 and the reverse primer C13C4.5gDNAR/GFPtail, labeled primer 1 and 2 in Figure 5. This resulted in the genomic coding DNA of the *C13C4.5* gene along with a region containing the promoter sequence. Successful amplification of this region was confirmed by the presence of a 6kb band which is shown in lanes 1-3 of Figure 6.

The GFP fragment was amplified using the primers GFPN-F and GFPD, labeled 3 and 4 in Figure 5. The resulting DNA fragment was confirmed as a 1.7kb product (Figure 7).

The GFP fragment and *pC13C4.5::C13C4.5* fragment were then used together as templates for a fusion PCR. Concentrations of both the GFP and *pC13C4.5::C13C4.5* fragments were determined by comparing the intensity of the bands seen on the agarose gel to the intensity of the ladders which have known concentrations. The ratios needed to have equal amounts of the two fragments were calculated. The fusion PCR was carried out using Long Template PCR with forward primer C13C4.5proF2 and reverse primer GFPD*, primer 5 and 6 in Figure 5. The resulting *pC13C4.5::C13C4.5::GFP* fusion PCR product is shown in Figure 8. The *pC13C4.5::C13C4.5::GFP* fusion product was then injected into a *C. elegans* worm using the microinjection technique to generate the transgenic strain which expressed the

pC13C4.5::C13C4.5::GFP construct. Alongside the GFP fusion construct a co-injection marker was injected which is constitutively expressed. The co-injection marker is used to identify the transgenic animals, as GFP may not always be easily observed. Five independent transgenic strains were identified, with MGL79 being the strain used for the characterization of the expression pattern of *C13C4.5.*

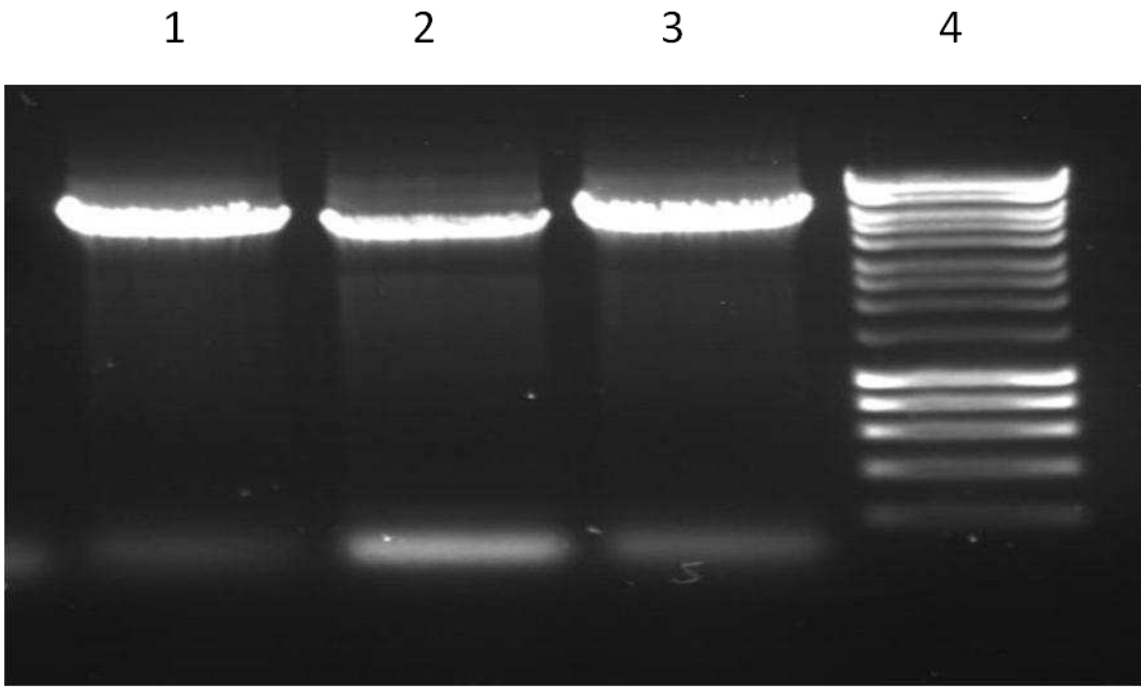


Figure 6: Amplification of C13C4.5 Promoter and Coding Regions. Shown in lanes 1-3 is the successful amplification of the 6kb *C13C4.5* genomic DNA inside a 1% agarose gel. Lane 4 is a molecular weight ladder.

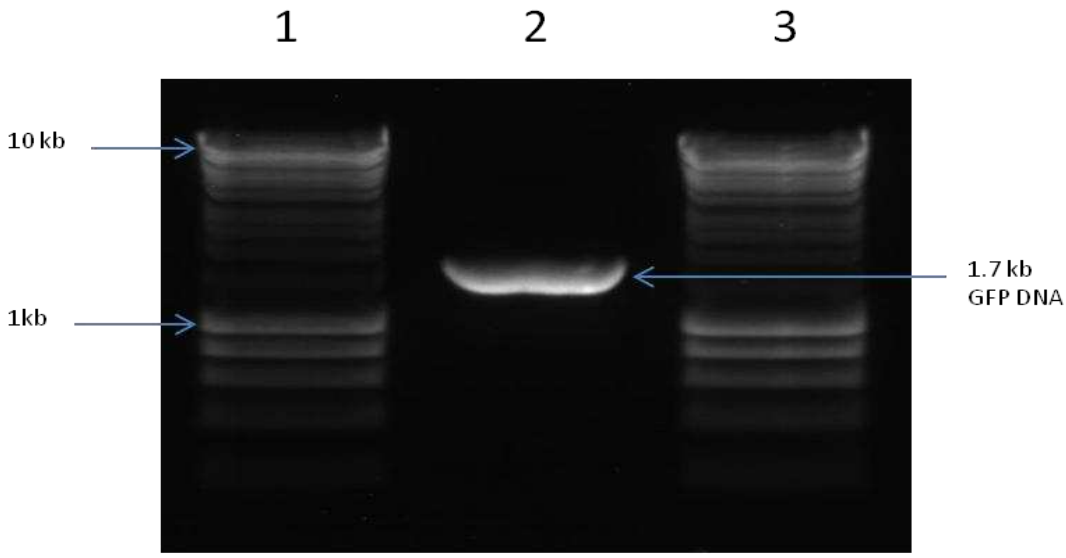


Figure 7: GFP DNA Shown on a 1% Agarose Gel After Amplification and Purification. Shown here in lane 2 is the 1.7kb GFP DNA after being PCR amplified and purified. This DNA is ready to be fused to the *pC13C4.5::C13C4.5* genomic DNA. Lanes 1 and 3 show the DNA ladder used to identify the size of DNA fragments.

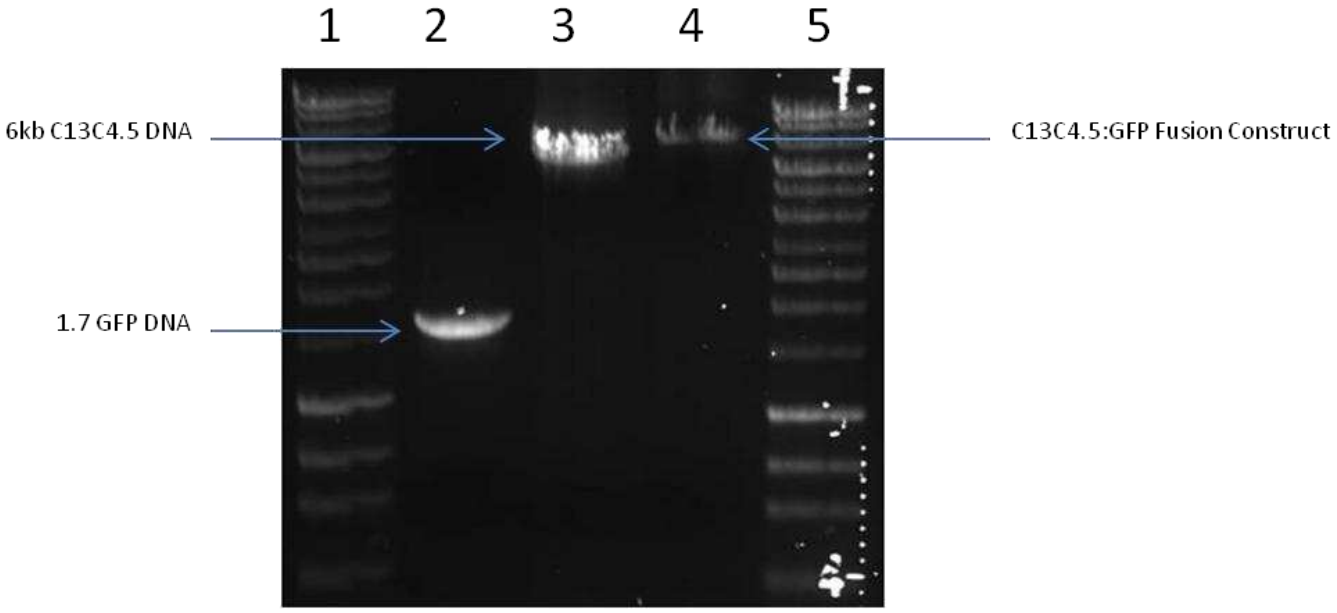


Figure 8: pC13C4.5::C13C4.5 Genomic DNA, GFP DNA, and pC13C4.5::C13C4.5::GFP Fusion DNA After PCR Amplification, Purification, and Fusion. The components of the fusion construct are shown on the 1% agarose gel. The GFP in lane 2 is fused with the *C13C4.5* DNA in lane 3 to form the 7.7 kb *pC13C4.5::C13C4.5::GFP* fusion construct shown in lane 4.

Characterization of the Expression Pattern of C13C4.5

The expression pattern of the *C13C4.5* transgenic worm strain was determined after exposure of the animal to either control or *let-363* RNAi. *let-363* encodes the *C. elegans* ortholog of the TOR subunit, a main inhibitor of autophagy. Inhibition of the TOR complex using *let-363* RNAi should promote autophagy (Melendez et al., 2009). When under the influence of control RNAi the expression pattern tended to be strongly localized to the lining of the lumen in the intestine, with diffuse patterning of the surrounding tissue. Exposure to *let-363* RNAi resulted in a similar pattern of expression but was distinguished by the presence of additional punctate expression in the intestinal cells (Figure 9).

Backcrossing of C13C4.5 Deletion Mutant to Wild Type

The strain RB1678[*C13C4.5*(*ok2087)V*] obtained from the CGC had to be backcrossed against the wild type N2 strain five times to remove mutations which may exist in addition to the *C13C4.5(ok2087)* deletion.

For the first backcross wild type males were mated with a mutant hermaphrodite worm, producing a F1 population in which all the progeny, both male and hermaphrodites, are heterozygous and have now been backcrossed once. These F1 heterozygous males were then mated with wild type hermaphrodites. The progeny of this cross will contain both homozygous wild type and heterozygous mutant males and thus PCR genotyping was used to identify the heterozygous males in each backcrossed

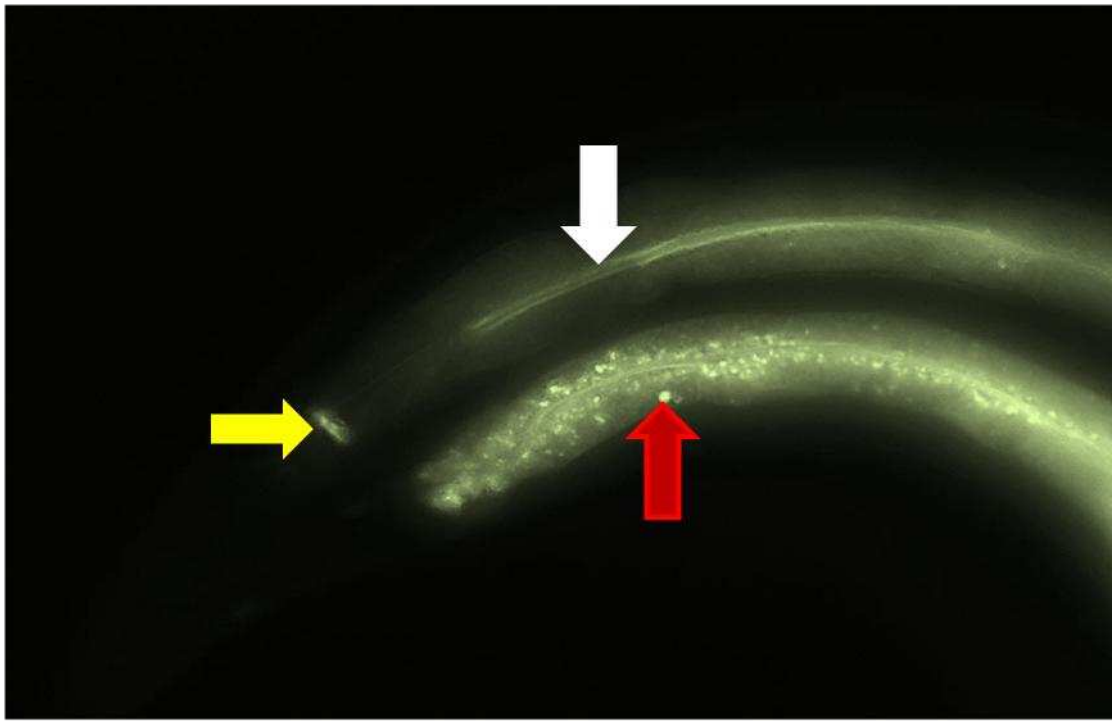


Figure 9: Expression Pattern of C13C4.5 under Control RNAi and RNAi Which Promotes Autophagy. The top transgenic animal shows an expression pattern typical in worms on control RNAi**.** The white arrow points to the strong expression which lines the lumen of the worm. This expression is surrounded by diffuse expression around the intestine. The yellow arrow points to the pharyngeal grinder, which is also expressing the fusion construct. The bottom worm is typical of what is seen when the transgenic animal is exposed to *let-363* RNAi which promotes autophagy. In addition to the strong expression in the lumen, there is re-organization of the expression to a more punctuate expression. It is possible that the red arrow shows what may be localization of C13C4.5::*GFP* to autophagolysosomes which form during autophagy.

generation (Figure 10:A). Figure 10:B shows the results of genotyping individual males for one of the backcrosses. The heterozygous males were chosen to initiate the next backcross. After five backcross events heterozygous hermaphrodites were identified and allowed to self-fertilize, resulting in progeny which contained some animals homozygous for the *C13C4.5* deletion. The progeny population was screened for these homozygous worms using the PCR genotyping technique. This backcrossed deletion strain was named MGL69.

Generation of C13C4.5 / Daf-2 Double Mutant

The MGL97[*daf-2(e1368)*III; *C13C4.5(ok2087)V*] worm strain containing the *daf-2(e1368)* and *C13C4.5(ok2087)* mutations was generated to study the effects of the *C13C4.5* deletion in a dauer constitutive background. This worm strain was generated by crossing male animals from the MGL69 strain with homozygous hermaphrodites from the DR1572[*daf-2(e1368)III*] dauer constitutive mutant strain. Their F1 heterozygous, hermaphrodite progeny were self-fertilized generating the F2 population of worms, some of which contained the desired genotype.

Dauer Assay with the C13C4.5 / Daf-2 Double Mutant

To study the effect of the *C13C4.5* deletion on dauer formation, the MGL97 worm strain was grown at the non-permissive temperature, 25°C. The population of dauer vs. non-dauer worms was scored. Figure 11 shows the results from seven replicates for the *daf-2* control strain and 12 replicates for the MGL97 strain. The data indicates that there

is a higher proportion of the population which is non-dauer for the MGL97 strain in comparison to the control *daf-2* strain.

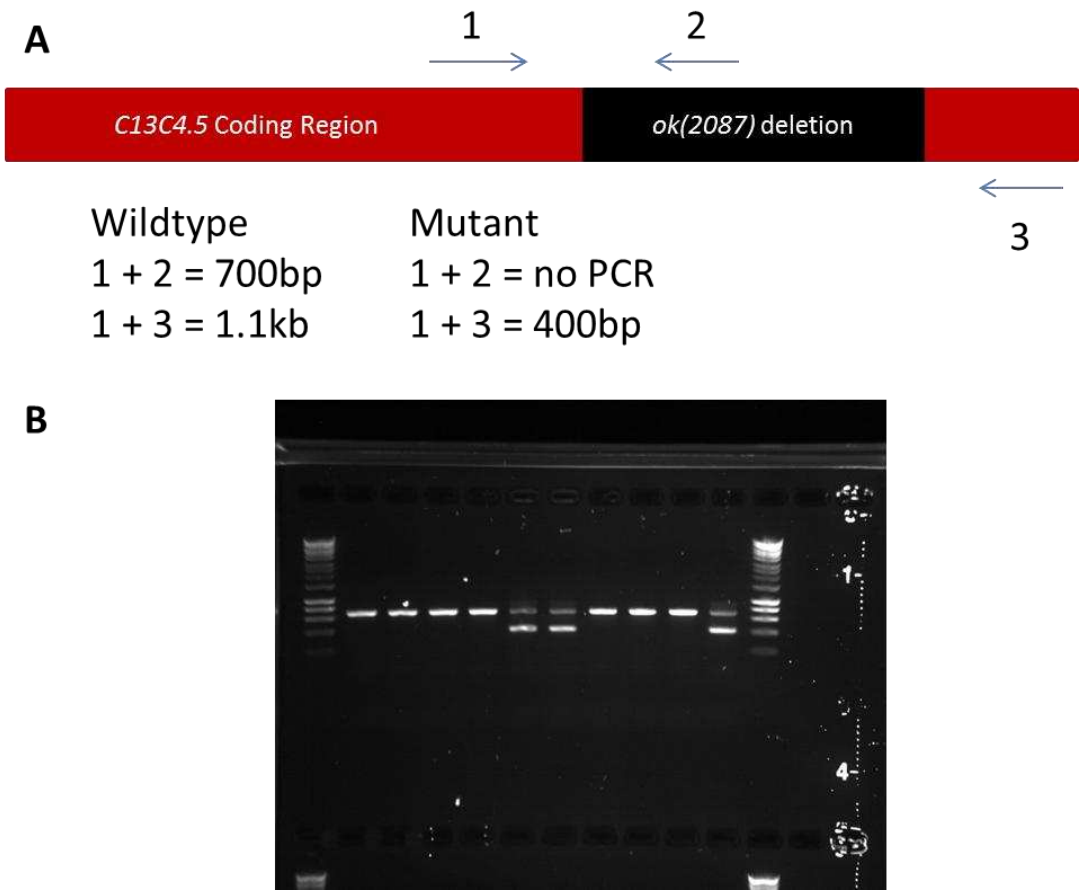


Figure 10: Genotyping Strategy and Results for C13C4.5 Backcross. (A) To genotype for the 900bp *C13C4.5* deletion allele *ok(2087)* primers 1 (C13diF), 2 (C13diR), and 3 (C13dR) are used. In a wild type animal the primers 1 + 2 generate a 700 bp produce while 1 + 3 generates a 1.1 kb product. Due to the higher efficiency of PCR for the smaller fragment it is preferentially amplified. An animal with the *C13C4.5(ok2087)* deletion cannot prime off of primer 2, so primers 1 and 3 will be used and generate a 400 bp fragment. (B) Genotyping of the third backcross of C13C4.5. Homozygous wild type animals have only one band at 700bp, as can be seen in lanes 2-5, and 8-10. Heterozygous animals have both the 700bp and 400bp band, as can be seen in lanes 6, 7, and 11.

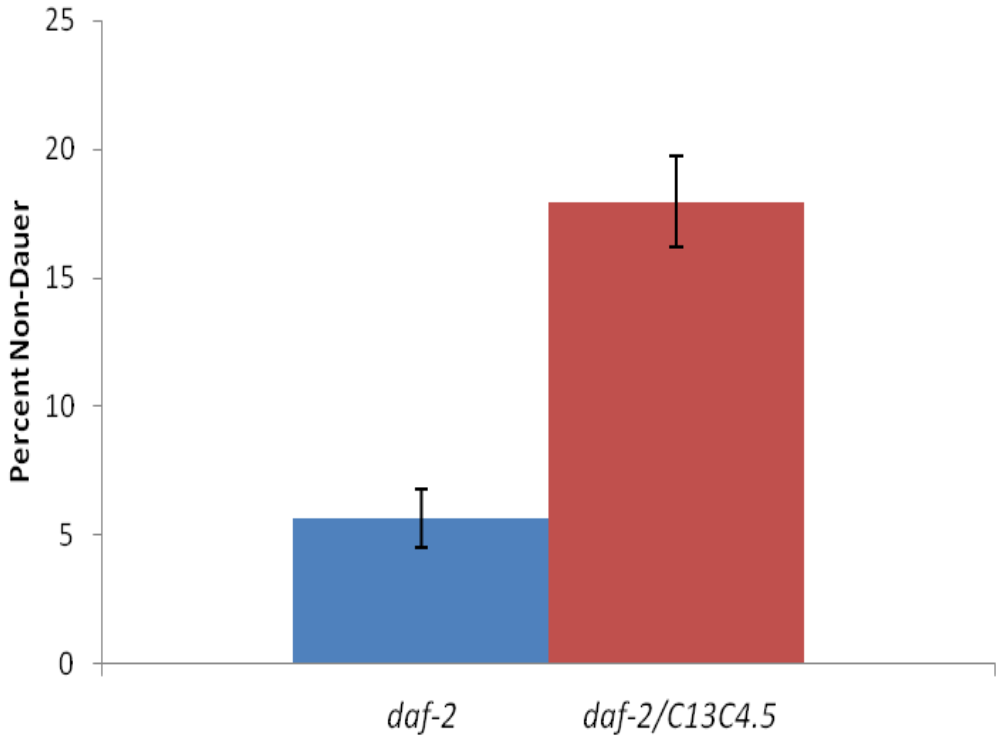


Figure 11: Dauer Assay of daf-2/C13C4.5 Double Mutant (MGL97). The dauer assay for the MGL97 strain shows a marked increase in the number of non-dauer animals in comparison to the *daf-2* control when the animals grow at the non-permissive temperature.

DISCUSSION

Spinster genes have been implicated in the transport of sugars out of autophagolysosomes, and the proper completion of autophagy in *D. melanogaster*. The *spinster* genes in *C. elegans*, identified by sequence homology, have not yet been characterized. The data suggests that of the four candidate *spinster* gene orthologs identified by sequence homology in *C. elegans*, *C13C4.5* is involved in dauer formation and may be a good functional ortholog of the *D. melanogaster* sugar transporter. It remains to be determined whether *C13C4.5* affects dauer formation through its involvement in the process of autophagy.

Identifying the Strongest Spinster Gene Ortholog Candidate

C. elegans spinster gene orthologs were first identified by sequence homology by Nakano and coworkers in 2001. These annotations were verified in this study and two additional candidates were identified by sequence comparison. In order to examine the loss of function phenotypes of these genes we used RNA interference. Two RNAi libraries were available: the Open Reading Frame (ORF) and MRC libraries. The ORF libraries target the open reading frame and tend to be more specific to the gene they knockdown while the MRC library contains genomic fragments PCR amplified by specific primers. Due to the method by which these libraries are constructed there are often errors in the RNAi clones and thus it is important to sequence individual clones. Sequencing of the four clones from the ORF library revealed that none of the clones matched their annotated sequence. By contrast, there was high sequence homology for

the MRC library strains with their target genes and thus the MRC library strains were used for RNAi knockdown on the spinster gene candidates. The MRC library contained feeding strains for *C13C4.5, C39E9.10,* and *F09A5.1* but not for *Y111B2A.19.* The strategy for the RNAi experiments was based on the published information that autophagy is required for entry into the dauer larval stage (Melendez et al., 2003), and on the fact that in *D. melanogaster spinster* is required for autophagy (Rong et al., 2011). Therefore a *spinster* ortholog in *C. elegans*, when knocked down, should not allow for a dauer constitutive mutant to enter into the dauer stage.

The data from the RNAi knockdown dauer assays show that only *C13C4.5* knockdown prevented entry into the dauer stage in approximately 50% of the population. There was a weak effect with the knockdown of *F09A5.1*. This implicated *C13C4.5* as being the strongest candidate for the *spinster* ortholog, so its expression pattern in the worm was determined by generating a transgenic animal expressing a GFP fusion protein.

Examining the Expression Pattern of C13C4.5

GFP is widely used as a means of examining the expression pattern of a gene of interest and is widely used in *C. elegans*. Generation of transgenic animals expressing GFP is particularly easy since a PCR fusion product can be used to generate an extrachromosomal array rather than generating an expression plasmid (Boulin et al., 2006). A *pC13C4.5::C13C4.*5::*GFP* fusion construct was made by amplification of *C13C4.5* promoter and genomic DNA, amplification of GFP DNA, and then fusion of these two DNA fragments. This fusion product was then injected along with a co-

injection marker, *unc25*::mRFP into the gonad of a worm to form a transgenic line which expressed the GFP tagged spinster protein. The co-injection marker drives expression from the constitutive *unc-25* promoter and thus is always expressed inside the worm at all stages. The fusion of GFP to the promoter and coding regions of a gene forms a transcriptional reporter which would show in which tissues the protein is generally expressed. The fusion of GFP to the promoter and coding region forms a translational reporter which is able to mark the specific cell structures which the tagged protein interacts with. Without the use of the translational reporter it would have been impossible to see the strong lining of the lumen or the punctate pattern. To identify a worm strain which has successfully taken up the fusion product the worms are screened for the presence of the co-injection marker. This is done because the co-injection marker is always expressed and is easily visible at all stages of development while the GFP tagged protein, due to its expression pattern being unknown, may not be easy to identify at certain stages of development or under some environmental conditions.

After screening for the presence of transgene, the strain is maintained by continually moving the transgenic animals from their non-transgenic siblings onto new petri dishes. The strain used for the expression patterning was grown on control RNAi and RNAi which promoted autophagy. The RNAi used to knockdown autophagy was the *let-363* strain. *let-363* is the TOR ortholog in *C. elegans,* so its knockdown should induce autophagy in the animals. Upon exposure to the different RNAi there was a clear distinction between the expression on the control and the pro-autophagy RNAi. In the control conditions GFP was present along the length of the intestinal lumen. On the pro-

autophagy RNAi the expression was also observed in the intestine but was much more punctate, or spotted, than the control RNAi. Given that the data in *D. melanogaster* showed a similar expression pattern it can be speculated that this expression pattern is due to localization of *C13C4.5* to autophagolysosomes.

To further support the expression evidence which implicates *C13C4.5* in autophagy, co-localization experiments should be performed with the known autophagosome marker *LGG-1* (Melendez et al., 2003). If the two gene products do co-localize then the evidence for *C13C4.5* being a *spinster* ortholog and acting in a function similar to that of the *D. melanogaster* spinster gene will be strengthened. There already exists a transgenic line which has *LGG-1* tagged with GFP. In order to study the co-localization of the *C13C4.5* protein to areas tagged by LGG-1 a transgenic strain with mCherry tagged *C13C4.5* would need to be generated and then crossed into the *LGG-1::GFP* transgenic line.

The Effect of the C13C4.5 Deletion Mutant on Dauer Formation

RNAi knockdown of the *C13C4.5* gene gave circumstantial indications as to its role as a *spinster* gene ortholog. To confirm this role *C13C4.5* deletion mutant was obtained from the *C. elegans* Knockout Consortium via the *Caenorhabditis* Genetics Center at the University of Minnesota. The deletion mutant was first outcrossed to the wild type N2 worm strain to remove any other mutations, and then crossed into the *daf-2(e1368)* dauer constitutive mutant to create a worm strain which should arrest into the dauer phase at 25°C, but also contain the *C13C4.5* deletion. The deletion removed 900bp

of the *C13C4.5* gene which possibly resulted in a null-mutant and thus it was hypothesized that it might have a stronger effect on dauer formation than RNAi.

The backcross of the *C13C4.5* deletion strain was done using a strategy which differs from the normal strategy. The normal backcrossing method is to cross a homozygous mutant worm with a homozygous wild type worm, resulting in a heterozygous hermaphrodite population. These worms then self-fertilize and produce a F2 population which contains animals that are either homozygous for the mutation, heterozygous, or homozygous wild type in the ratio of 1:2:1. The animals which are homozygous for the mutations are selected and then the cross is repeated. This standard method generally takes two months to complete five backcrosses. The method of backcrossing which was used here is different. Instead of needing two generation to complete one cross, it only requires one. This allows for a quicker completion of the five backcrosses and is accomplished by using male heterozygous worms instead of hermaphrodite heterozygous worms to produce the next population to be genotyped. The backcrossed *C13C4.5* deletion strain was named MGL69.

Next the MGL69 strain and the DR1572[*daf-2(e1368)III*] dauer constitutive strain were crossed into one another to create the MGL97 strain. Doing this generated a worm which contained the *daf-2* mutation and the *C13C4.5* deletion mutation. It was grown at the non-permissive temperature of 25°C and its population ratios of dauer versus non-dauer were recorded. The results showed a much weaker non-entry into dauer phenotype. While not as strong as the RNAi knockdown of the gene it still showed a higher proportion of the population not entering into the dauer phenotype than the control strain

and therefore supports the idea that *C13C4.5* is having an effect on dauer formation. However, the weaker phenotype indicated that the RNAi may be having effects outside of the *C13C4.5* knockdown. There is a high level of homology between the spinster genes (~50%). Due to this homology is it possible that the some of the RNAi fragments generated in the process of gene knockdown were able to target another of the spinster candidates, thereby knocking down multiple spinster genes.

Future work could focus on the effects of multiple knockdowns of these *C. elegans* orthologs. If the RNAi of *C13C4.5* is knocking down more than just the *C13C4.5* gene due to the high level of homology between the four *spinster* gene orthologs then it is possible that there is a redundancy built into the *spinster* gene system. The RNAi could be accounting for this redundancy, but the deletion could not. Multiple gene knockdowns/knockouts will give a better picture of how these genes effects the dauer formation and autophagy systems. To do this the MGL97 double mutant could be grown on *F09A5.1* RNAi or *C39E9.10* RNAi and a dauer assay done to assess the ability of this multiple *spinster* gene knockdown to prevent entry into the dauer stage.

CONCLUSION

Loss of function of the *spinster* gene ortholog *C13C4.5* prevents dauer formation in a dauer constitutive mutant which is consistent with a role in autophagy. Additionally, when TOR is inhibited *C13C4.5* appears to localize in punctate structures in the intestine that resemble autophagolysosomes. The function that *C13C4.5* serves in a more specific sense is assumed by sequence homology, but not known. Future experiments could be to use the MGL97 double mutant and cross the transgenic MGL79 strain into it. The goal would be to see if the transgenic GFP tagged C13C4.5 rescues the ability of the worms to enter into the dauer stage. Through better understanding of *spinster* in *C. elegans*, the processes which regulate autophagy can be expanded upon. In doing so it may facilitate our understanding of the regulation of autophagy in higher organisms. Since autophagy has already been shown to be important in apoptosis in humans, and apoptosis is known to be involved in important cellular features such as proper development and functioning of the immune system, embryonic development, as well as being implicated in human neurodegenerative diseases and many types of cancer, better understanding of autophagy may also lead to better understanding of human diseases (Elmore, 2007).

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