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Raymarie Gomez-Vazquez

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CHARACTERIZATION OF ONE OF THE REF-1 FAMILY MEMBERS,
HLH-25, IN *C. ELEGANS*

by

RAYMARIE GÓMEZ-VÁZQUEZ

Under the Direction of Casonya M. Johnson

The REF-1 family proteins are distinguished by the presence of two basic helix-loop helix domains. The REF-1 family members are considered functional homologs of the Hairy/Enhancer of Split in humans. HLH-25 is one of the six members of the REF-1 family. HLH-25 has not been studied extensively. In preliminary studies from our laboratory, genes identified by microarray analysis of *hlh-25* mutants were essential for embryogenesis, larval development, and growth. Thus, the present study was designed to further characterize HLH-25 and to more precisely define its role during embryonic and larval development. The gene encoding HLH-25 is actively expressed in embryos, larvae and adults. In the absence of *hlh-25*, animals show a 54% embryonic lethality, a reduced brood size, an increased number of unfertilized eggs, a slower movement rate, a longer life span, and a longer dauer recovery. The human tumor suppressor PTEN homolog, *daf-18* is one of the HLH-25 target genes.

INDEX WORDS: HES; bHLH; *daf-18*; Dauer; Longevity; Embryogenesis

CHARACTERIZATION OF ONE OF THE REF-1 FAMILY MEMBERS,
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RAYMARIE GÓMEZ-VÁZQUEZ

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2014

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May 2014

DEDICATION

Every success I have achieved in my life depends largely on the encouragement and guidance from my family and friends; hence this thesis is dedicated to them.

First of all, I would like to dedicate this to the one I love, my beloved husband Alfredo Matos Marin. You always encourage me to strive towards my goal, and I cannot say thank you enough for your tremendous support and the help you have shown me. Without your encouragement and motivation I would not have made it this far. Thank you for all of the sacrifices that you have made for me.

Next, I would like to dedicate this to my dear mother, María M. Cruz whose love for me knew no bounds and, who taught me the value of hard work. Thank you for your support, cheers, and prayers. I am grateful to have a big supportive family, and so I would like to give a special thank you to my siblings and their spouses: Jennifer, Pamela, Orlando, Danny, Dennis, David, Robert, Cesar, Carol, Cecibel, and Gema; my grandmother Barbara Vázquez, my stepfather Danny Cruz, my mother-in law Nylma Marin, and father-in-law Alfredo Matos. Thank you for your support and encouragement.

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1 INTRODUCTION

1.1 Transcription Factors

Transcription factors are a diverse family of proteins that bind to the promoter region, a specific DNA section upstream of a gene, in order to regulate its expression. Transcription factors can also bind to multi-subunit protein complexes to manipulate transcription either negatively or positively [2-7], and they determine how cells function by determining the time and place of their gene expression.

Transcription factors that control homeotic genes (i.e. genes that control the pattern of body formation) usually are vital for the normal development of the organism. In *Drosophila*, the transcription factor Bicoid, for instance, is necessary for the embryonic development of the anterior half of the embryo. Embryos with a mutation in Bicoid grow into larva with posterior structures at both ends and with two spiracles, instead of one, at the posterior end [8, 9]. Another function of transcription factors is to determine whether a gene functions or not at a given time. For example, HES mutations in the developing mouse embryo cause neural progenitor cells to prematurely differentiate causing neural defects [10] and sometimes small and deformed brain structures [11].

Transcription factors are often grouped into families based on their sequence similarity and their structure. Examples of different transcription factor families include winged helix, zinc fingers, homeodomain proteins, zinc-finger proteins, and basic helix loop helix proteins. These different families also typically have characteristic functions. For instance, in humans, the transcription factor T18 is part of the zinc-finger family. T18 acts as a breast cancer tumor suppressor [12]. In plants, many transcription factors from the family of basic

region/leucine zipper motif (bZIP) proteins, like the bZIP protein *lotus*, regulate processes including photomorphogenesis, leaf and seed development [13, 14].

1.2 The Basic Helix Loop Helix (bHLH) superfamily

Members of the basic helix-loop-helix (bHLH) transcription factor family have two highly conserved domains, the basic domain and the helix-loop-helix domain. The basic domain is located at the N-terminus; this domain binds to specific sequences of DNA upstream of the promoter region [15]. The second domain contains an HLH domain located at the C-terminus formed by two amphipathic helices connected by a loop region consisting of amino acids [16]. The HLH domain interacts with other proteins. These domains are approximately 60 amino acids long [15]. The HLH domain binds to other proteins to form homodimeric and heterodimeric complexes [17]. For example, bHLH proteins LYL1 form a heterodimeric complex with TCF3 in hematolymphoid cells to regulate blood vessel maturation and hematopoiesis [18]. bHLH transcription factors also have the ability to differentially regulate transcription based dimerization. For instance, in the fungal plant *Fusarium oxysporum*, the bHLH protein FoSTUA differentially regulates the development of two kinds of asexual pores, macroconidia and microconidia. FoStuA acts as a positive regulator for macroconidia and as a negative regulator for chlamydospores during development [19].

Different attempts have been made to subcategorize bHLH proteins. Atchley and Fitch [20], subdivided the bHLH proteins into four groups based on their binding to DNA at the hexanucleotide E-box, the amino acid patterns in other components of the motif, and the presence/absence of a leucine zipper. Group A proteins bind to the E-box (CAGCTG) usually to activate transcription. They have a distinctive pattern of amino acids at sites 5, 8, and 13. This

group has only small aliphatic residues. An example of a group A protein in mammals is the MyoD protein family. Group B proteins bind to the G-box (CACGTG). This group has arginine at site 13, a basic amino acid at site 5, and an E-box configuration at sites 5–8-13. Protein families with a LZ motif are included in group B. Sequences with an LZ have a very high frequency of N residues (93%). An example of a group B protein in *Drosophila* is the Hairy and Enhancer of split bHLH. Group C has the PAS domain, which is a protein-protein interaction region. Proteins with a PAS domain usually function as signal sensors [21]. For instance, human PAS proteins include hypoxia-inducible factors and voltage-sensitive ion channel proteins [22]. Group D proteins lack the DNA binding basic region, and thus, are unable to bind DNA, but they can form heterodimers with other bHLH proteins usually as negative regulators. An example of group D in mammals is the ID protein family [23, 24].

The superfamily bHLH is found in many organisms including yeast, mice, worms, and humans. bHLH transcription factors are involved in the regulation of many developmental processes including, cardiovascular development [25], mouse brain development [26, 27], neurogenesis [28], cell cycle regulation, and embryogenesis [10, 29]. They typically act in cascades, one after the other, to cause increasing degrees of specialization. For example, the regulation of the cell elongation (i.e. cell specialization) in *Arabidopsis* depends on a chain of antagonistic switches comprised of transcription factors, PREs, IBH1, and HBI1 [30].

Transcriptional regulators are required for the development of differentiated neurons. Many proteins are involved in neuron differentiation and HLH proteins regulate parts of this process. In mammals, different bHLH proteins work in a cascade to control different steps behind neurogenesis. In mice, two bHLH transcription factors, *Ascl1* and *Hand2*, are necessary at every step of the cascade for proper parasympathetic and sympathetic neuron development,

respectively [31]. *Ascl1* has also been implicated in the differentiation of the P19 EC cells into neuronal cells, and reduction of *Ascl1* causes delayed neurogenesis of the P19 EC cells [32]. Hand 2 impacts development of the enteric nervous system (ENS). Mutations in *Hand2* affect both neural precursor and neuron numbers, such as complete loss of neuronal nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP) neurons [33].

1.3 *Hairy/Enhancer of Split (HES)*

The Hairy/Enhancer of Split (HES) proteins form a sub-family within the bHLH superfamily. The founding members of the HES family were first described in *Drosophila* where it was demonstrated that HES proteins are direct targets of Notch signaling [34-36]. Notch signaling is a conserved cell to cell communication necessary for proper development in many organisms. Notch signaling has been implicated in the development of embryogenesis, the nervous system, the cardiovascular system, and the endocrine system [11, 34, 37, 38]. In humans, for instance, a mutation in *NOTCH1* causes T-cell acute lymphoblastic leukemia [39].

Proteins of the HES family have three structural domains: bHLH, Orange, and WRPW. The bHLH domain contains a highly conserved proline residue that is typically absent in other members of the bHLH family. The Orange domain consists of two amphipathic helices and regulates the selection of bHLH heterodimer partners [11, 35]. For example, the Orange domain in *Xenopus* is required for heterodimerization of *XHRT1* with *Xhairy2b* [40]. The WRPW domain is found at the C-terminal end of the protein and is required for repression of transcription. This domain consists of four amino acids Trp-Arg-Pro-Trp that mediates degradation of its own protein. For instance, WRPW recruits Groucho to actively repress transcription [41, 42].

Even though HES proteins are critical for normal function and play central roles in embryogenesis by maintaining progenitor cells in an undifferentiated state [11], little is known about how they work. HES mutations have been associated with lung and breast cancer in mammals [43, 44] and oocyte death [29].

1.4 REF-1 family proteins

The members of the REF-1 family in *C. elegans*, like the HES family, are also a direct target of the Notch signaling [45], but they also act in Notch-independent functions [46]. Both families share a sequence similarity at the bHLH domain [2], and they regulate a variety of target genes that influence embryo development. Therefore, the REF-1 family members are considered functional homologs of the HES family.

Unlike other bHLH members, the REF-1 family proteins are distinguished by the presence of two basic helix-loop helix domains instead of one [2], and unlike the HES proteins, REF-1 proteins do not have a conserved Orange domain [47]. The six members of the REF-1 family are REF-1(HLH-24), HLH-25, HLH-26, HLH-27, HLH-28 and HLH-29. The REF-1 family also lacks the WRPW domain, but at the C-terminal of each member has a slightly different pentapeptide repeat sequence: REF-1 has FRPWE; HLH-25 has LDIIN; HLH-26 has IDIVG; HLH-27 has VDISN; and HLH-28 and HLH-29 have IDIIG (Figure 1). These pentapeptide repeat sequences have similar net charges to those of the WRPW sequences, and the sequences in REF-1 has been shown to interact with the *C. elegans* Groucho homolog [45].

Alignment of bHLH domains from *Drosophila* HES-6, the *C. elegans* REF-1 family and the *C. briggsae* showed that the first bHLH domain in the REF-1 family proteins is more closely related to each other than to the second bHLH domain, and the first bHLH domain is also significantly more similar to the bHLH domain in the Hairy/E(spl)/LIN-22 [2]. Therefore, we

postulated that the second bHLH domain in the REF-1 family could functionally replace the Orange domain and may act to provide stability during protein-protein interactions.

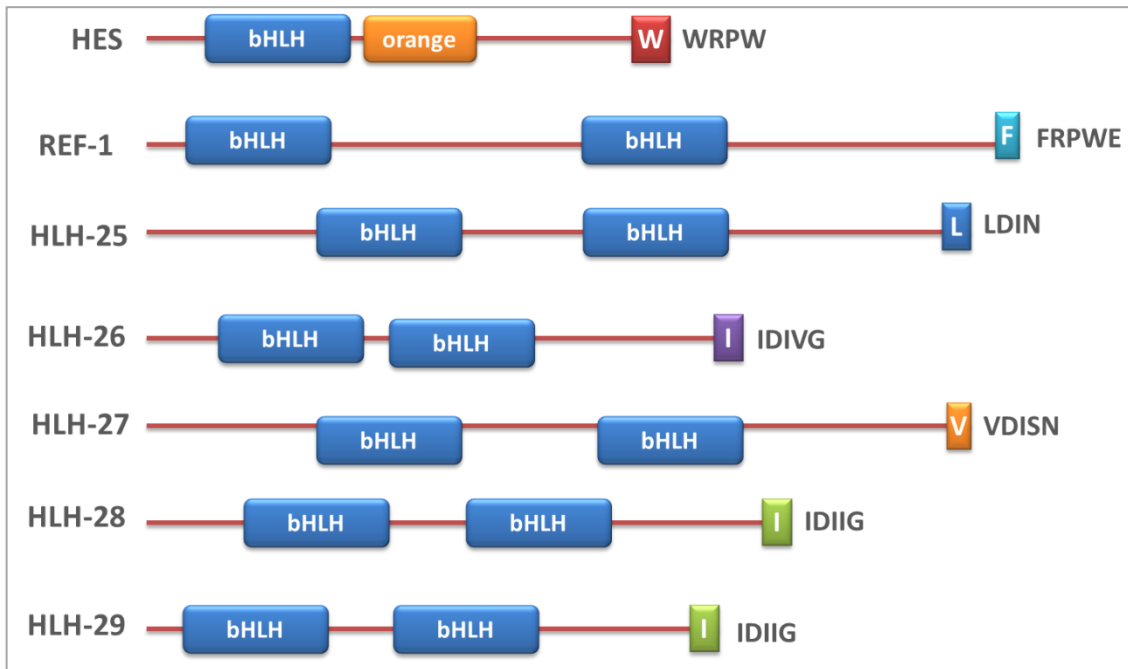


Figure 1 Domain Organization of HES and REF-1 family members.

This figure was adapted from Dawson et al. 1995 and Neves et al. 2005. Unlike HES, the REF-1 family lacks the Orange domain present in HES. It is postulated that the second basic helix-loop-helix domain can functionally substitute for the orange domain.

REF-1 was the first member to be studied. It has been shown that REF-1 regulates HOX genes, genes that control the body plan of the embryo; thus making it an important transcription factors needed for development during embryogenesis [48]. Mutations to *ref-1* affect cell fate decisions in different body regions along the *C. elegans* AP body axis, giving rise to aberrant physical phenotypes such as irregular head shapes and multivulva [48]. REF-1 also affects the V ray lineage in *C. elegans* males resulting in a partial transformation of the ray identity from V6 to V5 [48, 49].

The *hll-28* gene is nearly identical to *hll-29* and their gene products are identical [50]. Therefore they are usually studied together. They are expressed in all cells of the early MS and

E lineages. Both of these members are also involved in embryonic development and reproduction. RNAi against *hlh-29/hlh-28* affects the embryonic viability, adult egg-laying and organismal homeostasis [51]. Also, HLH-29 regulates the ability of oocytes to enter and exit the spermatheca, within the IP₃ signaling pathway [52]

Even though HLH-26 and HLH-27 are known to activate in response to Notch signaling during embryogenesis [2], they have not yet been studied extensively. However, *hlh-27* and *hlh-25* are almost identical and may work redundantly in the mesoderm and endoderm networks in *C. elegans* [46, 53].

HLH-25 is expressed during embryonic development in response to Notch signaling [45], in Abp granddaughters (beginning after eight-cell stage) and in four of the EMS granddaughters (MSaa, MSap, MSpa, Mspp) [45]. HLH-27 is also expressed in the early MS lineage [46, 53]. *hlh-25* is one of the MED-1 target genes which participates in specifying the mesendoderm [54]. It has been shown that overexpression of *hlh-25* restores muscle differentiation in development in a small proportion of embryos with *skn-1* and *pal-1* mutations [46, 53]. Since the cascade controlling muscle development includes members of the bHLH family, MyoD, myogenin, Myf5, and MRF4, it is believed that HLH-25 may be involved in muscle development [46, 53].

Using enriched GO annotations, previous studies have shown that HLH-25 DNA binding specificities are associated with candidate target genes required for cytoskeleton, reproduction, cuticle/molting, secretion, cell division, locomotion, signaling, development, and metabolism [55]. In preliminary studies from our laboratory, genes identified by gene expression microarray analysis of *hlh-25* mutants were grouped under regulation of growth, embryonic development ending in birth or egg hatching, nematode larval development, positive regulation of growth, post-embryonic development, and body morphogenesis.

Grove *et al* [56] also used protein binding microarray (PBMs) assays to identify the DNA binding sequence preferences of the bHLH dimers. They found that HLH-25 and the other REF-1 family proteins can bind to DNA as homodimers, and that HLH-25 recognizes five different consensus sequences. Interestingly, the REF-1 family member HLH-29 shared two of the five HLH-25 recognition sequences (table 1) [55].

Table 1 DNA Consensus Sequences

Protein	CACGCG	CATGCG	CATACG	CACACG	CACGCT
HLH-25	X	X	X	X	X
HLH-27	X	X	X		
HLH-29	X	X			

We compared the target genes identified by the gene expression microarray studies from our lab with those identified by PBM. A number of targets were on both lists. One target gene found on both lists was *daf-18*, the *C. elegans* ortholog of PTEN. In humans, PTEN is a tumor suppressor in human cancers [26]. Mutations to PTEN have resulted in the development of glioblastoma, colon cancer, breast cancer, and prostate cancer [57-60]. PTEN is a phosphatase protein involved in the regulation of the cell cycle and prevents cells from growing too rapidly by preventing phosphorylation of the Akt/PKB signaling pathway which is responsible for cell growth regulation [61-63]. In *C. elegans*, DAF-18, like PTEN, is a phosphatase protein involved in cell regulation. DAF-18 also prevents phosphorylation of the AKT-1/AKT-2 complex in the insulin pathway [64] which plays a role in, development, metabolism, and longevity [65-68].

1.5 *Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans*) are transparent free-living, non-parasitic microscopic nematodes measuring about 1 mm in length as adults. These round worms live in soil environments and feed on bacteria [69-72]. In laboratories, *C. elegans* grow in Petri-dish plates and feed on *E. coli*. There are two sexes, a self-fertilizing hermaphrodite and male. *C. elegans* have a short life span of roughly one month.

The *C. elegans* life cycle is divided into the following stages: embryos, four larvae stages: L1, L2, L3, L4, and adults (Figure 2). When these animals encounter a hostile environment such as high

temperatures, little or no food, or crowdedness, they

can go to an alternate stage called the dauer stage. During the dauer stage, *C. elegans* do not age, eat or reproduce and their locomotion is reduced. Surprisingly, worms that encounter this stage can live up to four months, four times longer than non-dauer animals. Morphologically they are long, thin, and covered in a thick cuticle. When dauer-stage animals find a suitable environment they resume their normal life cycle and molt directly to an L4 within a few hours [73].

C. elegans hermaphrodites have a “U” shape gonad. The gonad consists of two ovaries, oviducts, spermatheca, and one uterus. There is a distal end (DTC) of the germline on each side of the gonad where the somatic cells are situated (Figure 3). As these cells move through the gonad they go through a series of phases. Once they reach the loop (bend of the gonad) they are surrounded by plasma membrane in preparation for compartmentalization to form oocytes. Once

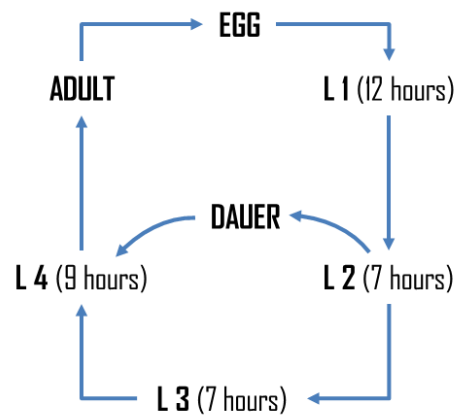


Figure 2 *C. elegans* Life Cycle at 25°C.
<http://avery.rutgers.edu/WSSP/StudentScholars/project/introduction/worms.html>

fertilized, the oocytes undergo meiosis and are pushed to in the uterus, and the eggs are laid after approximately thirty divisions. The fertilized egg forms an eggshell that protects it from the external environment [69, 72]. If plenty of food is present, hermaphrodites who run out of sperms may lay an average of 31 unfertilized eggs per worm [74].

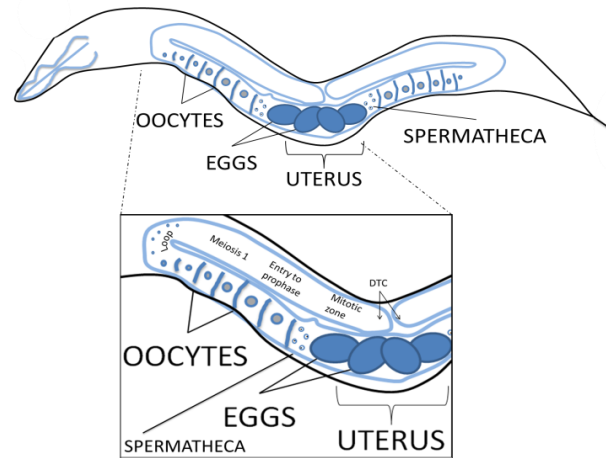


Figure 3 *C. elegans* Hermaphrodites

1.6 Objective

Since previous studies suggest that HLH-25 may be critical for embryonic and larval development, my objective is to further characterize HLH-25 and to precisely define its role during embryonic and larval development. In order to achieve this objective, the following aims were developed: to determine the temporal and spatial expression profile of HLH-25, to identify phenotypes of *hlh-25 mutants*, to define the *hlh-25* transcriptional network, and to determine if HLH-25 is required for embryonic cell division. The key challenge in this genetic research is to understand how the HLH-25 transcription factor affects the performances of its target genes by examining phenotypes caused by *hlh-25* mutations.

2 EXPERIMENTAL METHODS

2.1 *Nematodes Strains and Maintenance*

The wild-type strain used in this work was Bristol N2. VC1220 [*hlh-25* (ok1710) II.] and RB712 (*daf-18*(ok480) IV.), was received from the Caenorhabditis Genetics Center (CGC). The *daf-18* transgenic reporter strain used was **FS84** (*daf-2*(e1370) ; *daf-18*(mg198); fsEx84(*daf-18* promoter::*daf-18* cDNA::*unc-54* 3'UTR 20ng/ul + *daf-18* promoter::GFP::*unc-54* 3'UTR 20ng/ul (in pPD95.75) + pRF4 100ng/ul) kindly provided by Dr. Florence Solari from Claude Bernard University Lyon from the European Molecular Biology Laboratory. **IC748**, quIS18 [*daf-18* genomic(+)] was provided by Dr. Ian D. Chin-Sang from Queen's University, Kingston, ON Canada. **GR1673** (*akt-2*::GFP; pRF4 (*rol-6*)) was provided by Dr. Gary Ruvkun from Harvard Medical School.

The *hlh-25* transgenic reporter strain used was **CMJ3001** (*Phlh-25*::GFP(A); pRF4 (*rol-6*). Other strains used were **CMJ4001** (*daf-18* promoter::*daf-18* cDNA::*unc-54* 3'UTR 20ng/ul + *daf-18* promoter::GFP::*unc-54* 3'UTR 20ng/ul (in pPD95.75) + pRF4 100ng/ul; *hlh-25* (ok1710) II.),*hlh-25*(VC1220); **CMJ4004** (*Phlh-25*::GFP); *Pmyo-2*::mCherry::*unc-54*utr), and **CMJ4003**(α -tubulin::GFP; *hlh-25* (ok1710) II.)

Animals were maintained on nematode growth medium (NGM) with OP50 as a food source, previously described [75]. For synchronization, embryos were collected by treatment with sodium hypochlorite, as previously described, [76, 77], with the following exceptions. Only 500 μ L of 5M KOH and 1mL of household bleach were added and the worms were shaken vigorously for no more than six minutes.

2.2 Constructs and Transgenic Lines

To generate *Phlh-25::GFP*, 1570 bp upstream of the first ATG of *hlh-25* in the coding sequence was amplified by PCR (see appendix A for primers) using Phusion[®] High-Fidelity PCR Master Mix. For the HLH-25 target genes *daf-18*, *vha-1*, *akt-2*, *pqn-95*, *ima-1*, *imp-2*, *ntl-4*, *thoc-2*, and *ran-1*, 2,000 bp upstream of the first ATG in the coding sequence was amplified using Phusion[®] High-Fidelity PCR Master Mix. All PCR was done using the DNA Engine Dyand Peltier Thermal Cycler and the products purified using the Qiagen QIAquick PCR Purification Kit. The transgenes were cloned using standard techniques (Figure 4) [78, 79].

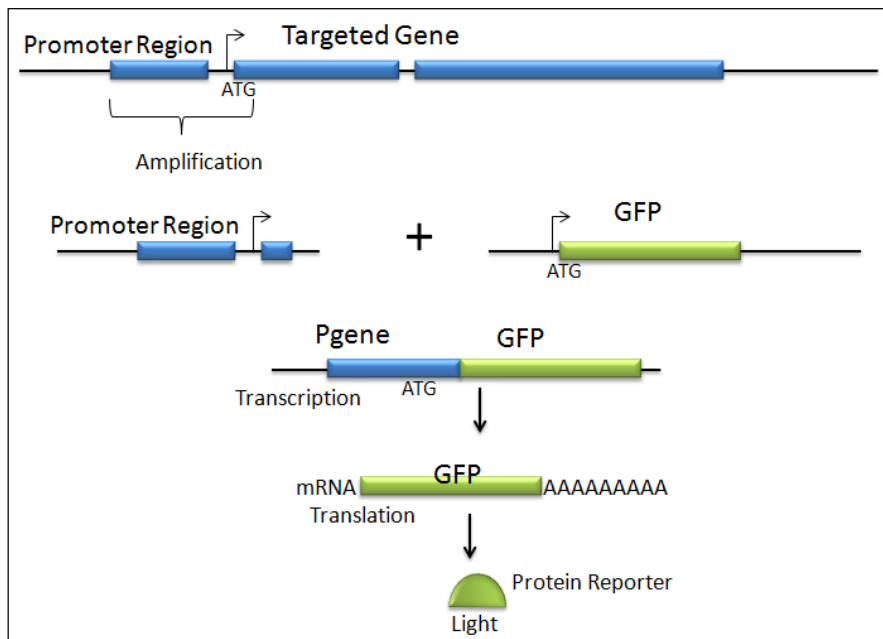


Figure 4 Transcription Reporter

Schematic image of a transgenic DNA construct where the (GFP) gene is under the control of the selected gene's promoter, producing a green fluorescent protein when translated into the region where the gene is expressed. Plasmid 1490: pPD95.67 (Fire Vector)

2.3 Nematode Transformation

Microinjection has been previously described [80, 81]. The *Phlh-25::GFP* DNA construct was microinjected as a “simple” array and was co-injected with *rol-6(su1006)* as a marker which induces a dominant “roller” phenotype [82] in a 1:1 ratio. The *HLH-25* target in a DNA constructs were microinjected directly into the syncytial gonad as “complex” arrays in low copy number to prevent transgene silencing [81, 83]. The DNA solution consisted of the following components: 100 ng/ μ L of genomic DNA cut with PvuII, 1ng/ μ L of linearized construct, and 1ng/ μ L of marker *rol-6(su1006)*. A PCR product of genomic DNA from 1541bp upstream to 770 bp downstream of the *hlh-25* coding sequence was co-injected with *Pmyo-2::mCherry*.



Figure 5 Microinjection.

A) Site for microinjection of the DNA in the cytoplasm of the syncytial gonad. B) Sample needle used for microinjection.

2.4 *Microscopy*

The transgenic worms were exposed to ultra-violet (UV) to analyze their expression and position by comparing GFP (green fluorescent protein) and DIC (differential interference contrast) images. Animals were anesthetized with 0.2% levamisole [84], mounted on 2.0% agarose pads and imaged using a Nikon Eclipse 90i microscope equipped with a Nikon Coolsnap CCD camera.

2.5 *Embryonic Lethality and Unfertilized Oocytes Assay*

Twenty virgin L4 stage animals of each strain were singled out and placed on fresh plates every 24 hours. Embryonic lethality was determined by counting the number of non-hatched embryos divided by the total progeny (non-hatched and hatched) and subtracting the number from 100% to obtain the lethality percentage. Embryos that did not hatch after 24 hours were scored as dead. The p-value of lethality was calculated using two-way ANOVA. The unfertilized oocytes average was determined by moving another twenty L4 to a new plate daily and counting the unfertilized eggs until death or nineteen days after egg laying was reached, whichever occurred first. The percentage and p-value was calculated using the Graphpad Software Package.

2.6 *Life Span Assay*

Assays were conducted at 20°C. During the egg laying period, these worms were transferred every two days to a new fresh NGM plates. The numbers of surviving, dead and missing worms were counted each day. Animals were scored as dead when they no longer responded to the touch of the platinum wire or were censored if missing [85, 86]. The Graphpad

Software Package was used for statistical analysis and to calculate p-value, means and percentiles.

2.7 *Dauer Recovery Assay*

In order to promote dauer formation more than 200 embryos were placed on each plate containing very little food (10 μ L OP50) at 27° C for a minimum of 96 hours. Three day old dauers were singled out to a plate with plenty of food (OP50, 75 μ L) and moved to 20° C. Dauer recovery was examined by monitoring five characteristics: fat accumulation, pharyngeal pumping, visibility of crescent in vulvae, visibility of embryos, and visibility of eggs laid (Figure 26) [70, 87].

2.8 *Mobility Assays*

2.8.1 *Locomotion and Thrashing Assay*

Dauer and L4s were used to examine movement. The locomotion rate on solid media was quantitated by counting body bends for one minute in a plate with no food [88]. The thrashing rate was obtained by counting each lateral movement made by the worms when swimming in M9 buffer over a period of one minute [89].

2.9 *Total RNA Isolation*

Embryos, L4, and dauer animals were frozen at -80°C for a minimum of twenty- four hours. Each pellet was resuspended in 100 μ L of freshly made lysis buffer containing 0.5% SDS, 5% 2-mercaptoethanol, 10 mM EDTA, 10 mM TrisHCl (pH 7.5), and 10 μ L of Proteinase

K [(Thermo E00491) (19.2mg/mL)]. The samples were incubated at 55°C shaking at 900 rpm for 1 hour. RNA was extracted using a Quiagen RNeasy Plus Kit and with following the protocol from the RNeasy Microhandbook for animal tissue.

2.10 RTqPCR

cDNA was synthesized using the high capacity cDNA Reverse Transcription Kit (Applied Biosciences catalog #43674966), and following the instructions from the manufacturer. cDNA synthesis reactions were performed in 20 µL reaction volumes containing 0.5 µg of total RNA. Reverse transcriptase PCR assays were performed with Taqman Gene Expression Assays (Applied Biosystems) for detection of amplicon, specific for each target gene, using relative quantitation against the endogenous control gene *pmp-3* [90]. For primer sequences see appendix B.

2.11 Strain Crossing

Fifty *hlh-25* young adult hermaphrodites were heat shocked at 35° C for 3 hours and singled out in plates to screen for males [91]. Transgenic strains *akt-2::GFP*, *daf-18::GFP*, and α -tubuline were crossed with *hlh-25* animals. Each plate contained six males and one adult hermaphrodite. To select progeny with the transgene and homozygous deletion of *hlh-25*, PCR was used to amplify the *hlh-25* gene using internal primers: Left 5' ACCAAACCGGAGTTCTCAAA 3'; Right 5' AGAATGGGACATCCCACAAA 3'. Deletion was confirmed by agarose gel electrophoresis. The internal wild-type amplicon is 2,113 bp and the deletion size is 1,550 bp.

2.12 Live Embryo Imaging

Gravid adult worms were placed in a drop of M9 buffer over a glass slide. An incision was made with a scalpel (or razor blade) at the vulva to release the eggs, (Figure 6). Vaseline® Petroleum Jelly was applied on the edges of a cover slip that was then placed over the glass slide containing the embryos to lock moisture in. Less than

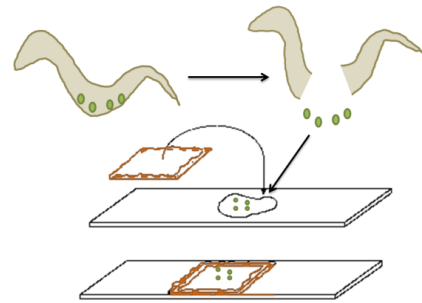


Figure 6 Release of embryos

ten eggs were placed per slide to prevent hypoxia. A time-lapse recording of epifluorescence pictures at time intervals of three minutes was taken to track the first divisions of the embryo with 40X or 60X objective [92].

3 RESULTS

3.1 *Temporal and Spatial expression profile of HLH-25*

Even though it was previously shown that HLH-25 is expressed in response to Notch signaling during embryogenesis, its full expression profile was unknown. As a first step to understanding HLH-25 and to determine the full expression of *hlh-25* in live animals, we generated a transcriptional reporter. This reporter consisted of the gene encoding the green fluorescent protein (GFP) under the control of the *hlh-25* promoter. Using DIC imaging and epifluorescent microscopy, I observed the transcriptional reporter expression in embryos, larvae, including dauer larvae, and adult animals. According to our microarray analysis, HLH-25 regulates many important genes needed for development. Thus, I expected to observe expression not only in embryos, but also in the larvae stages.

3.1.1 *HLH-25 is expressed in embryos*

I examined eggs *in utero*, immediately after fertilization, or after manual extraction from adult hermaphrodites. I first detected expression of *Phlh-25::GFP* after the ten-cell stage (Figure 7). This result is consistent with previous reported expression after the first embryonic Notch signaling event in *C. elegans* [45]. As the cells continued to divide mitotically, the expression expanded to all cells on the outer surface of the embryo. During normal gastrulation, which initiates at the 26-cell stage, wild-type animals progress from the bean stage to the tadpole stage, and precursor cells of the gut, germline, pharynx and body wall muscles and mesoderm migrate from the outer surface towards the interior of the embryo through the entry zone. I found that *hlh-25* expression correlated with migrating cells. Expression of *Phlh-25::GFP* during the bean

stage was located only on the outer surface of the embryo (figure 7 -8); however, during the comma stage the expression expanded. When the embryos reached the tadpole stage, I observed expression in the entry zone along the ventral side, in part of the anterior where the future buccal opening, or mouth, will form, and in the posterior region of the animal, where the future tail will form. Interestingly, I detected no embryonic expression immediately prior to hatching, a result which suggests that *hlh-25* is actively silenced at some stage during embryogenesis.

3.1.2 *HLH-25 is expressed in larval stages and in adults*

After hatching, if food is available, *C. elegans* go through four larval stages before reaching adulthood. During the first larval stage, L1, somatic gonad precursors start dividing. At the end of L1 stage, five of the eight types of motor neurons are made, one of which is the ventral nerve cord (VNC). The second larval stage is L2. During this stage, the somatic gonad precursors continue dividing and give rise to the distal tip cells that are required for gonad elongation. At the third larval stage, L3, the arms of the somatic gonad elongate, the spermathecae are formed, and sperm production begins. In the fourth and final larval stage, L4, sperm production stops and meiosis in the germline begins. Animals enter adulthood after exiting the L4 stage. During adulthood, the reproductive system is complete; oocytes are made and reproduction commences. I examined synchronized cultures of *C. elegans* hermaphrodites through all of the larval stages, and in adults, to characterize *hlh-25* expression. I did not detect expression from the *Phlh-25::GFP* reporter in L1 stage animals. However, during the L2, L3, and L4 stages, I observed expression in unidentified head and tail neurons, and in head and body-wall muscles (figure 9). I also detected expression in the midbody mechanosensory PDE neuron. In adults, I continued to detect expression in the head and tail neurons, but not in the

head or body-wall muscles (figure 10). I also detected weak expression in the ventral nerve cord of some adults.

3.1.3 *HLH-25 is expressed in the dauer stage*

The dauer stage is an alternative stage that promotes survival when *C. elegans* encounter a hostile environment. During this stage, animals grow a thicker cuticle that seals the buccal cavity, slows metabolism and inhibits reproduction. To complete the expression profile of HLH-25, I also examined *Phlh-25::GFP* expression during the dauer stage. I detected strong *Phlh-25::GFP* expression in the head neurons and VNC of every dauer animal, and occasionally, in the synaptic branches between VNC and the dorsal nerve cord (Figure 11-12). Because the transgene expression was mosaic and highly variable, I was unable to identify which cell bodies of the VNC were expressing the reporter.

3.2 *Phenotypes of hlh-25 animals*

To further characterize HLH-25, I sought to identify phenotypes of *hlh-25 animals*. Because *hlh-25* is expressed in embryos, neurons and muscles, I believe these results are indicative of a possible role for HLH-25 in directing embryogenesis and body movement. Thus, I examined *C. elegans* embryonic lethality, quantity of eggs laid, and movement.

3.2.1 *HLH-25 affects embryogenesis*

Embryos take approximately ten hours to develop following fertilization, going through a series of division and folding before hatching. *hlh-25* is expressed in the embryos in response to Notch signaling, and expression continues throughout embryogenesis but ceases just before

hatching. As a measure of the requirement for HLH-25 during embryogenesis, I examined embryonic viability in mutant and wild-type animals.

Homozygous deletion of *hlh-25* gave rise to embryonic lethality. I observed 54% lethality in *hlh-25* mutant embryos versus 14% embryo lethality in wild types embryos (Figure 13a), representing a 3.79 fold increase (P-value = 6.32E-14). These results correlate with a previous genome-wide RNAi study which showed a 40% embryonic lethality for animals subjected to *hlh-25* RNAi. Deletion of *hlh-25* also resulted in a reduced quantity of eggs laid. *hlh-25 animals* laid a total average of 208 eggs, while wild-type animals laid an average of 249 (P-value = 0.0565) (Figure 13b).

3.2.2 *hlh-25 animals have an increased unfertilized egg laying behavior*

Wild-type *C. elegans* hermaphrodites produce approximately 300 sperm between L3 and L4 stages. As the animals progress through L4, they cease sperm production and switch to producing oocytes at late L4/early adulthood. As adults, fertilization is initiated when mature oocytes are fertilized by sperm that reside in the spermatheca. Normally at 20°C, the onset of fertilization and egg production begins at approximately 65 hours after hatching (~ 3days) and continues until approximately 128 hours after hatching (~5.5 days) [93, 94]. Since oocyte production is not the limiting factor, wild-type hermaphrodites that run out of sperm lay some unfertilized oocytes, usually at day six. If plenty of food is present hermaphrodites may lay an average of 31 unfertilized eggs per worm

I found that *hlh-25 animals* lay more unfertilized oocytes than wild-type animals, and that the production of unfertilized oocytes extended much further than in wild-type animals. As shown in figure 14, both wild-type and *hlh-25 animals* begin to lay unfertilized oocytes as early

as the second day of egg laying; however, *hlh-25* animals lay significantly more unfertilized oocytes each day. *hlh-25* animals laid an average of 133 unfertilized oocytes from egg laying day 2 (4 days after hatching) to egg laying day 13 (15 days after hatching), while wild-type animals laid an average of 27 unfertilized oocytes and stopped laying unfertilized eggs completely by egg laying day 8 (10 days after hatching).

3.2.3 *hlh-25* animals have a slower movement rate

Because the HLH-25 expression pattern is seen in both neurons and in muscles, and because a number of the genes predicted to be HLH-25 targets are associated with GO term locomotion, I hypothesized that that HLH-25 could be required for normal body movement.

hlh-25 animals have a normal sinusoidal movement, and their forward and reversal movement on solid media appears normal [95]. Although I did not assay reversal frequency, *hlh-25* animals appear to have the same backing up phenotype as wild-type animals. Therefore, I used two different assays to detect more subtle movement defects in *hlh-25* animals at dauer and L4 stages: locomotion on solid media and thrashing in M9 solution. In the locomotion assay, I counted the number of body bends per minute, and in the thrashing assay, I counted each lateral movement over a period of one minute. The results for both assays were generally the same: *hlh-25* animals moved slower than wild type animals at both developmental stages. Specifically, the *hlh-25* L4 stage and dauer stage animals moved with an average of 28 and 21 fewer body bends-per-minute than wild-type animals, respectively. Likewise, *hlh-25* animals thrashed with an average of 23 and 31 fewer bends per minute than wild-type animals at the L4 stage and the dauer stage, respectively (Figure 15-16).

3.2.4 *HLH-25 affects organization and formation of the germline*

The hermaphroditic reproductive system consists of two, U-shaped gonad arms connected to a uterus and one spermatheca on each side (figure 3). In wild type animals, oocyte production starts in the distal tip of the gonad arm and continues throughout the syncytial section, where the multinucleated mass of cytoplasm has not separated into individual cells. In the syncytial section, the germline nuclei go through mitosis and meiosis while being surrounded by the cytoplasmic membrane. Oocytes start to compartmentalize when they reach the loop of the gonad, and achieve full compartmentalization as they move closer to the spermatheca. In wild type animals, fully cellularized (i.e. separated into distinct cells) oocytes have the appearance of squared compartments with a narrow gap.

A number of the HLH-25 target genes are known to affect either mitotic or meiotic cell division, including *ran-1*, *mex-5*, *mex-6*, and *pos-1*. Often animals with mutations in these genes show germline abnormalities that include abnormally shaped or endomitotic oocytes. I examined the gonad arms of young adult hermaphrodites and found that in approximately 75% of *hlh-25* animal; at least one gonad arm contained irregularly shaped oocytes. After compartmentalization, the oocytes were more round or tear-drop in shape, and were not tightly packed (Figure 17).

3.2.5 *HLH-25 has a role in Early Embryonic Cell Division*

The *C. elegans* lineage in early embryonic development begins with P₀ which divides in AB and P₁. AB further divides into Aba and ABp; P₁ further divides into EMS and P₂. These first divisions during embryogenesis require the presence of some of the HLH-25 targets like *ran-1*, *mex-5*, *mex-6*, and *pos-1*. However, *hlh-25* is not expressed until ABp and EMS

granddaughters in response to the Notch signaling [45]. Since *C. elegans* germline cells have the ability to silence transgene arrays [96], I wondered if HLH-25 is needed for early cell divisions even though I did not observe expression at early stages.

To determine whether HLH-25 is required for early cell division, I compared the first five embryonic cleavages from wild type and *hlh-25* animals using α -tubulin::GFP. Images were taken every three minutes to create a time lapse video. In vivo time-lapse imaging of seven different embryos reveals that *hlh-25* animals have similar first embryonic cleavages to wild types (Figure 18).

3.3 *HLH-25 transcriptional network*

I used two different approaches to validate the HLH-25 transcriptional network: reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) and transcriptional reporters. RT-qPCR allows quantification of mRNA levels in whole animals. Transcriptional reporters allow temporal and spatial measurement of the promoter activity in live animals.

3.3.1 *Confirmation of HLH-25 target genes by RT-qPCR*

Our previous microarray analysis shows that HLH-25 regulates 634 genes of which 510 were up-regulated and 124 were down-regulated. Two of the GO term annotations assigned to these genes were “embryonic development ending in birth or egg hatching” and “post-embryonic development”. These results suggest that HLH-25 plays an important role in embryonic and nematode larval development.

The microarray analysis was done using young adult worms, which may have contained developing embryos in their uterus. Since the microarray analysis included genes necessary for embryonic and larval development, I wondered if HLH-25 regulated genes in both embryos and in larval/adult animals. To address this, I monitored the change in RNA levels in embryos and L4 stage larvae. The L4 stage was used to validate the microarray results, while the other stages were selected to correlate with the *hlh-25* gene expression profile. For some genes, I also measured expression in dauer larvae. I selected 12 genes to validate, and for all 10 of them, in the L4 stage expression, they were up-regulated or down-regulated to the same extent as indicated by the microarray analysis (Figure 19, table 2). As described below, the selected genes are important during embryogenesis and at other stages in the life cycle.

The gene *ran-1* encodes the *C. elegans* Ran GTPase ortholog [97] which, among other proteins, comprises the Ran GTPase system. The GTPase cycle is known to regulate transport of proteins across the nuclear envelope [98, 99], chromosome positioning and nuclear envelope assembly [100]. Previous studies have shown that during embryogenesis *ran-1* knock-out causes improper mitotic spindle formation, irregular chromatin structures, and fission of the pronucleus during meiosis [97]. This gene was up-regulated in L4-stage animals, but was down-regulated in embryos.

Genes *mex-5*, *mex-6*, and *pos-1* encode two CCH-finger proteins that are important for the establishment of embryonic polarity [101]. These three genes are maternally transcribed. MEX-5 and MEX-6 establish soma/germline asymmetry in the early embryo. POS-1 is needed for fate specification of germ cells, pharynx, and intestine during embryogenesis [102]. These three proteins are needed for the first two cell divisions to occur properly [103]. Mutation to any

of these genes results in embryonic lethality [77, 103-108]. These three genes were up-regulated in L4 stage animals and down-regulated during the embryonic stage.

The genes *spn-4*, *cpg-2*, *emb-9*, *fat-2*, *sca-1*, *lin-37*, *lin-54* are necessary for embryogenesis. Mutations to all of these genes results in embryonic lethality. EMB-9 is essential for embryonic morphogenesis, gonad elongation, and for larval development; [109]. These genes were up-regulated at the L4 stage. *spn-4*, *cpg-2*, *emb-9*, *fat-2*, *sca-1* were down-regulated during the embryo stage.

The genes *acs-2*, *mtl-1*, and *daf-18* are necessary for homeostasis and stress adaptation [64, 110, 111]. Mutations to either *acs-2* or *mtl-1* results in reduced brood size [112, 113]. *acs-2* and *mtl-1*, were down-regulated in the microarray, but up-regulated in my RT-qPCR results at the L4 stage. *daf-18* was up-regulated at all stages tested.

3.3.2 Confirmation of HLH-25 target genes by Transcriptional Reporters

As a second step to validate *hlh-25* transcriptional network, I generated transcriptional reporters for five randomly selected HLH-25 target genes with the goal of establishing their expression pattern in the presence and absence of *hlh-25*. I used the green fluorescent protein gene fused to the promoters of these selected genes to analyze *hlh-25* dependent expression in live animals. The genes chosen were selected because they were strongly up-regulated or down-regulated in *hlh-25* animals, according to the gene expression microarray, and because they had potential roles in development. Genes *imp-2*, *ima-1*, *pqn-95*, when mutated, result in embryonic lethality [106, 107]. In addition to acting within the insulin signaling pathway, the genes *akt-2* and *daf-18* have antagonistic affects on life span [114-116].

Although, I successfully cloned the transgenes *Pimp-2::GFP*, *Pdaf-18::GFP*, *Pima-1::GFP*, *Ppqn-95::GFP*, and *Pakt-2::GFP*, I did not obtain stably transgenic lines. The constructs were linearized, blunt ended, and injected as a low copy number to prevent transgene silencing [83]. I learned, practiced and successfully injected the constructs into the animals. As a result, I obtained F1 progeny that carried the injection marker and displayed the “roller” phenotype; however the injection marker was never transmitted to F2 progeny. Therefore, I obtained strains containing *akt2::GFP* and *daf-18::GFP* (fsEx84) through other sources. Transgenic lines carrying *akt2::GFP* and *daf-18::GFP* (fsEx84) were kindly provided Dr. Gary Ruvkun from Harvard Medical School and Dr. Solari Florence from Claude Bernard University Lyon.

To analyze *hlh-25* dependent expression of *akt-2* and *daf-18*, I mated *hlh-25* males with hermaphrodites carrying these constructs. Homozygous deletion of *hlh-25* was verified by PCR. I compared the transgene expression in these newly made strains in expression to transgenic wild-type animals at larval and adult stages.

3.3.2.1 Validation of *akt-2* as a target of HLH-25*akt-2::GFP*

As shown in figure 20, *akt-2::GFP* is expressed in wild-type animals in head and tail neurons, the ventral nerve cord, muscles, spermatheca, and in the head posterior bulb. I did not detect expression in the embryos of adult hermaphrodites. In *hlh-25 animals*, the spatial *akt-2::GFP* expression was indistinguishable from the pattern in wild-type animals. We were not able to reliably quantify the GFP levels to determine if there was a detectable difference in the level of expression.

3.3.2.2 *daf-18* expression increases in the absence of HLH-25

In wild-type animals, *daf-18::GFP* expression was first detected at the pretzel stage and continued after hatching, throughout adulthood. The post-embryonic expression was localized only to the head neurons (Figure 22). In *hlh-25* animals, both the spatial distribution and the level of *daf-18::GFP* expression increased significantly, and correlated with cells or tissues that were found to express the *Phlh-25::GFP* in the head and tail neurons, muscles throughout the body, pharyngeal muscles, spermatheca and ventral nerve cord (Figure 21).

3.3.3 Genetic validation that *daf-18* is a target of HLH-25

The results presented thus far suggest that HLH-25 transcriptionally represses *daf-18*. Based on these data, I hypothesized that *hlh-25* animals would have phenotypes that are similar to animals that overexpress *daf-18*. Animals with loss of function alleles of *daf-18* have shorter lifespans and are defective in dauer formation [114, 115]. Likewise, animals that overexpress *daf-18* have longer lifespans, are more prone to form dauers, and have difficulty exiting the dauer stage [65, 117]. Therefore, I tested the lifespan and dauer exit phenotypes of *hlh-25* animals.

3.3.4 HLH-25 animals have a longer life span

I measured the mean life span of wild-type and *hlh-25* animals at 20 °C. As indicated in figure 23, *hlh-25* animals have a median life span of 19 days, while wild-type animals have a life span of 16 days (table 3). The extended lifespan of *hlh-25* animals is similar to the

previously reported median lifespan of 17.4 days for *daf-18;daf-2* animals expressing an extrachromosomal copy of *daf-18* [117] .

3.3.5 *HLH-25 is necessary for proper dauer exit timing*

When starvation-induced dauer larvae are placed into favorable growth conditions, they take approximately 60 minutes to commit to exit the dauer state at 25° C and about ten hours to molt into the L4 stage [87, 118-120]. Within 60 minutes, the first biological sign of dauer exit is the increase in fat accumulation or lipophilicity, and pharyngeal pumping starts after approximately 3 hours [121]. Between nine and twelve hours after the decision to exit dauer, vulva formation commences and is visible as the vulval crescent. Finally, between 12 and 24 hours, recovering dauers begin to create oocytes and fertilization commences.

In order to determine if dauer recovery depends on HLH-25 regulation of *daf-18*-expression, I compared the dauer recovery phenotypes of wild type animals with: *daf-18* mutants, animals over-expressing *daf-18* (*daf-18^{GOF}*) or *hlh-25* animals. Because deletion of *hlh-25* increases *daf-18* expression, I expected *hlh-25* animals and *daf-18^{GOF}* animals to recover similarly.

Because the time that it takes for dauer to recover varies and depends on their age and the environmental factor that caused them to become dauers [87, 118-120], I induced dauer formation under the same conditions for all strains simultaneously, and used three day old dauers for the assays.

In my dauer recovery assay, wild-type animals took an average of 12 hours (80%) to reach L4. As expected, dauer recovery was similar in *daf-18* animals but was longer in *daf-18^{GOF}* animals. At 12 hours, 65% of the *daf-18* mutants, but only 10% of the *daf-18^{GOF}* animals,

had reached L4. Interestingly, the dauer recovery timing of the *hlh-25* mutant animals was very similar to the *daf-18*^{GOF}. After 12 hours of recovery only 35% of the *hlh-25* mutant animals reached L4. To rescue the wild type phenotype, I reintroduced *hlh-25* into *hlh-25* animals (*hlh-25R*). Animals carrying *hlh-25* as an extrachromosomal array were able to recover similarly to the wild type animals. They took an average of 12 hours to reach L4 (Figure 24, 26).

3.3.6 *DAF-18* does not affect movement

Although a role for *daf-18* in locomotion and movement has not been previously described, I wondered if the locomotion defect of *hlh-25* animals is dependent on *daf-18*. Therefore, I compared the locomotion and thrashing of *daf-18* mutants to wild-type animals as described above in section 3.2.3. Our results showed that *daf-18* animals move at a similar rate as wild-type animals, and thrash slightly, but not significantly, more than wild-type with an average of fifteen more lateral movements than wild type per minute (Figure 25).

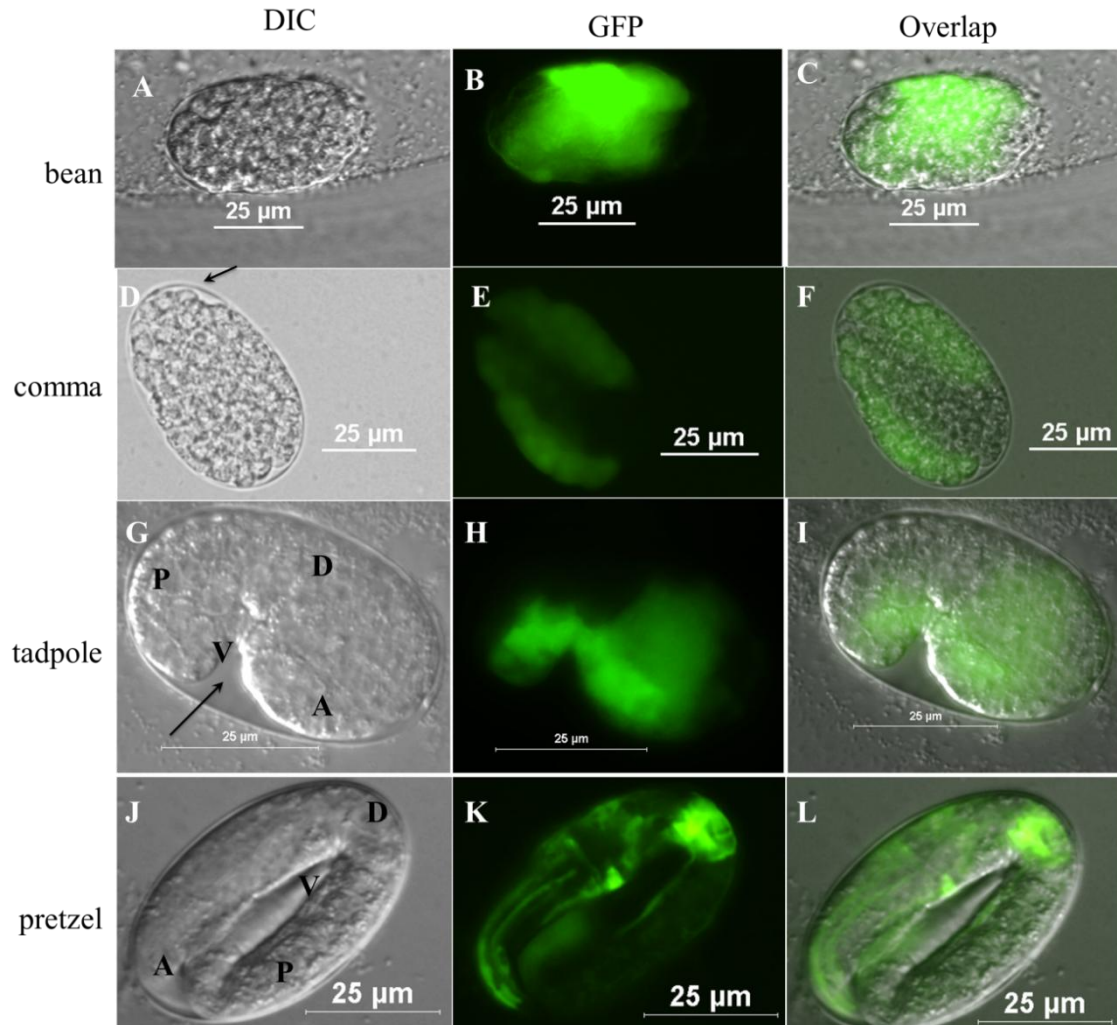


Figure 7 Embryonic Expression of *Phlh-25::GFP*.

The embryo developmental stage is named on the left. The entry zone is marked with an arrow. The plane of the embryo is marked as A: anterior, P: posterior, D: dorsal, V: ventral

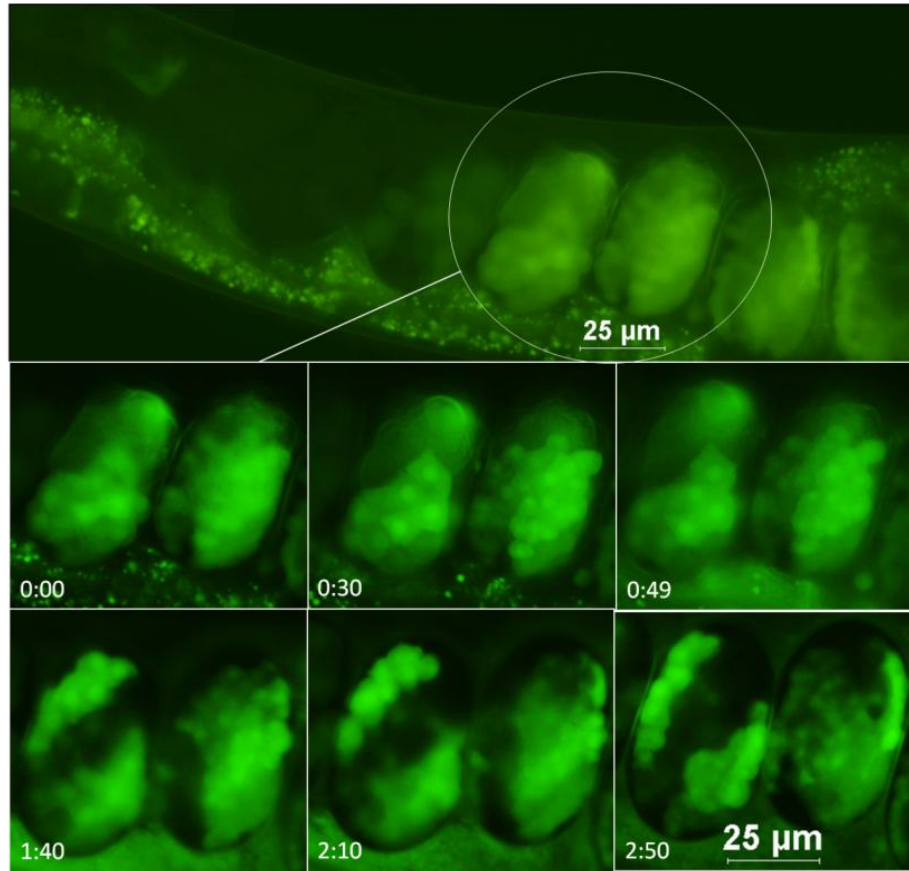


Figure 8 Embryonic Expression of *Phlh-25::GFP*.

This figure represents stills from a time lapse video of *Phlh-25::GFP* expression during progression from bean to comma stage while still in the uterus.

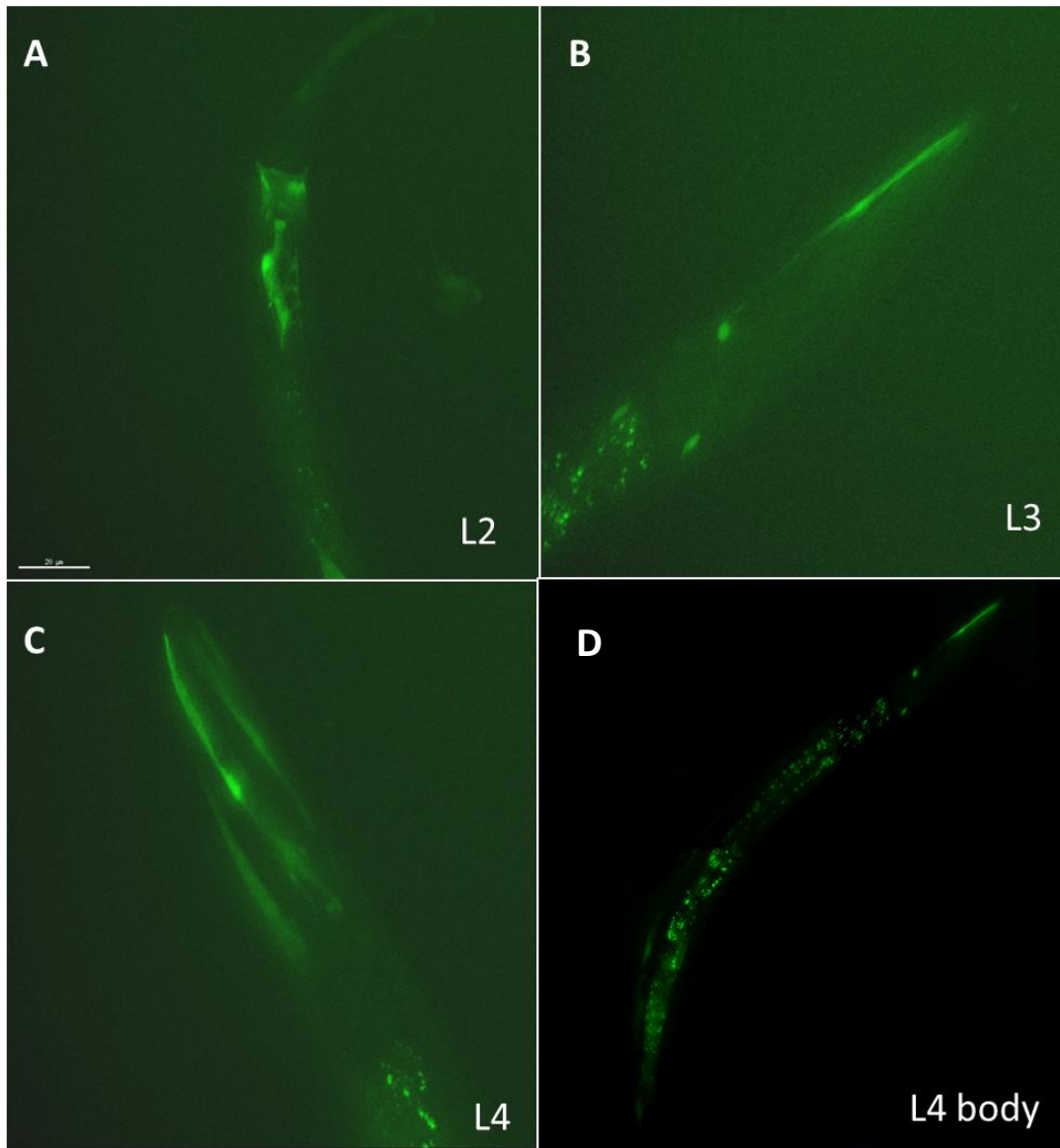


Figure 9 Larvae Expression of *Phlh-25::GFP*.
Expression of *Phlh-25::GFP* is seen (A) in tail neuron, (B-D) head muscles and (D) body muscles
Larvae stages are specified at the right bottom of each image. Bar marks 20 µm.

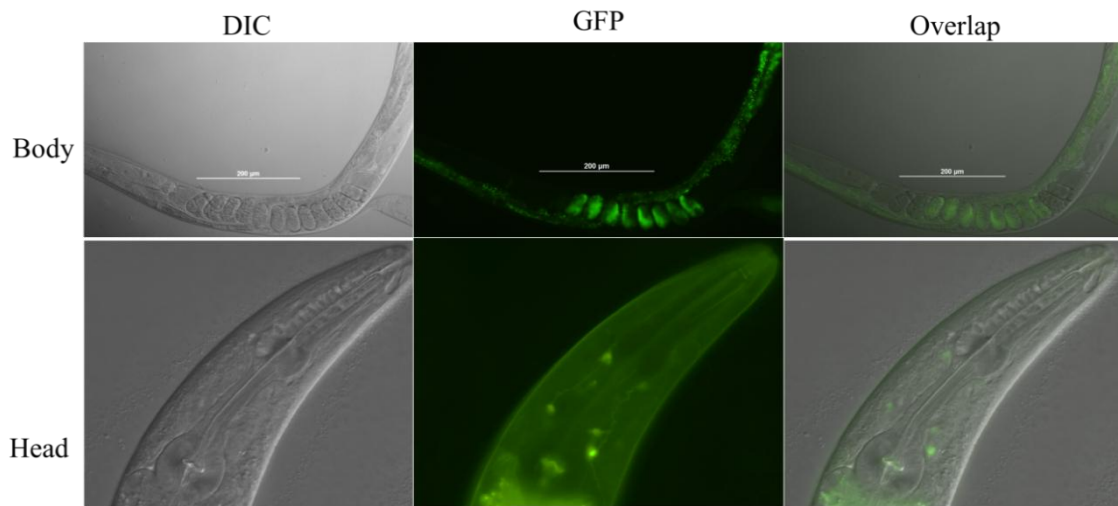


Figure 10 Adult Expression of *Phlh-25::GFP*.
HLH-25 expression observed in unidentified head neurons and embryos inside the adult hermaphrodite. Bar marks 25 µm.

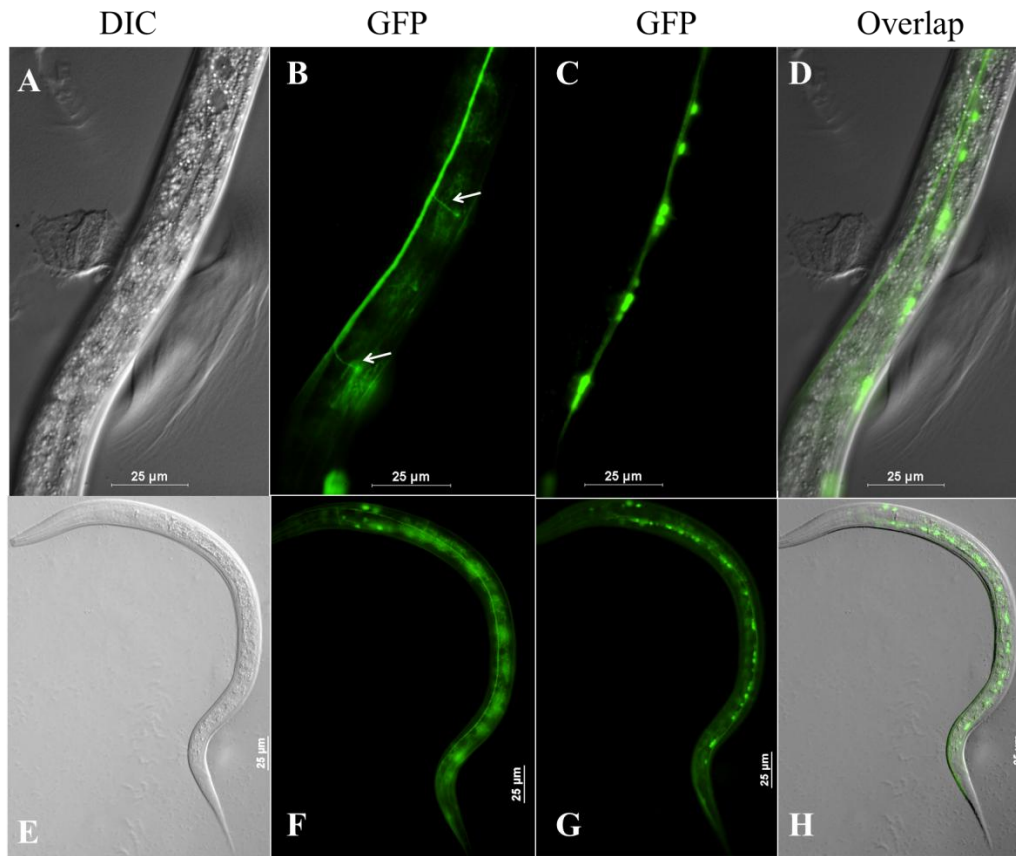


Figure 11 *Phlh-25::GFP* expression in dauer.

Expression is seen in the VNC and DNC. This image contains two focal plains of GFP expression in the same animal. (B) and F show the dorsal nerve cord; (C and G) show the ventral nerve cord. Arrows indicate the synaptic branches between VNC and the dorsal nerve cord.

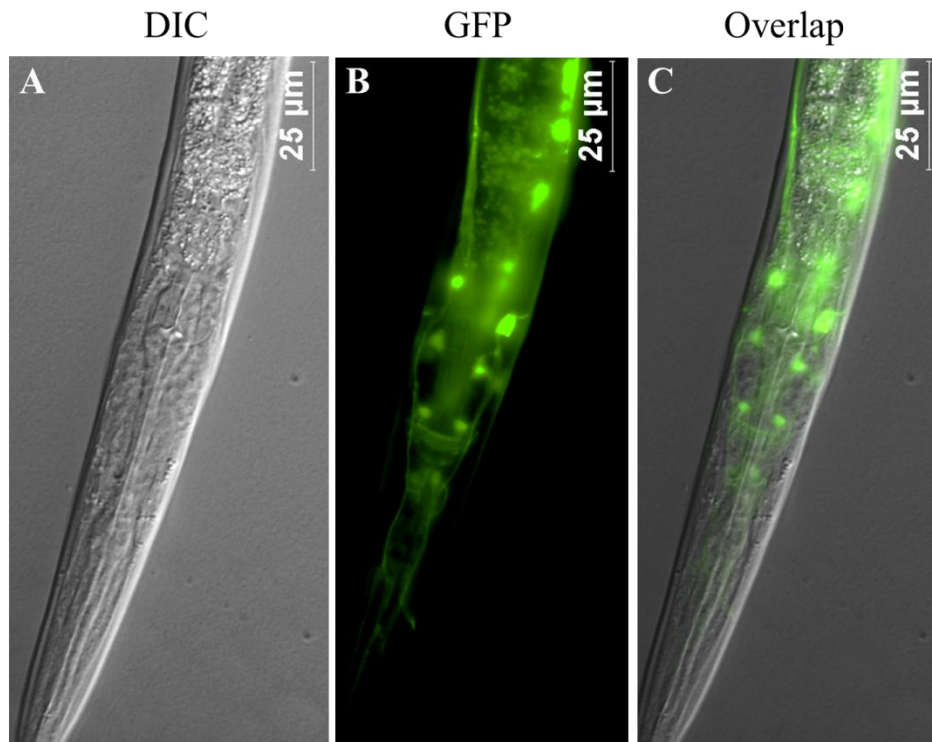


Figure 12 *Phlh-25::GFP* expression in the dauer head.

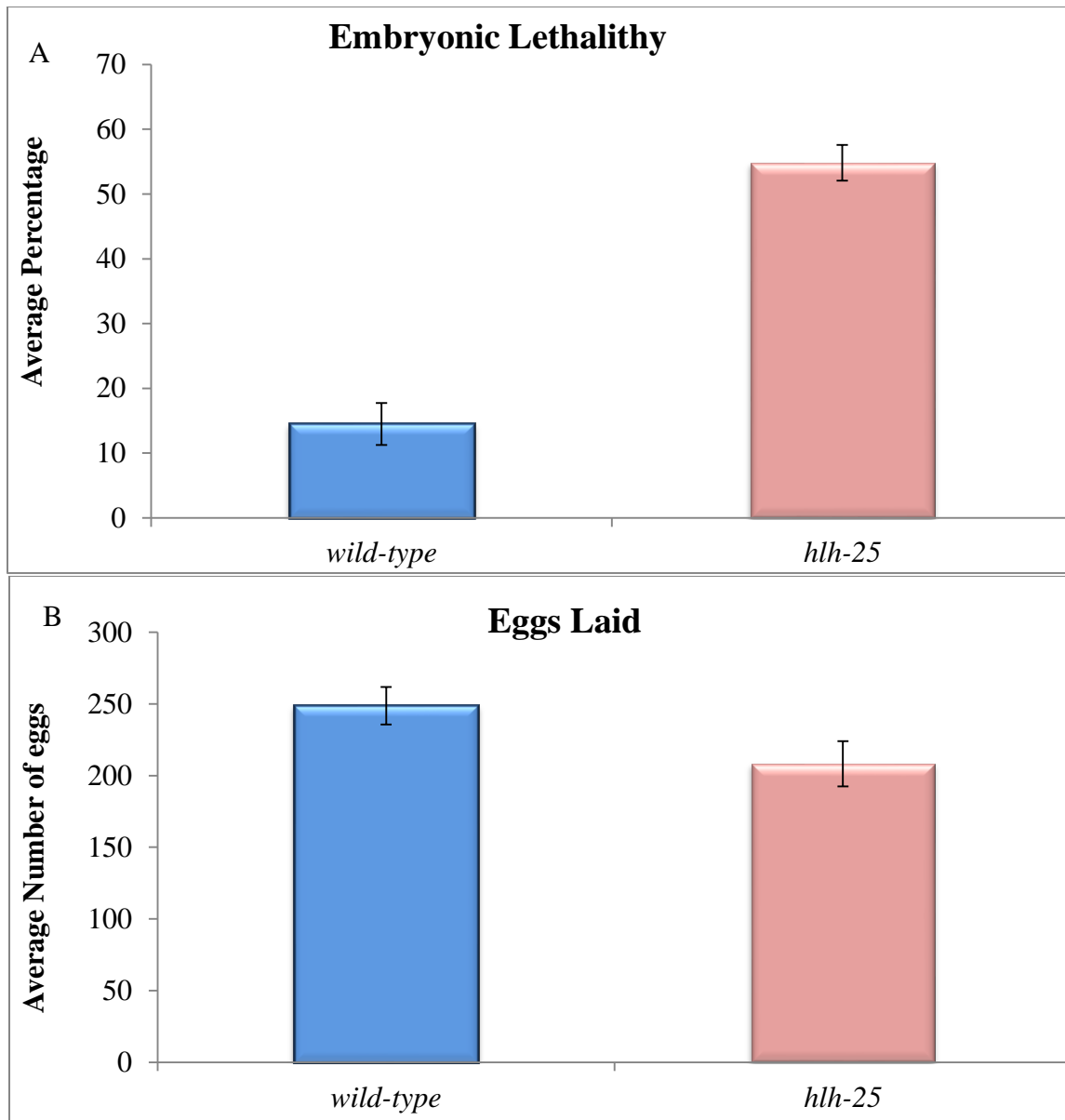


Figure 13 HLH-25 affects embryogenesis.

(A) *hlh-25* mutants have a 64% embryonic lethality versus a 14% embryo lethality in wild type embryos (P-value = 6.32E-14). (B) Deletion of *hlh-25* also resulted in a reduced quantity of eggs laid. *hlh-25* animals laid a total average of 208 eggs, while wild-type animals laid an average of 249 (P-value = 0.0565). Error bars represent SEM.

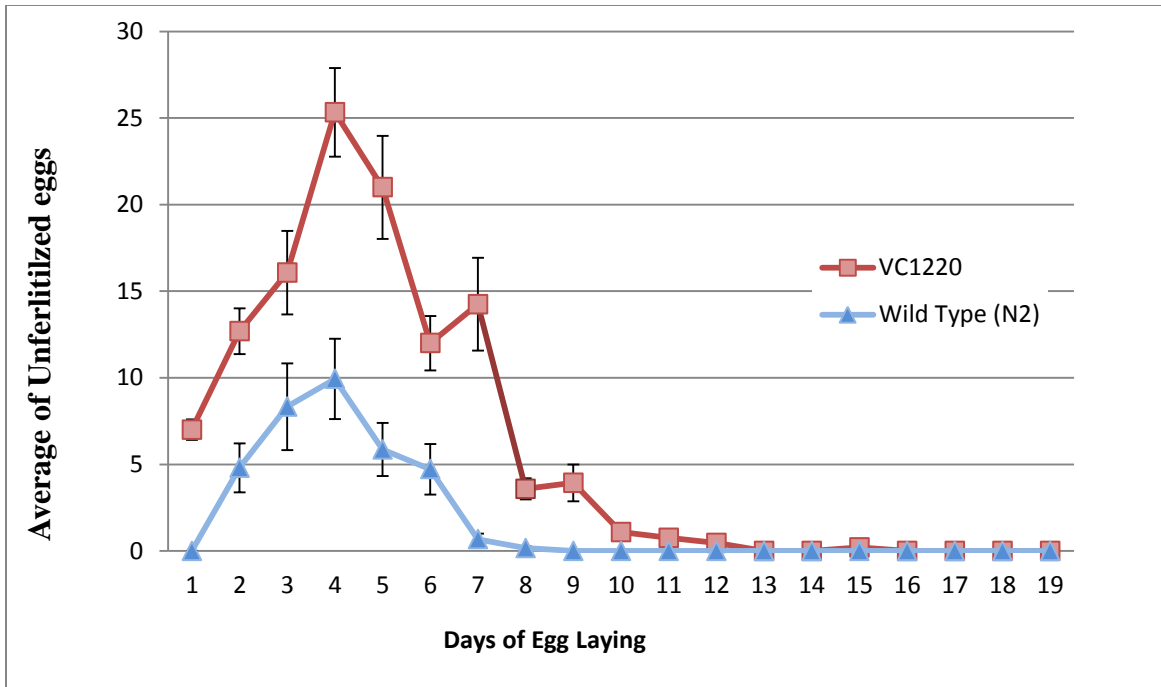


Figure 14 Unfertilized Egg Laying Behavior of *hllh-25* compared to wild-type.

Day "1" was assigned for when the egg-laying period started. *hllh-25* animals laid an average of 133 unfertilized oocytes while wild-type animals lay laid an average of 27 unfertilized oocytes. Error bars represent SEM

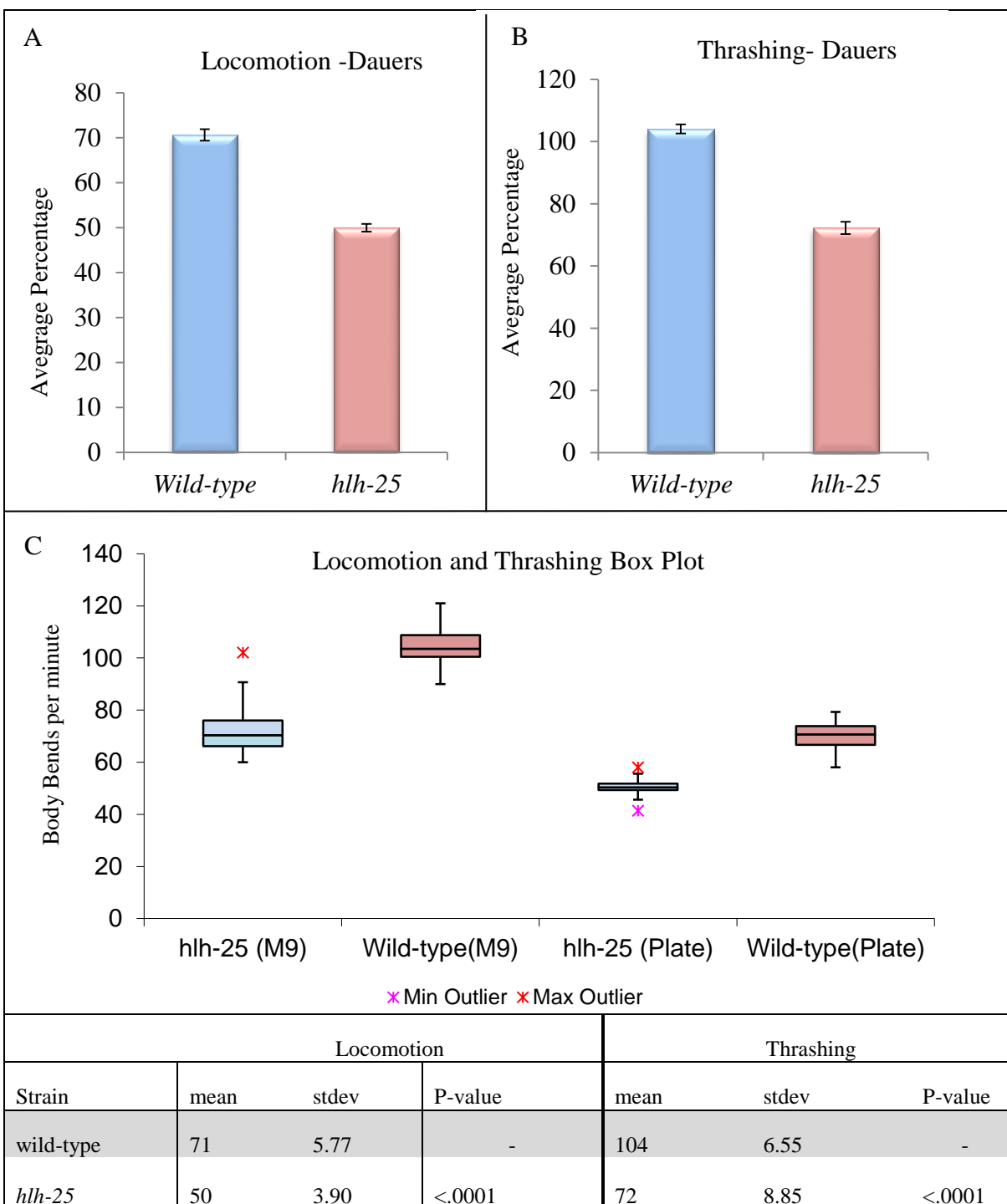


Figure 15 Dauer Mobility Assay

Error bars represent SEM. (C) The band near the middle of the box represents the median. Outliers are indicated with an asterisk.

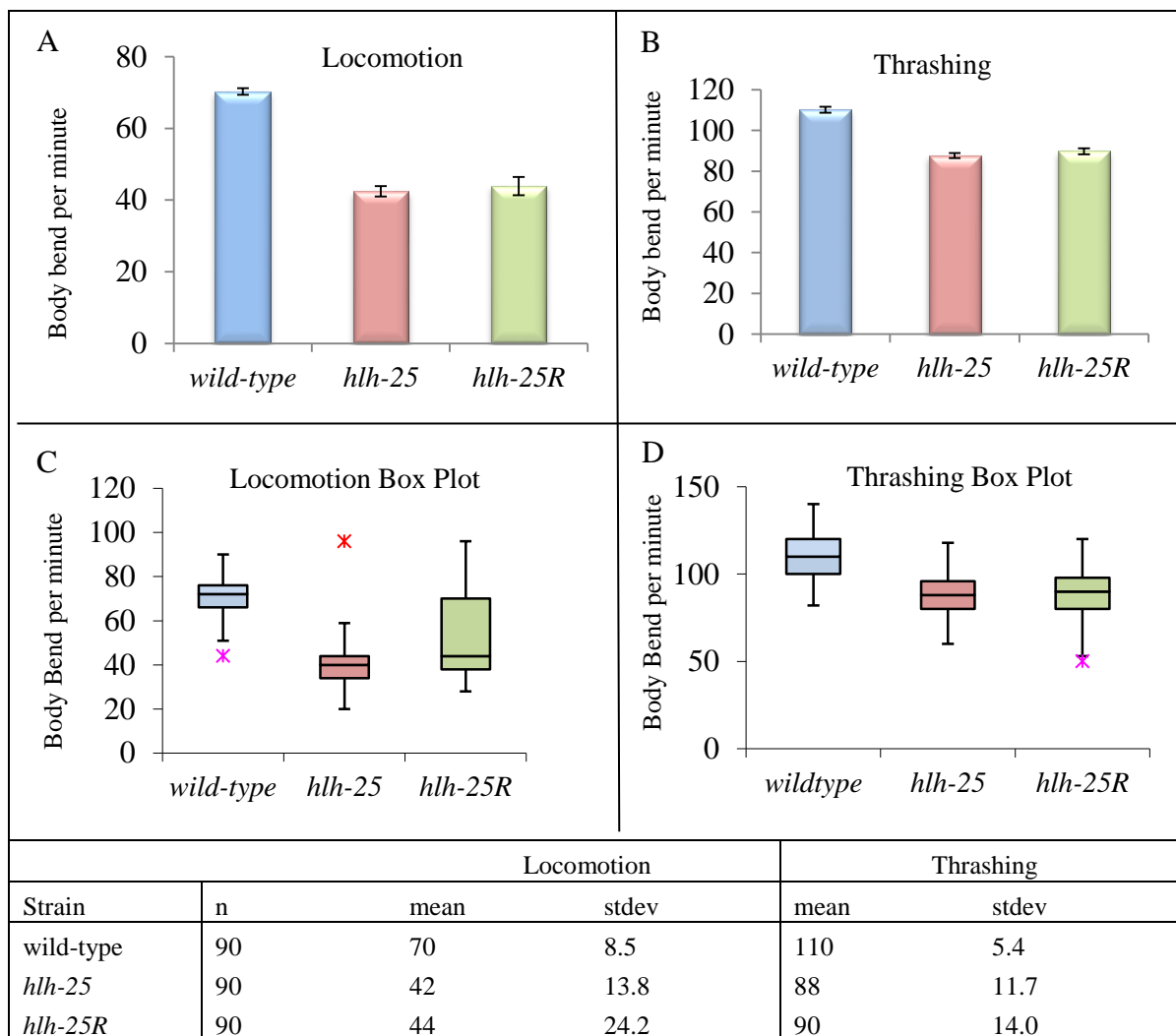


Figure 16 L4 Locomotion and Thrashing

Error bars represent SEM. (C-D) The band near the middle of the box represents the median.

Outliers are indicated with an asterisk.

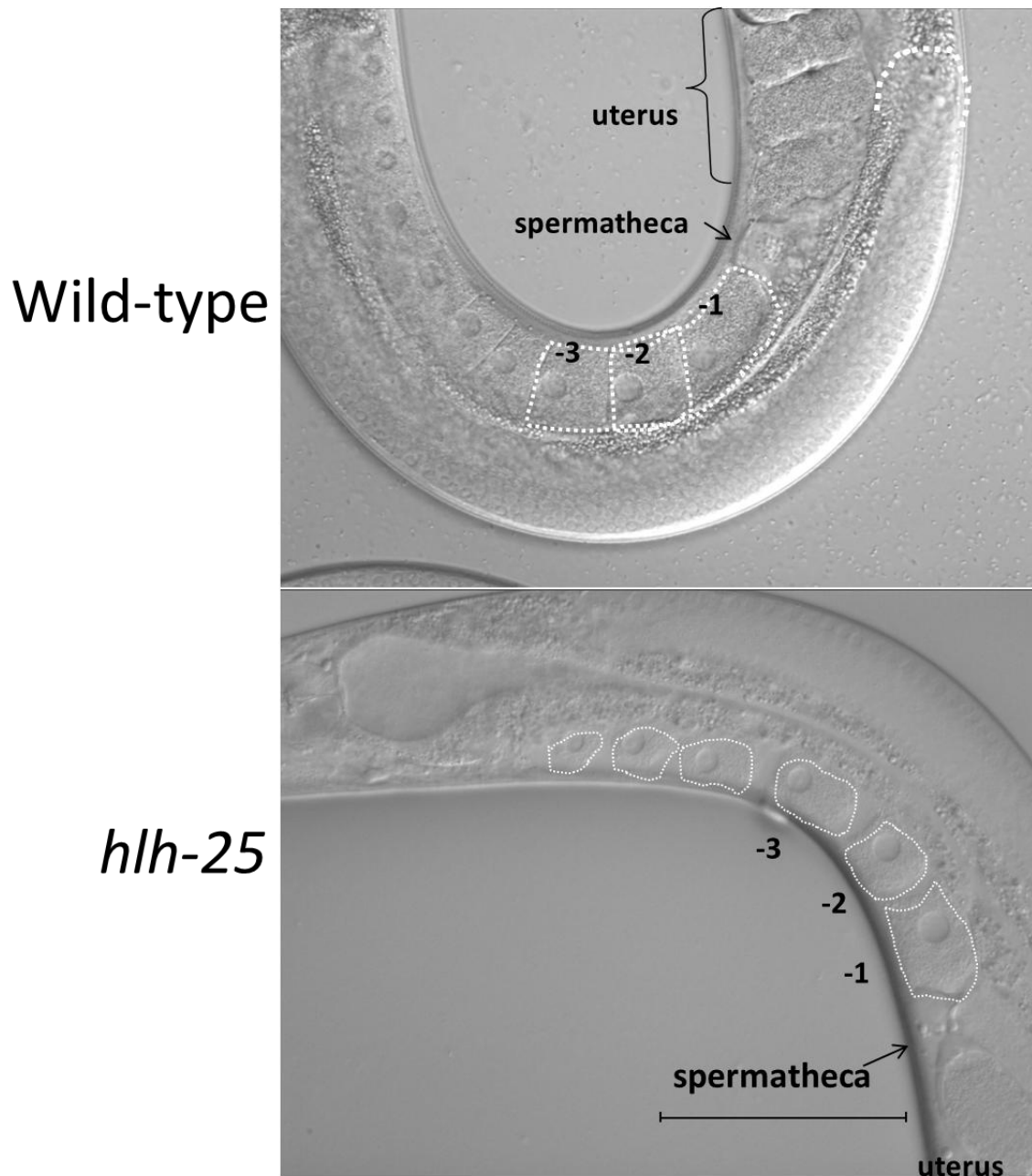


Figure 17 *hlh-25* depletion causes irregular shape of oocytes
 HLH-25 affects organization and formation of the germline. *hlh-25* animals contained fewer and irregularly shaped oocytes. The oocyte adjacent to the spermatheca is numbered -1 and the one farther away are -2, -3. Scale Bar: 100 μ m

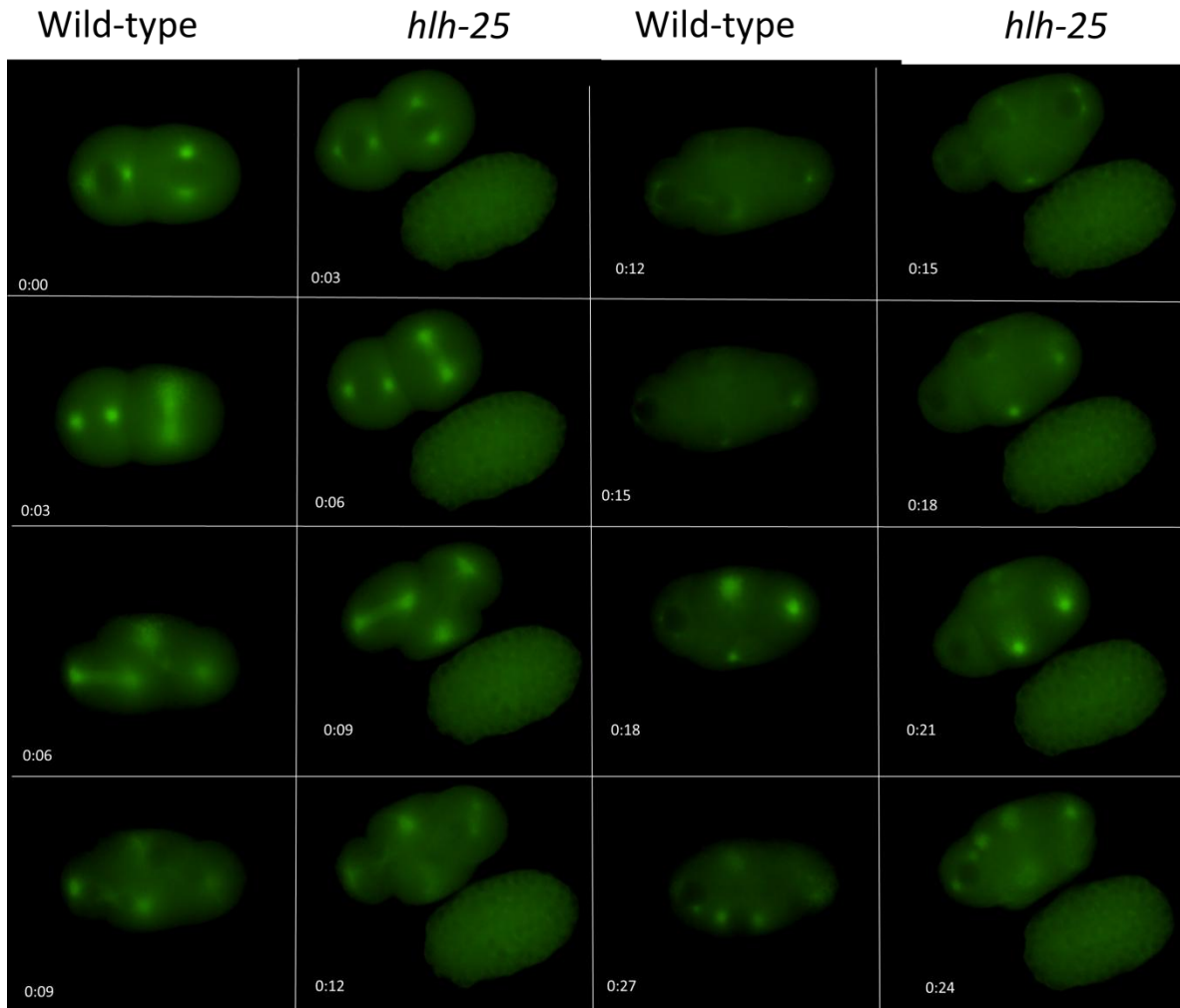


Figure 18 Early Embryonic Cell Division Time-lapse video.

In vivo time-lapse still image of α -tubulin::GFP (which expresses spindle formation) in wild-type and *hlh-25* embryos.

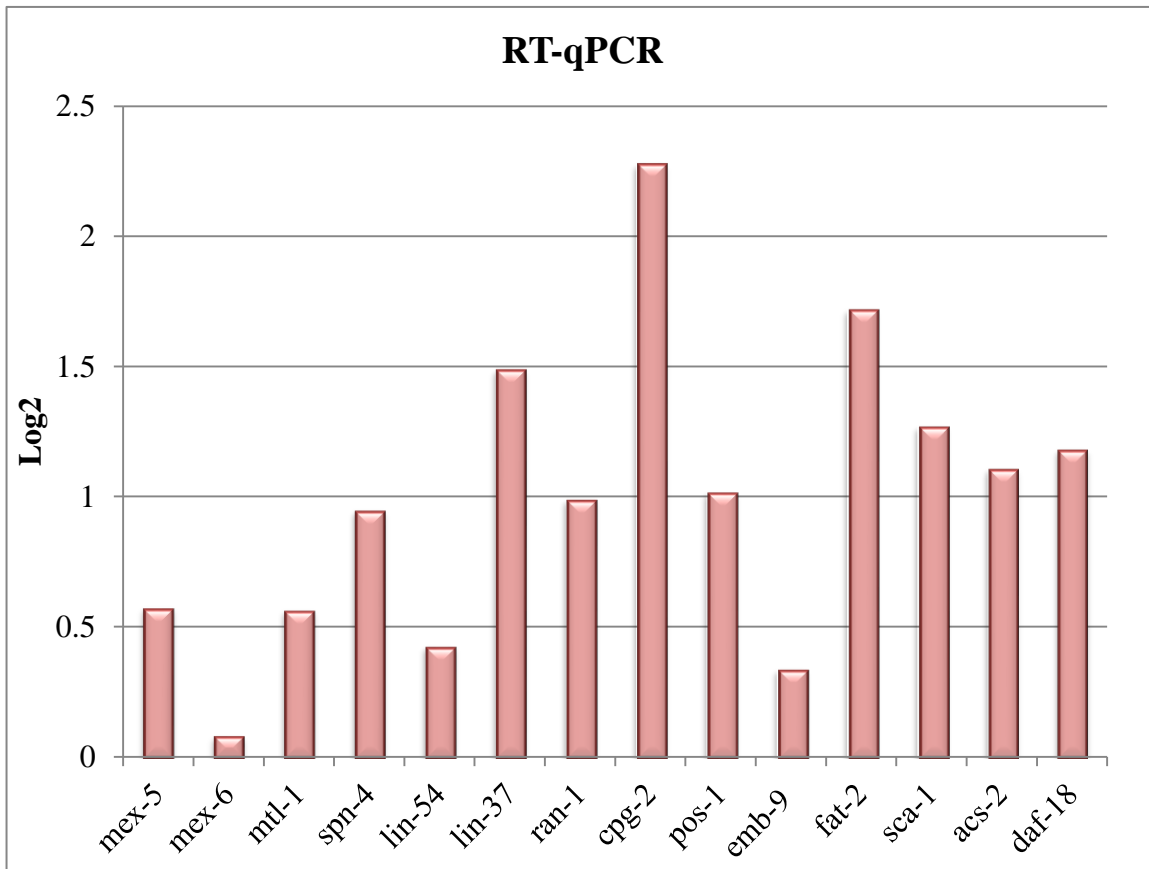


Figure 19 HLH-25 targets expression during L4 stage.

Graph represents relative gene expression levels. Transcript levels were quantified by RT-qPCR. Data are expressed as log₂ of fold change.

Table 2 RTqPCR Log₂ Comparison of *hlh-25* with wild-type

gene	Log ₂		
	embryo	L4	dauers
<i>mex-5</i>	-0.140859213	0.574520826	-
<i>mex-6</i>	-0.731609333	0.079969371	-
<i>mtl-1</i>	-1.438593174	0.56487424	-
<i>spn-4</i>	-0.755692286	0.947934039	-
<i>lin-54</i>	-	0.425533103	-
<i>lin-37</i>	-	1.48913987	-
<i>ran-1</i>	-0.284879656	0.989225894	-0.558373223
<i>cpg-2</i>	-1.768954799	2.277367657	-1.591294232
<i>pos-1</i>	-0.772183582	1.017858268	0.070098511
<i>emb-9</i>	-0.355788189	0.336756657	0.143902443
<i>fat-2</i>	-0.087547834	1.718457624	-1.883574631
<i>sca-1</i>	-0.244550149	1.270412924	-0.167984707
<i>acs-2</i>	-2.427682984	1.107159331	-1.373822577
<i>daf-18</i>	1.389777859	1.181618478	0.081026597

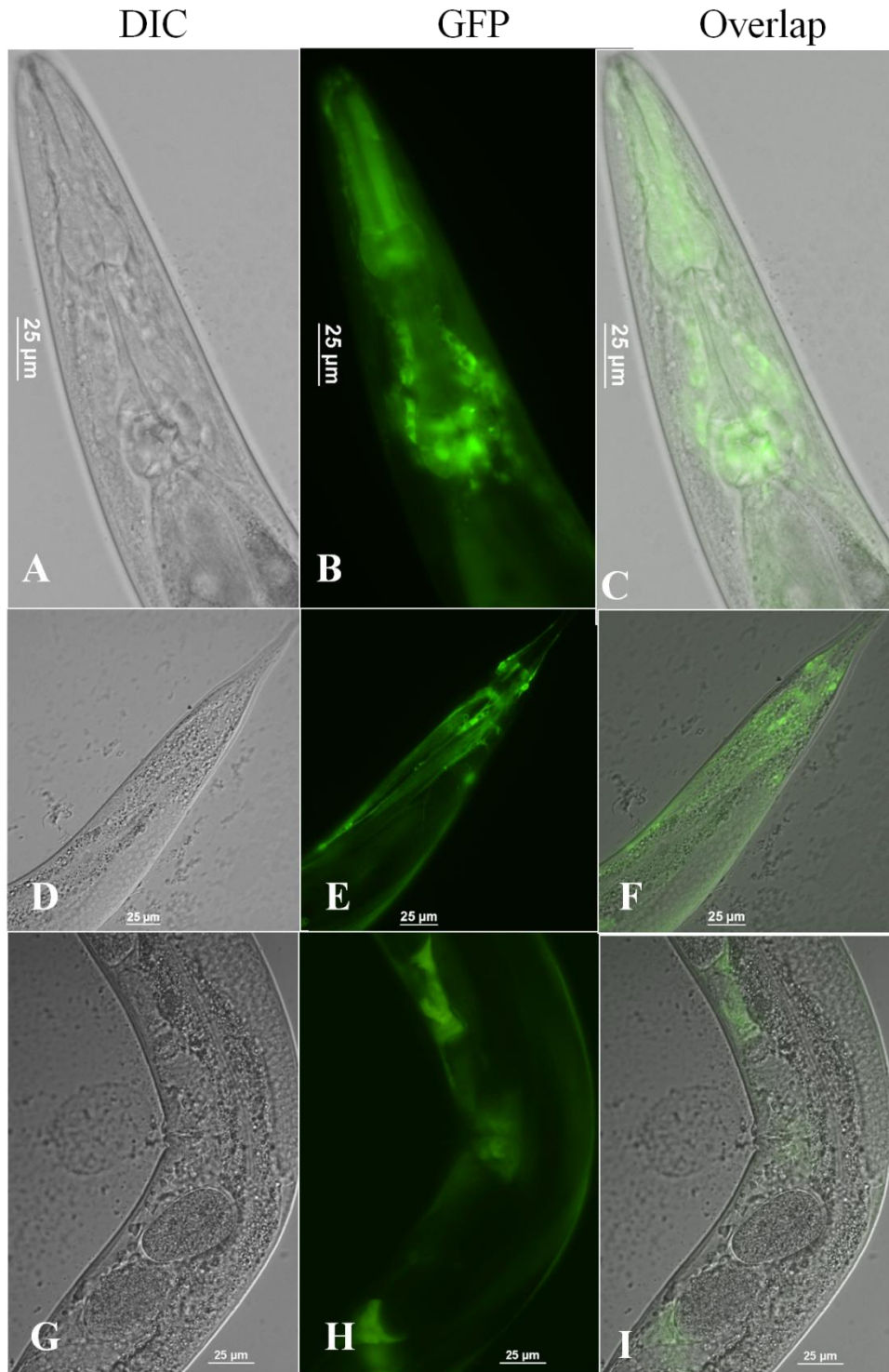


Figure 20 *akt-2::GFP* expression.

akt-2::GFP is expressed in wild-type animals in (A-C) the head and (D-F) the tail neurons, the (G-I) the ventral nerve cord, muscles, spermatheca, and in the (A-C) the head posterior bulb. In *hlh-25* animals, the spatial *akt-2::GFP* expression was indistinguishable from the pattern in wild-type animals (pictures not shown).

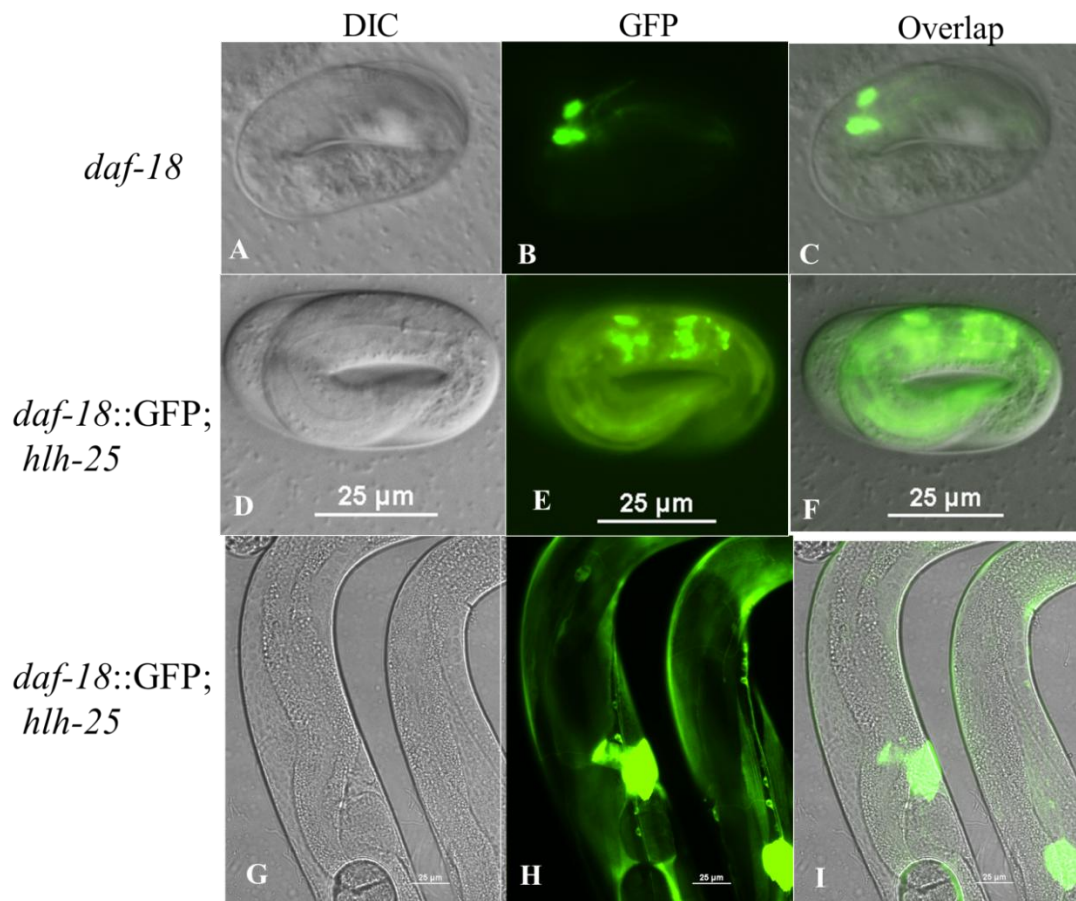


Figure 21 *daf-18* expression changes

In wild-type animals, *daf-18::GFP* expression was first detected at (A-C) the pretzel stage, but in a larvae, the expression was only seen in the head (figure 22 a-c). In the absence of HLH-25, not only the expression in embryos increased significantly (D-F), but also expression in the the head and tail neuron, muscles throughout the body, pharyngeal muscles, spermatheca and ventral nerve cord (G-I).

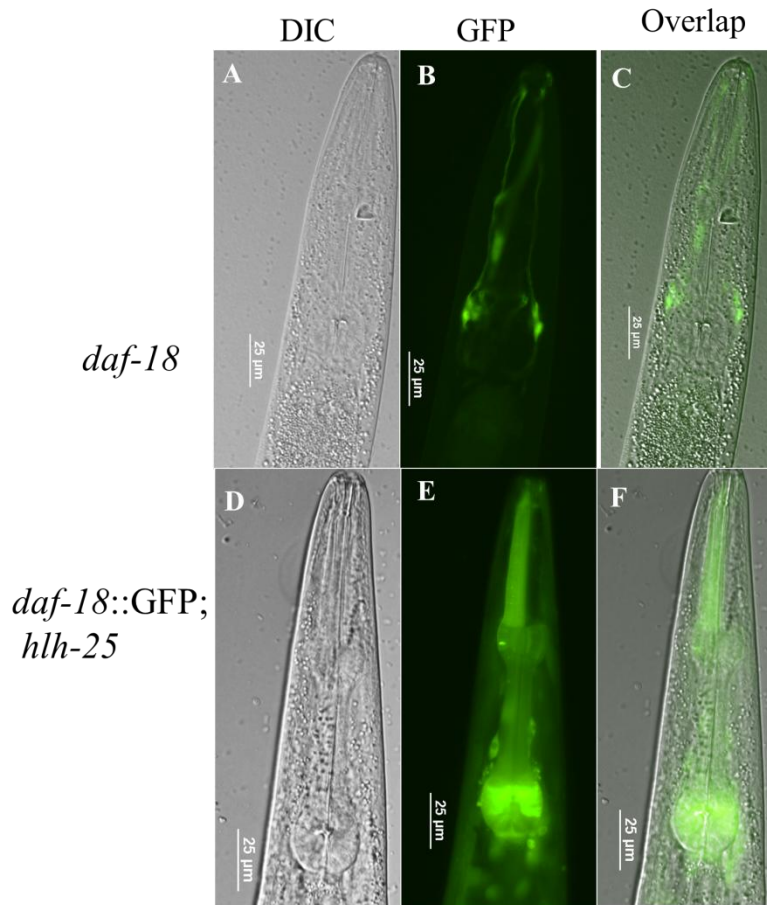


Figure 22 *daf-18* head expression changes
Expression was localized only to the head neurons (A-C), but in the absence of HLH-25, the expression increased in the head, (D-F).

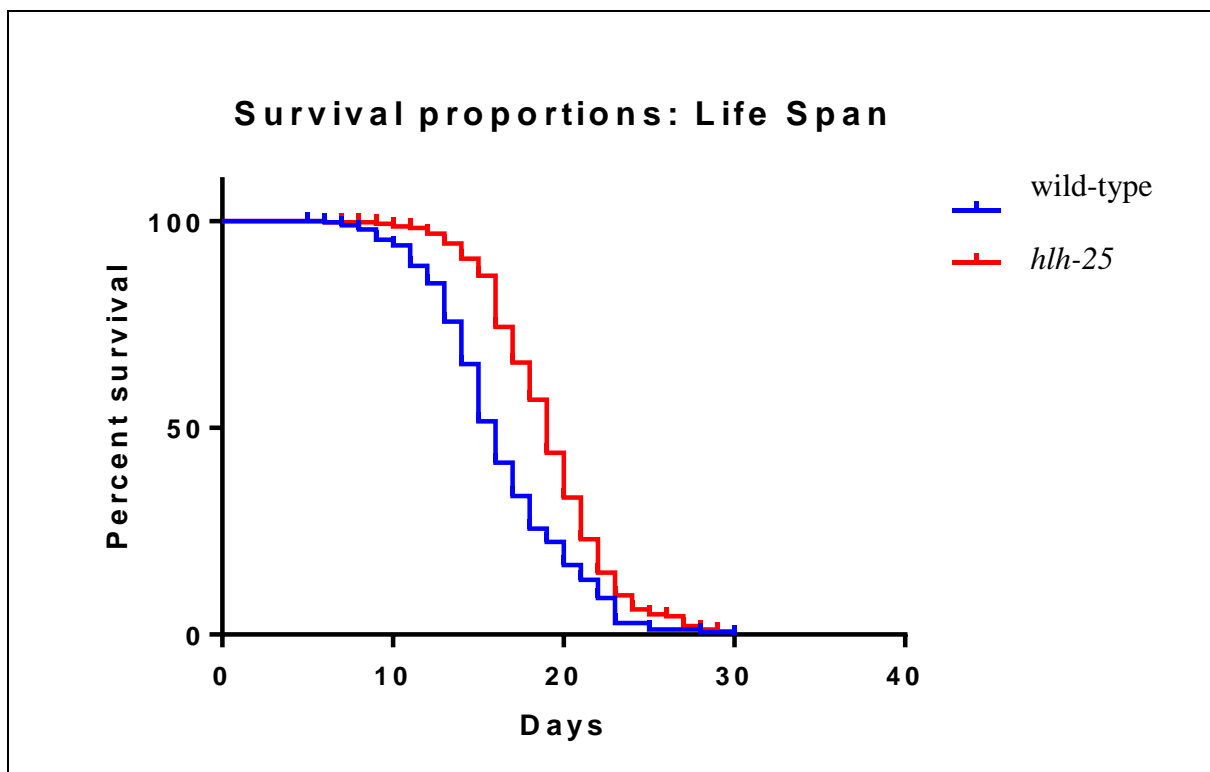


Table 3 Life Span Measurements

Strain	Biological Replicate	Total # of Animals to Die/or to be Censored	Median Life Span of All Animals (days)	P-value Log-Rank Test compared to N2 animals
wild-type	1	88/13	15	
	2	90/10	17	
	3	86/12	15	
	all	264/35	16	
<i>hhh-25</i>	1	93/7	20	< 0.0001
	2	93/7	19	0.0046
	3	96/4	19	< 0.0001
	all	282/18	19	< 0.0001

Figure 23 Life span curve of wild-type versus *hhh-25*.

Measurement and comparison of life span between the wild type and *hhh-25* on solid NGM (nematode growth medium) with OP50 *E. coli*.

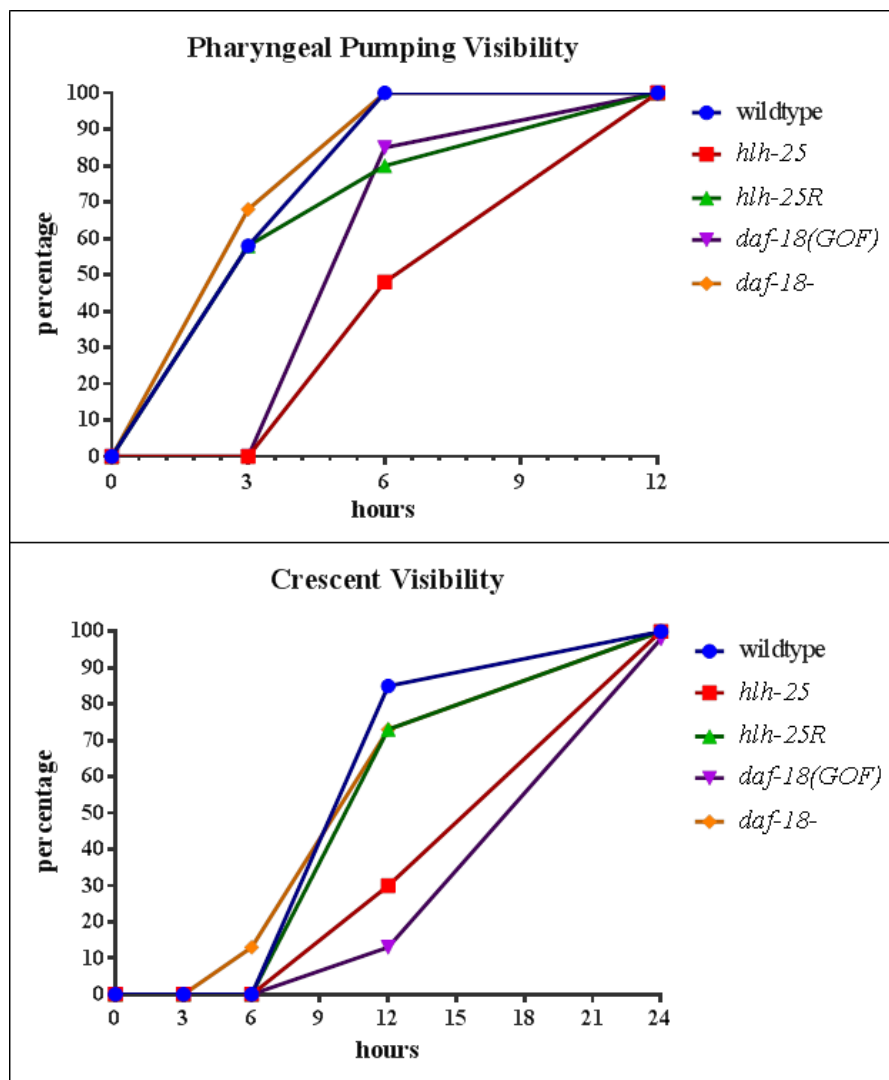


Figure 24 Dauer Recovery.

Visibility of A) pharyngeal pumping and B) crescent formation during dauer recovery was observed and recorded for twenty-four hours. 0 hrs marks the time dauers were placed under favorable conditions.

Table 4 Dauer Recovery

		wild-type	hhh-25	hhh-25R	daf18-	daf-18 ^{GOF}	hhh-25 vs daf-18 ^{GOF}
pumping	median	3	12	3	3	6	12
	p-value		< 0.0001	0.1174	0.3586	< 0.0001	0.0004
crescent	median	12	24	12	12	24	24
	p-value		< 0.0001	0.1745	0.9619	< 0.0001	0.0386

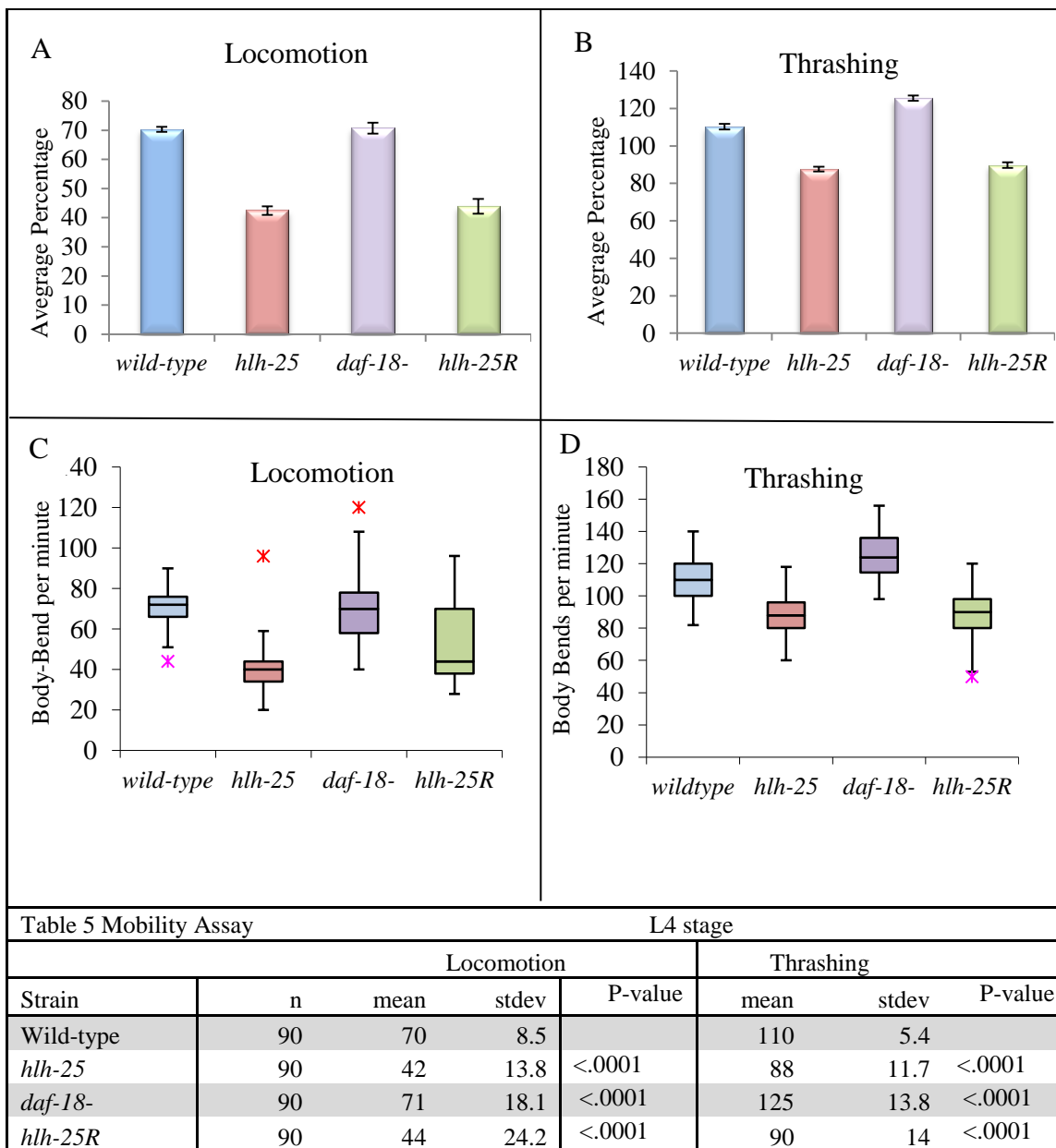


Figure 25 Locomotion Assay

Error bars represent SEM. (C, D) The band near the middle of the box represents the median. Outliers are indicated with an asterisk.

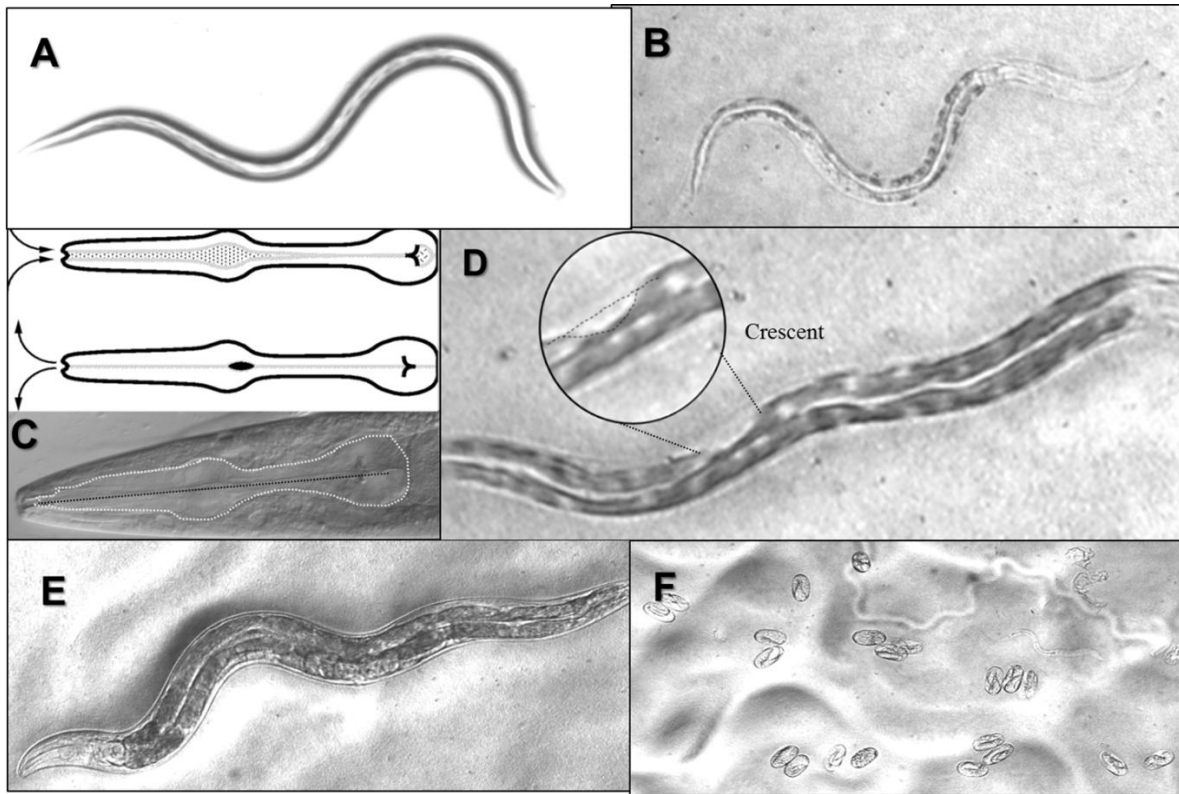


Figure 26 Characteristics of Dauer Recovery

Representative pictures of dauer recovery characteristics on NGM plates. (A) at “0” hour dauers are long, thin and transparent. The first sign of recovery is (B) fat accumulation in the intestines and pharyngeal pumping [1] followed by (C , D) the crescent formation. Once the dauers reach the adult stage (F) embryos are made and laid.

4 DISCUSSION

4.1 *HLH-25 Functions During Embryogenesis*

The experiments in this research lay a foundation for understanding the role of HLH-25 in *C. elegans* during embryonic and larval development. The key challenge in this genetic research was to understand how the HLH-25 transcription factor affects the performances of its target genes by examining phenotypes caused by *hlh-25* mutations. HLH-25 is actively expressed in embryos, larvae and adults. In the absence of *hlh-25*, animals show a 54% embryonic lethality, a reduced brood size, an increased number of unfertilized eggs, a slower movement rate, a longer life span, and a longer dauer recovery. The results presented offer excellent starting points to further characterize HLH-25, and raised some questions that can be further examined.

4.1.1 *Is hlh-25 a maternal effect gene?*

Maternal effects genes play important roles in the early processes of embryonic development of different organisms, such as sea urchins, nematodes, fruit flies, zebrafish, frogs and mice. Maternally synthesized proteins or mRNAs are placed in the oocytes and sometime after fertilization, those mRNAs are expressed in the developing embryo, or zygote. This event is known as the maternal to zygotic transition (MZT) [122]. During this period, maternal transcripts are eliminated and the zygotic genome becomes transcriptionally activate [123]. The timing of MZTs differs from cell to cell, which reflects the development of distinct cell types and causes the maternal transcripts to overlap with some of the early zygotic transcripts [123].

During *Drosophila* embryogenesis, maternal mRNAs, such as hunchback, bicoid, and nanos are required for early patterning of the embryo [9, 124-126]. Firstly, maternal hunchback is distributed evenly throughout the egg. Maternal nanos mRNAs and bicoid mRNAs are

localized to the posterior and anterior pole of the mature oocyte, respectively [127]. The nanos protein inactivates hunchback, preventing its translation in the posterior. Bicoid activates transcription of the hunchback gene which creates a concentration gradient of the hunchback expression along the embryo. Hunchback activates or represses several genes depending on its concentration. For instance, hunchback activates and represses kruppel [127, 128]. Kruppel is necessary for differentiation, growth, and development of the embryo. At high concentrations, hunchback represses kruppel, and at lower concentrations hunchback activates kruppel [128].

The maternal effect hunchback, along with other maternal effect genes, initiates the embryo pattern. Zygotic hunchback transcription creates a gradient of the hunchback protein which regulates genes necessary for embryo development [122]. Hunchback is necessary for proper morphology [122, 129-132]. *Drosophila* with hunchback mutations lack mouthparts and thorax structures [122, 129-132].

Different methods have been utilized to identify maternal genes in *Drosophila*, one of which is to isolate pole cells, progenitors of the germ-line stem cells, from blastodermal embryos by fluorescence-activated cell sorting (FACS) and then use these isolated cells in a microarray analysis [133, 134]. Another method is to use flow cytometry to sort GFP-labeled pole cells then use multidimensional protein identification technology (MuDPIT) [134-137] to identify proteins in both the GFP-positive and GFP-negative cells. Moreover, another method is to observe mRNA molecule localization by using fluorescent labeled probes [138].

In *C. elegans*, oocytes are loaded with a number of maternally synthesized proteins and mRNAs that are necessary for directing the first mitotic divisions. These include *mex-5*, *mex-6*, and *pos-1* mRNA, all of which are required to establish the overall polarity of the embryo, and *ran-1* mRNA, which is necessary for the first embryonic division. Mutation of *ran-1* disrupts

formation of the mitotic spindle and causes aberrant chromatin localization [97]. This raises the question about whether *hlh-25* is also a maternal effect gene.

Three observations from our experiments lead us to suggest that HLH-25 is not a maternal effect gene. First, movies show that the early embryonic divisions of *hlh-25* animals appear normal. Second, *hlh-25* is expressed in early embryos in response to Notch signaling in the Abp granddaughters (beginning after the eight-cell stage) and in four of the EMS granddaughters (MSaa, MSap, MSpa, Mspp). The maternal-to-zygotic transition (MZT) begins after the second cleavage (4 Cells) [123, 139], so HLH-25 is likely just becoming active after the Notch-dependent induction of transcription. Third, *hlh-25* expression has not been detected in the somatic gonad, the intestine, or the germline of adult animals, though it is possible that our transgene does not reflect the full expression pattern of the gene. In future studies to test more directly whether *hlh-25* is or not a maternal effect gene, methods described above should be used.

4.2 *Why is the morphology of oocytes irregular in hlh-25 animals? Why do hlh-25 animals have a high embryonic lethality, increased quantity of unfertilized eggs and reduced brood size?*

We have established that different HLH-25 target genes play different roles during embryogenesis. Some of these genes, such as *cpg-2* and *H02I12.5*, are necessary during oocyte formation [139], while others are necessary after fertilization, such as *ran-1*. When these important genes are mutated or down-regulated (as they are in *hlh-25* animals), embryos encounter different problems such as irregular oocyte formation and/or embryonic lethality. This may explain why *hlh-25* animals have irregular shaped oocytes, high embryonic lethality, an increased in unfertilized oocytes and reduced brood size.

hlh-25 embryos that die do so prior to the tadpole stage; however, it is unknown exactly at what cell stage embryos stop dividing and die. Using Hoechst dye blue fluorescent stain (DAPI) to stain DNA in the nucleus of the embryos could be a method for future studies to confirm at which cell stage embryos arrest. Also, since overexpression of PTEN/*daf-18* causes apoptosis of cells [140], future studies should use the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay to detect DNA fragmentation from apoptotic cells in *hlh-25* unhatched embryos. Furthermore, strains co-expressing fluorescent markers that target the plasma membrane (GFP fusion that binds PI4, 5P2) and the chromosomes (mCherry-histone H₂B) to observe plasma membrane and chromosome, respectively, should be used as a method to further study the gonad architecture in *hlh-25* animals.

4.3 HLH-25 Function in Movement

4.3.1 Are hlh-25 animals UNC variants?

C. elegans move in a wave-like motion that is described as sinusoidal movement [141]. This movement pattern is determined by antagonist movement of the ventral and dorsal body muscles, and it is controlled by distinct classes of motorneurons [142]. Some of these neurons form neuromuscular junctions with the ventral and dorsal body muscles, one of which is the ventral nerve cord (VNC) [142]. The expression of *hlh-25* in the ventral nerve cord led us to examine *hlh-25* animals for locomotion defects.

Even though *hlh-25* animals appear to have a sinusoidal movement, they move at a slower rate than wild-types. However, this phenotype could not be rescued. The failure to rescue the phenotype by re-introducing the transgene suggests that HLH-25 does not function in

locomotion. There are two other alternative possibilities. First, the *hlh-25* deletion was created using EMS (Ethyl methanesulfonate), which produces random mutations in the genetic material. Although, *hlh-25* animals were extensively outcrossed in order to reduce genetic abnormalities, there could be a mutation in a gene located close to *hlh-25* and on the same chromosome that is necessary for movement. Second, it is possible that the transgene is not expressed at high enough levels in the cells to rescue movement. Future studies can address these possibilities, by testing additional transgenic lines, which may express *hlh-25* in different cells, for the ability to rescue the movement phenotype.

4.4 Stage Dependent Regulation by HLH-25

4.4.1 Does HLH-25 act as both, a transcriptional activator and a transcriptional repressor?

Generally, transcription factors are characterized by their mechanism of transcriptional regulation. Transcription factors typically act to either repress or activate transcription: many may activate one group of genes while and to repress a different group of genes [143, 144]. Importantly, only a few transcription factors are known to act both as an activator and as a repressor of the same gene. For instance, Mcm1, a yeast transcription factor, affects both activation and repression of α -specific genes [145] in yeast mating-type switching. In *Arabidopsis*, the homeodomain-leucine zipper *ATHB2* and the basic helix–loop–helix (bHLH) *PIL1* transcription factor, activate genes implicated in the elongation response provoked by neighbor shade, and repress the same genes when neighbor shade is no longer detected [146, 147].

Our results show that in *C. elegans* a number of the genes that are activated by HLH-25 during embryogenesis are repressed by HLH-25 in during L4 stage. In order to attempt to

explain why *hlh-25* activates and represses some of its target genes but only represses *daf-18*, I looked for the *hlh-25* binding sites within 2000 base pairs upstream of the ATG start codon of the target genes previously validated by RTqPCR. As explained in section 1.3, HLH-25 recognizes five different consensus sequences. Genes *cpg-2* and *ima-1*, have three of the five HLH-25 consensus sequence binding site [55]. Genes *ran-1*, *pos-1*, *imp-2*, *pqn-95*, *acs-2* and *daf-18* only have one of the five HLH-25 consensus sequence binding sites (Table 5) [55]. Even though HLH-25 both activates and represses *acs-2* and only represses *daf-18*, these genes share the same HLH-25 consensus sequence binding site, “CACACG”. This result raises the question of whether HLH-25 regulates gene expression in a stage-dependent manner. To examine this, future studies should prepare microarrays comparing wild-types with *hlh-25* mutants at all stages.

Table 5 Location of the HLH-25 binding sites

Gene	Consensus Sequence	Location Upstream of the ATG	Gene	Consensus Sequence	Location Upstream of the ATG
<i>cpg-2</i>	CATACG	-129	<i>daf-18</i>	CACACG	-468
	CACACG	-608	<i>lin-34</i>	CATGCG	-1694
	CATGCG	-1614		CACGCT	-1533
<i>ima-1</i>	CACGCG	-264	<i>mtl-1</i>	CACACG	-398
	CATGCG	-311	<i>pqn-95</i>	CATGCG	-537
	CATACG	-991	<i>imp-2</i>	CATACG	-1512
<i>pos-1</i>	CACGCT	-636	<i>mex-5</i>	none	-
<i>ran-1</i>	CACGCT	-1948	<i>mex-6</i>	none	-
<i>sca-1</i>	none	-	<i>spn-4</i>	CACGCT	-415
<i>emb-9</i>	none	-	<i>acs-2</i>	CACACG	-281

4.5 *HLH-25 links Notch signaling to the PTEN pathway.*

DAF-18, in *C. elegans*, is the ortholog of the human tumor suppressor PTEN (phosphatase and tensin homolog), and HLH-25 is an ortholog of Hairy/Enhancer of Split (HES). Both of them are important for development. PTEN, helps regulate the cell cycle and cell division through the PI3-kinase pathway [26, 148, 149] by dephosphorylating PIP3 (phosphatidylinositol-3,4,5-trisphosphate) [150]. Either PTEN gain of function or loss of function causes irregularities in development. For instance, mutations to PTEN have been associated with the autosomal-dominant disorder Cowdens-Disease and with different types of cancer such as prostate cancer and malignant glioma. Overexpression of PTEN induces apoptosis and cell cycle arrest. For instance, overexpression of PTEN in MCF-7 breast cancer cells, causes G1 cell arrest and cell death [140]. Furthermore, HES proteins are important regulators of a variety of genes that influence cell proliferation and differentiation in embryo development [38]. HES mutations have been associated with lung and breast cancer in mammals [43, 44] and with oocyte death [29].

HES and HLH-25 are direct targets of Notch signaling [34-36, 45]. Our findings show that *daf-18* expression is regulated by HLH-25, providing a link between Notch signaling and PTEN mediated control of cell proliferation. Future studies in the lab can further exploit the relationship between HLH-25 and *daf-18* by analyzing the expression of *daf-18::GFP* in animals lacking the Notch signaling, LIN-12/Notch.

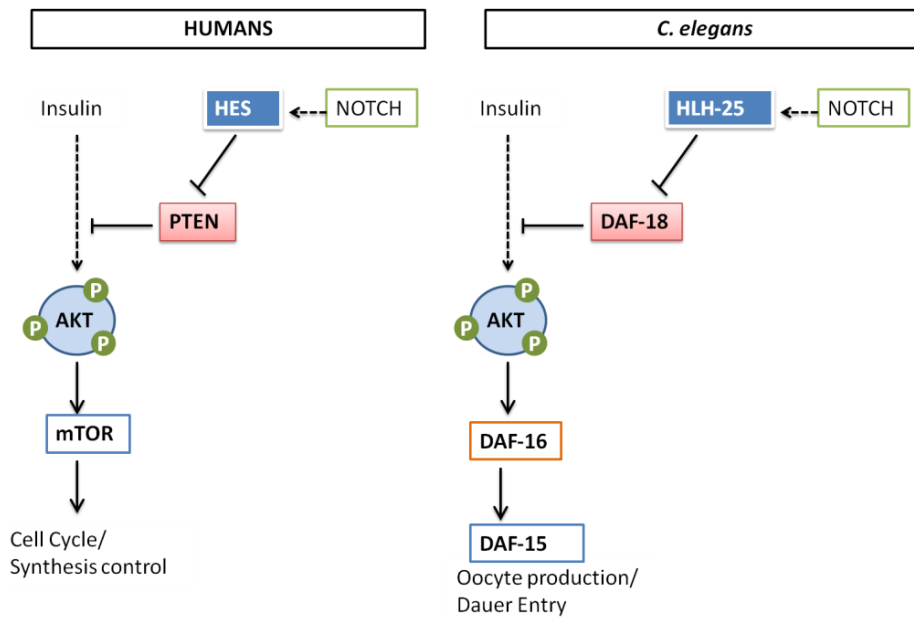


Figure 27 Role comparisons between humans and *C. elegans*.

This diagram is based on previous experiments and the one presented here. In humans, HES is activated by Notch signaling. HES represses PTEN. PTEN prevents AKT complex phosphorylation allowing mTOR to enter the nucleus to control cell cycle. Similarly, in *C. elegans*, HLH-25 is activated by Notch signaling. HLH-25 represses *daf-18*/PTEN. *daf-18*/PTEN prevents AKT complex phosphorylation allowing DAF-16 to enter the nucleus.

REFERENCES

1. Avery LaY, Y.J.: **C. elegans feeding**: The C. elegans Research Community, WormBook; May 21, 2012.
2. Latchman DS: **Transcription factors: an overview**. *The international journal of biochemistry & cell biology* 1997, **29**(12):1305-1312.
3. Karin M: **Too many transcription factors: positive and negative interactions**. *The New biologist* 1990, **2**(2):126-131.
4. Roeder RG: **The role of general initiation factors in transcription by RNA polymerase II**. *Trends in biochemical sciences* 1996, **21**(9):327-335.
5. Nikolov DB, Burley SK: **RNA polymerase II transcription initiation: a structural view**. *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(1):15-22.
6. Brivanlou AH, Darnell JE, Jr.: **Signal transduction and the control of gene expression**. *Science* 2002, **295**(5556):813-818.
7. Mitchell PJ, Tjian R: **Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins**. *Science* 1989, **245**(4916):371-378.
8. Driever W, Nüsslein-Volhard C: **The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner**. *Cell*, **54**(1):95-104.
9. Berleth T, Burri M, Thoma G, Bopp D, Richstein S, Frigerio G, Noll M, Nusslein-Volhard C: **The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo**. *The EMBO journal* 1988, **7**(6):1749-1756.

10. Axelson H: **The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1.** *Cancer letters* 2004, **204**(2):171-178.
11. Kageyama R, Ohtsuka T, Kobayashi T: **The Hes gene family: repressors and oscillators that orchestrate embryogenesis.** *Development* 2007, **134**(7):1243-1251.
12. Jandrig B, Seitz S, Hinzmann B, Arnold W, Micheel B, Koelble K, Siebert R, Schwartz A, Ruecker K, Schlag PM *et al*: **ST18 is a breast cancer tumor suppressor gene at human chromosome 8q11.2.** *Oncogene* 2004, **23**(57):9295-9302.
13. Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F: **bZIP transcription factors in Arabidopsis.** *Trends in plant science* 2002, **7**(3):106-111.
14. Nijhawan A, Jain M, Tyagi AK, Khurana JP: **Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice.** *Plant physiology* 2008, **146**(2):333-350.
15. Ferré-D'Amaré A, Prendergast G, Ziff E, Burley S: **Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain.** *Nature* 1993, **363**(6424):38-45.
16. Ferré-D'Amaré AR, Pognonec P, Roeder RG, Burley SK: **4 Structure and function of the b/HLH/Z domain of USF.** *The EMBO Journal* 1994, **vol. 13 no. 1 pp**:180 - 189.
17. Murre C, McCaw PS, Vaessin H, Caudy M, Jan LY, Jan YN, Cabrera CV, Buskin JN, Hauschka SD, Lassar AB *et al*: **Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence.** *Cell* 1989, **58**(3):537-544.

18. Miyamoto A, Cui X, Naumovski L, Cleary ML: **Helix-loop-helix proteins LYL1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells.** *Molecular and cellular biology* 1996, **16**(5):2394-2401.
19. Ohara T, Tsuge T: **FoSTUA, encoding a basic helix-loop-helix protein, differentially regulates development of three kinds of asexual spores, macroconidia, microconidia, and chlamydospores, in the fungal plant pathogen Fusarium oxysporum.** *Eukaryotic cell* 2004, **3**(6):1412-1422.
20. Atchley W, Fitch W: **A natural classification of the basic helix-loop-helix class of transcription factors.** *Proc Natl Acad Sci U S A* 1997, **94**(10):5172-5176.
21. Ponting CP, Aravind L: **PAS: a multifunctional domain family comes to light.** *Current biology : CB* 1997, **7**(11):R674-677.
22. Taylor BL, Zhulin IB: **PAS domains: internal sensors of oxygen, redox potential, and light.** *Microbiology and molecular biology reviews : MMBR* 1999, **63**(2):479-506.
23. Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H: **The protein Id: a negative regulator of helix-loop-helix DNA binding proteins.** *Cell* 1990, **61**(1):49-59.
24. Atchley WR, Fitch WM: **A natural classification of the basic helix-loop-helix class of transcription factors.** *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(10):5172-5176.
25. Wiese C, Heisig J, Gessler M: **Hey bHLH factors in cardiovascular development.** *Pediatric cardiology* 2010, **31**(3):363-370.
26. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R *et al*: **PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer.** *Science* 1997, **275**(5308):1943-1947.

27. Gray PA, Fu H, Luo P, Zhao Q, Yu J, Ferrari A, Tenzen T, Yuk DI, Tsung EF, Cai Z *et al*: **Mouse brain organization revealed through direct genome-scale TF expression analysis.** *Science* 2004, **306**(5705):2255-2257.
28. Verzi MP, Anderson JP, Dodou E, Kelly KK, Greene SB, North BJ, Cripps RM, Black BL: **N-twist, an evolutionarily conserved bHLH protein expressed in the developing CNS, functions as a transcriptional inhibitor.** *Developmental biology* 2002, **249**(1):174-190.
29. Manosalva I, Gonzalez A, Kageyama R: **Hes1 in the somatic cells of the murine ovary is necessary for oocyte survival and maturation.** *Developmental biology* 2013, **375**(2):140-151.
30. Bai MY, Fan M, Oh E, Wang ZY: **A triple helix-loop-helix/basic helix-loop-helix cascade controls cell elongation downstream of multiple hormonal and environmental signaling pathways in Arabidopsis.** *The Plant cell* 2012, **24**(12):4917-4929.
31. Rohrer H: **M Brain Research.** In.
32. Voronova A, Fischer A, Ryan T, Al Madhoun A, Skerjanc IS: **Ascl1/Mash1 is a novel target of Gli2 during Gli2-induced neurogenesis in P19 EC cells.** *PloS one* 2011, **6**(4):e19174.
33. Lei J, Howard MJ: **Targeted deletion of Hand2 in enteric neural precursor cells affects its functions in neurogenesis, neurotransmitter specification and gangliogenesis, causing functional aganglionosis.** *Development* 2011, **138**(21):4789-4800.

34. Jennings B, Preiss A, Delidakis C, Bray S: **The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the *Drosophila* embryo.** *Development* 1994, **120**(12):3537-3548.
35. Dawson SR, Turner DL, Weintraub H, Parkhurst SM: **Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression.** *Molecular and cellular biology* 1995, **15**(12):6923-6931.
36. Alifragis P, Poortinga G, Parkhurst SM, Delidakis C: **A network of interacting transcriptional regulators involved in *Drosophila* neural fate specification revealed by the yeast two-hybrid system.** *Proceedings of the National Academy of Sciences of the United States of America* 1997, **94**(24):13099-13104.
37. Artavanis-Tsakonas S, Rand MD, Lake RJ: **Notch signaling: cell fate control and signal integration in development.** *Science* 1999, **284**(5415):770-776.
38. Kageyama R, Ohtsuka T: **The Notch-Hes pathway in mammalian neural development.** *Cell research* 1999, **9**(3):179-188.
39. Weng AP, Aster JC: **Multiple niches for Notch in cancer: context is everything.** *Current opinion in genetics & development* 2004, **14**(1):48-54.
40. Taelman V, Van Wayenbergh R, Solter M, Pichon B, Pieler T, Christophe D, Bellefroid EJ: **Sequences downstream of the bHLH domain of the *Xenopus* hairy-related transcription factor-1 act as an extended dimerization domain that contributes to the selection of the partners.** *Developmental biology* 2004, **276**(1):47-63.
41. Fisher AL, Ohsako S, Caudy M: **The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and**

- protein-protein interaction domain. *Molecular and cellular biology* 1996, **16**(6):2670-2677.**
42. Kang SA, Seol JH, Kim J: **The conserved WRPW motif of Hes6 mediates proteasomal degradation.** *Biochemical and Biophysical Research Communications* 2005, **332**(1):33-36.
43. Shinga J, Itoh M, Shiokawa K, Taira S, Taira M: **Early patterning of the prospective midbrain-hindbrain boundary by the HES-related gene XHR1 in Xenopus embryos.** *Mechanisms of development* 2001, **109**(2):225-239.
44. Natsugoshi R, Sato Y, Matsumoto T, Kageyama T, Hattori M, Iyoda A, Satoh Y, Ryuge S, Masuda N, Jiang SX *et al*: **The balance between the expressions of hASH1 and HES1 differs between large cell neuroendocrine carcinoma and small cell carcinoma of the lung.** *Lung cancer* 2011, **74**(3):405-410.
45. Neves A, Priess JR: **The REF-1 family of bHLH transcription factors pattern C. elegans embryos through Notch-dependent and Notch-independent pathways.** *Developmental cell* 2005, **8**(6):867-879.
46. Broitman-Maduro G, Maduro MF, Rothman JH: **The Noncanonical Binding Site of the MED-1 GATA Factor Defines Differentially Regulated Target Genes in the C. elegans Mesendoderm.** *Developmental cell* 2005, **8**(3):427-433.
47. Neves A, Priess J: **The REF-1 family of bHLH transcription factors pattern C. elegans embryos through Notch-dependent and Notch-independent pathways.** *Dev Cell* 2005;8(6):867-879.

48. Alper S, Kenyon C: **REF-1, a protein with two bHLH domains, alters the pattern of cell fusion in *C. elegans* by regulating Hox protein activity.** *Development* 2001, **128**(10):1793-1804.
49. Lanjuin A, Claggett J, Shibuya M, Hunter CP, Sengupta P: **Regulation of neuronal lineage decisions by the HES-related bHLH protein REF-1.** *Developmental biology* 2006, **290**(1):139-151.
50. Broitman-Maduro G, Maduro MF, Rothman JH: **The noncanonical binding site of the MED-1 GATA factor defines differentially regulated target genes in the *C. elegans* mesendoderm.** *Developmental cell* 2005, **8**(3):427-433.
51. McMiller TL, Sims D, Lee T, Williams T, Johnson CM: **Molecular characterization of the *Caenorhabditis elegans* REF-1 family member, hlh-29/hlh-28.** *Biochimica et biophysica acta* 2007, **1769**(1):5-19.
52. White A, Fearon A, Johnson CM: **HLH-29 regulates ovulation in *C. elegans* by targeting genes in the inositol triphosphate signaling pathway.** *Biology open* 2012, **1**(3):261-268.
53. Molkenkin JD, Olson EN: **Defining the regulatory networks for muscle development.** *Current opinion in genetics & development* 1996, **6**(4):445-453.
54. Maduro MF, Rothman JH: **Making Worm Guts: The Gene Regulatory Network of the *Caenorhabditis elegans* Endoderm.** *Developmental biology* 2002, **246**(1):68-85.
55. Grove CA, De Masi F, Barrasa MI, Newburger DE, Alkema MJ, Bulyk ML, Walhout AJ: **A multiparameter network reveals extensive divergence between *C. elegans* bHLH transcription factors.** *Cell* 2009, **138**(2):314-327.

56. Christian A. Grove FDM, M. Inmaculada Barrasa, Daniel E. Newburger, Mark J. Alkema, Martha L. Bulyk, and Albertha J.M. Walhout: **A Multiparameter Network Reveals Extensive Divergence between *C. elegans* bHLH Transcription Factors.** *Cell* 2009;138(132): 314–327.
57. Li J, C Y, D L, K P, S B, SI W, J P, C M, Rodgers L MR, SH B *et al*: **PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer.** *Science* 1997;275 (5308): 1943–5307.
58. Chen Z, LC T, D S, HK L, ZA D, M N, JA K, HI S, T L, W G *et al*: **Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient inumorigenesis.** *Nature* 2005:436.
59. Day F, RN J, L L, D M, A S, M C, S L, C T, J T, J D *et al*: **PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer.** . *Clin Cancer Res* 2013;19(12):3285-3296. .
60. Shideng B, Ouyang G, Bai X, Huang Z, Ma C, Liu M, Shao R, Anderson RM, Rich JN, Wang aX-F: **Periostin potently promotes metastatic growth of colon cancerby augmenting cell survival via the Akt/PKB pathway.** *Cancer Cell* 2004;5(4):329-339.
61. Chu E, Tarnawski A: **PTEN regulatory functions in tumor suppression and cell biology.** *Med Sci Monit* 2004;10 (10): RA235–241.
62. Simpson L, Parsons R: **PTEN: life as a tumor suppressor.** *Exp Cell Res* 2001;264: 229–241.
63. Song G, Ouyang G, Bao S: **The activation of Akt/PKB signaling pathway and cell survival.** *J Cell Mol Med* 2005;9 (1): 59–71.

64. Paradis S, Ruvkun G: **Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor.** *Genes Dev* 1998;12(16):2488-2498.
65. Solari F, Bourbon-Piffaut A, Masse I, Payrastra B, Chan AM, Billaud M: **The human tumour suppressor PTEN regulates longevity and dauer formation in Caenorhabditis elegans.** *Oncogene* 2005;(24) 20–27.
66. Mihaylova T, Borland CZ, Manjarrez L, Stern MJ, Sun H: **The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway.** *Proc Natl Acad Sci U S A*, 1996;(96).
67. E.B. Gil EML, L.X. Liu, C.D. Johnson, J.A. Lees: **Regulation of the insulin-like developmental pathway of Caenorhabditis elegans by a homolog of the PTEN tumor suppressor gene.** *Proc Natl Acad Sci U S A* 1999(96):2925–2930.
68. Ogg S, Ruvkun G: **The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway** *Cell Mol* 1998: (2) 887–893.
69. von Ehrenstein G, Schierenberg E: **Nematodes as Biological Models**, vol. 1: Academic Press, New York; 1980.
70. D.L. Riddle TB, B.J. Meyer, J.R. Priess . **Celegans II.** Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1997.
71. Sulston J, Hodgkin J: **In: The Nematode Caenorhabditis elegans:** New York: Cold Spring Harbor Laboratory Press; 1988.
72. Bird AF, Bird J: **The structure of nematodes** S. Diego, CA,,: Academic Press); 1991.

73. Cassada RC, Russell RL: **The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*.** *Developmental biology* 1975, **46**(2):326-342.
74. Goranson NC, Ebersole JP, Brault S: **Resolving an adaptive conundrum: reproduction in *Caenorhabditis elegans* is not sperm-limited when food is scarce.** *Evolutionary Ecology Research* 2005, **7**:325-333.
75. Sulston J: **The nematode *C. elegans* 1988.** New York.: Cold Spring Harbor Laboratory Press; 1988.
76. Stiernagle T: **Maintenance of *C. elegans*.** *WormBook : the online review of *C elegans* biology* 2006:1-11.
77. Kamath RS, Ahringer J: **Genome-wide RNAi screening in *Caenorhabditis elegans*.** *Methods (San Diego, Calif)* 2003, **30**(4):313-321.
78. Chen I, Dubnau D: **DNA uptake during bacterial transformation.** *Nature reviews Microbiology* 2004, **2**(3):241-249.
79. Lacks S: **Binding and Entry of DNA in Bacterial Transformation.** In: *Microbial Interactions*. Edited by Reissig JL, vol. 3: Springer US; 1977: 177-232.
80. Roella M: **Microinjecting Worms.** In. Cold Spring Harbor, NY, USA.; 1999.
81. Mello CC, Kramer JM, Stinchcomb D, Ambros V: **Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences.** *EMBO J* 1991, **10**:3959–3970.
82. Kramer JM, French RP, Park EC, Johnson JJ: **The *Caenorhabditis elegans* rol-6 gene, which interacts with the *sqt-1* collagen gene to determine organismal morphology, encodes a collagen.** *Mol Cell Bio* 1990, **10**:2081–2089.

83. Kelly WG, Xu S, Montgomery MK, Fire A: **Distinct requirements for somatic and germline expression of a generally expressed *Caenorhabditis elegans* gene.** *Genetics* 146 1997: 227–238.
84. Govindan JA, Nadarajan S, Kim S, Starich TA, Greenstein D: **Somatic cAMP signaling regulates MSP-dependent oocyte growth and meiotic maturation in *C. elegans*.** *Development* 2009, **136**(13):2211-2221.
85. Sutphin GL, Kaeberlein M: **Measuring *Caenorhabditis elegans* life span on solid media.** *Journal of visualized experiments : JoVE* 2009(27).
86. Larsen PL, Clarke CF: **Extension of life-span in *Caenorhabditis elegans* by a diet lacking coenzyme Q.** *Science* 2002, **295**(5552):120-123.
87. Golden JW, Riddle DL: **A pheromone influences larval development in the nematode *Caenorhabditis elegans*.** *Science* 1982, **218**(4572):578-580.
88. Sawin ER, Ranganathan R, Horvitz HR: ***C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway.** *Neuron* 2000, **26**(3):619-631.
89. Li C, Kim K: **Neuropeptides.** *WormBook : the online review of *C elegans* biology* 2008:1-36.
90. Hoogewijs D, Houthoofd K, Matthijssens F, Vandesompele J, Vanfleteren JR: **Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*.** *BMC molecular biology* 2008, **9**:9.
91. Barr MM, Garcia LR: **Male mating behavior.** *WormBook : the online review of *C elegans* biology* 2006:1-11.

92. Verbrugghe KJ, Chan RC: **Imaging C. elegans embryos using an epifluorescent microscope and open source software.** *Journal of visualized experiments : JoVE* 2011(49).
93. Byerly L, Cassada RC, Russell RL: **The life cycle of the nematode Caenorhabditis elegans. I. Wild-type growth and reproduction.** *Developmental biology* 1976, **51(1):23-33.**
94. Byerly L, Scherer S, Russell RL: **The life cycle of the nematode Caenorhabditis elegans. II. A simplified method for mutant characterization.** *Developmental biology* 1976, **51(1):34-48.**
95. Parashar A, Lycke R, Carr JA, Pandey S: **Amplitude-modulated sinusoidal microchannels for observing adaptability in C. elegans locomotion.** *Biomicrofluidics* 2011, **5(2):24112.**
96. Kelly WG, Xu S, Montgomery MK, Fire A: **Distinct requirements for somatic and germline expression of a generally expressed Caenorhabditis elegans gene.** *Genetics* 1997, **146(1):227-238.**
97. Askjaer P, Galy V, Hannak E, Mattaj IW: **Ran GTPase cycle and importins alpha and beta are essential for spindle formation and nuclear envelope assembly in living Caenorhabditis elegans embryos.** *Molecular biology of the cell* 2002, **13(12):4355-4370.**
98. Carazo-Salas RE, Guarguaglini G, Gruss OJ, Segref A, Karsenti E, Mattaj IW: **Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation.** *Nature* 1999, **400(6740):178-181.**

99. Kutay U, Izaurralde E, Bischoff FR, Mattaj IW, Gorlich D: **Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex.** *The EMBO journal* 1997, **16**(6):1153-1163.
100. Bamba C, Bobinnec Y, Fukuda M, Nishida E: **The GTPase Ran regulates chromosome positioning and nuclear envelope assembly in vivo.** *Current biology : CB* 2002, **12**(6):503-507.
101. Schubert CM, Lin R, de Vries CJ, Plasterk RH, Priess JR: **MEX-5 and MEX-6 function to establish soma/germline asymmetry in early C. elegans embryos.** *Molecular cell* 2000, **5**(4):671-682.
102. Tabara H, Hill RJ, Mello CC, Priess JR, Kohara Y: **pos-1 encodes a cytoplasmic zinc-finger protein essential for germline specification in C. elegans.** *Development* 1999, **126**(1):1-11.
103. Piano F, Schetter AJ, Morton DG, Gunsalus KC, Reinke V, Kim SK, Kemphues KJ: **Gene clustering based on RNAi phenotypes of ovary-enriched genes in C. elegans.** *Current biology : CB* 2002, **12**(22):1959-1964.
104. Dudley NR, Labbe JC, Goldstein B: **Using RNA interference to identify genes required for RNA interference.** *Proceedings of the National Academy of Sciences of the United States of America* 2002, **99**(7):4191-4196.
105. Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, Brehm M, Alleaume AM, Artelt J, Bettencourt P, Cassin E *et al.*: **Full-genome RNAi profiling of early embryogenesis in Caenorhabditis elegans.** *Nature* 2005, **434**(7032):462-469.

106. Simmer F, Moorman C, van der Linden AM, Kuijk E, van den Berghe PV, Kamath RS, Fraser AG, Ahringer J, Plasterk RH: **Genome-wide RNAi of *C. elegans* using the hypersensitive *rrf-3* strain reveals novel gene functions.** *PLoS biology* 2003, **1**(1):E12.
107. Rual JF, Ceron J, Koreth J, Hao T, Nicot AS, Hirozane-Kishikawa T, Vandenhautte J, Orkin SH, Hill DE, van den Heuvel S *et al*: **Toward improving *Caenorhabditis elegans* phenome mapping with an ORFeome-based RNAi library.** *Genome research* 2004, **14**(10B):2162-2168.
108. Huang NN, Mootz DE, Walhout AJ, Vidal M, Hunter CP: **MEX-3 interacting proteins link cell polarity to asymmetric gene expression in *Caenorhabditis elegans*.** *Development* 2002, **129**(3):747-759.
109. Zhou XR, Green AG, Singh SP: ***Caenorhabditis elegans* Delta12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Delta12 and Delta15 positions.** *The Journal of biological chemistry* 2011, **286**(51):43644-43650.
110. Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C: **Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*.** *Nature* 2003, **424**(6946):277-283.
111. Ashrafi K, Chang FY, Watts JL, Fraser AG, Kamath RS, Ahringer J, Ruvkun G: **Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes.** *Nature* 2003, **421**(6920):268-272.
112. Swain SC, Keusekotten K, Baumeister R, Sturzenbaum SR: ***C. elegans* metallothioneins: new insights into the phenotypic effects of cadmium toxicosis.** *Journal of molecular biology* 2004, **341**(4):951-959.

113. Birkenfeld AL, Lee HY, Guebre-Egziabher F, Alves TC, Jurczak MJ, Jornayvaz FR, Zhang D, Hsiao JJ, Martin-Montalvo A, Fischer-Rosinsky A *et al*: **Deletion of the mammalian INDY homolog mimics aspects of dietary restriction and protects against adiposity and insulin resistance in mice.** *Cell metabolism* 2011, **14**(2):184-195.
114. Mouchiroud L, Molin L, Kasturi P, Triba MN, Dumas ME, Wilson MC, Halestrap AP, Roussel D, Masse I, Dalliere N *et al*: **Pyruvate imbalance mediates metabolic reprogramming and mimics lifespan extension by dietary restriction in *Caenorhabditis elegans*.** *Aging cell* 2011, **10**(1):39-54.
115. Padmanabhan S, Mukhopadhyay A, Narasimhan SD, Tesz G, Czech MP, Tissenbaum HA: **A PP2A regulatory subunit regulates *C. elegans* insulin/IGF-1 signaling by modulating AKT-1 phosphorylation.** *Cell* 2009, **136**(5):939-951.
116. Tullet JM, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R, Blackwell TK: **Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*.** *Cell* 2008, **132**(6):1025-1038.
117. Brisbin S, Liu J, Boudreau J, Peng J, Evangelista M, Chin-Sang I: **A role for *C. elegans* Eph RTK signaling in PTEN regulation.** *Developmental cell* 2009, **17**(4):459-469.
118. Golden JW, Riddle DL: **A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply.** *Journal of chemical ecology* 1984, **10**(8):1265-1280.
119. Golden JW, Riddle DL: **The *Caenorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature.** *Developmental biology* 1984, **102**(2):368-378.

120. Golden JW, Riddle DL: **A pheromone-induced developmental switch in *Caenorhabditis elegans*: Temperature-sensitive mutants reveal a wild-type temperature-dependent process.** *Proceedings of the National Academy of Sciences of the United States of America* 1984, **81**(3):819-823.
121. Proudfoot L, Kusel JR, Smith HV, Harnett W, Worms MJ, Kennedy MW: **Rapid changes in the surface of parasitic nematodes during transition from pre- to post-parasitic forms.** *Parasitology* 1993, **107** (Pt 1):107-117.
122. Associates GS: **The Origins of Anterior-Posterior Polarity.** *Developmental Biology*. Sunderland (MA): Sinauer; 2000.
123. Tadros W, Lipshitz HD: **The maternal-to-zygotic transition: a play in two acts.** *Development* 2009, **136**(18):3033-3042.
124. Driever W, Ma J, Nusslein-Volhard C, Ptashne M: **Rescue of bicoid mutant *Drosophila* embryos by bicoid fusion proteins containing heterologous activating sequences.** *Nature* 1989, **342**(6246):149-154.
125. Driever W, Nusslein-Volhard C: **The bicoid protein is a positive regulator of hunchback transcription in the early *Drosophila* embryo.** *Nature* 1989, **337**(6203):138-143.
126. Driever W, Thoma G, Nusslein-Volhard C: **Determination of spatial domains of zygotic gene expression in the *Drosophila* embryo by the affinity of binding sites for the bicoid morphogen.** *Nature* 1989, **340**(6232):363-367.
127. Reinitz J, Mjolsness E, Sharp DH: **Model for cooperative control of positional information in *Drosophila* by bicoid and maternal hunchback.** *The Journal of experimental zoology* 1995, **271**(1):47-56.

128. Stanojevic D, Hoey T, Levine M: **Sequence-specific DNA-binding activities of the gap proteins encoded by hunchback and Kruppel in Drosophila.** *Nature* 1989, **341**(6240):331-335.
129. Hulskamp M, Pfeifle C, Tautz D: **A morphogenetic gradient of hunchback protein organizes the expression of the gap genes Kruppel and knirps in the early Drosophila embryo.** *Nature* 1990, **346**(6284):577-580.
130. Lehmann R, Nusslein-Volhard C: **hunchback, a gene required for segmentation of an anterior and posterior region of the Drosophila embryo.** *Developmental biology* 1987, **119**(2):402-417.
131. Schulz C, Tautz D: **Autonomous concentration-dependent activation and repression of Kruppel by hunchback in the Drosophila embryo.** *Development* 1994, **120**(10):3043-3049.
132. Struhl G, Johnston P, Lawrence PA: **Control of Drosophila body pattern by the hunchback morphogen gradient.** *Cell* 1992, **69**(2):237-249.
133. Shigenobu S, Arita K, Kitadate Y, Noda C, Kobayashi S: **Isolation of germline cells from Drosophila embryos by flow cytometry.** *Development, Growth & Differentiation* 2006, **48**(1):49-57.
134. Siddiqui NU, Li X, Luo H, Karaiskakis A, Hou H, Kislinger T, Westwood JT, Morris Q, Lipshitz HD: **Genome-wide analysis of the maternal-to-zygotic transition in Drosophila primordial germ cells.** *Genome biology* 2012, **13**(2):R11.
135. Kislinger T, Cox B, Kannan A, Chung C, Hu P, Ignatchenko A, Scott MS, Gramolini AO, Morris Q, Hallett MT *et al*: **Global survey of organ and organelle protein**

- expression in mouse: combined proteomic and transcriptomic profiling.** *Cell* 2006, **125**(1):173-186.
136. Taylor P, Nielsen PA, Trelle MB, Horning OB, Andersen MB, Vorm O, Moran MF, Kislinger T: **Automated 2D peptide separation on a 1D nano-LC-MS system.** *Journal of proteome research* 2009, **8**(3):1610-1616.
137. Washburn MP, Wolters D, Yates JR, 3rd: **Large-scale analysis of the yeast proteome by multidimensional protein identification technology.** *Nature biotechnology* 2001, **19**(3):242-247.
138. Weil TT, Parton RM, Davis I: **Making the message clear: visualizing mRNA localization.** *Trends in cell biology* 2010, **20**(7):380-390.
139. Baugh LR, Hill AA, Slonim DK, Brown EL, Hunter CP: **Composition and dynamics of the *Caenorhabditis elegans* early embryonic transcriptome.** *Development* 2003, **130**(5):889-900.
140. Weng L, Brown J, Eng C: **PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways.** *Human molecular genetics* 2001, **10**(3):237-242.
141. Sulston JE, Horvitz HR: **Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*.** *Developmental biology* 1977, **56**(1):110-156.
142. White JG, Southgate E, Thomson JN, Brenner S: **The structure of the nervous system of the nematode *Caenorhabditis elegans*.** *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 1986, **314**(1165):1-340.

143. Balderas-Martinez YI, Savageau M, Salgado H, Perez-Rueda E, Morett E, Collado-Vides J: **Transcription factors in Escherichia coli prefer the holo conformation.** *PloS one* 2013, **8**(6):e65723.
144. Boyle P, Despres C: **Dual-function transcription factors and their entourage: unique and unifying themes governing two pathogenesis-related genes.** *Plant signaling & behavior* 2010, **5**(6):629-634.
145. Elble R, Tye BK: **Both activation and repression of a-mating-type-specific genes in yeast require transcription factor Mcm1.** *Proceedings of the National Academy of Sciences of the United States of America* 1991, **88**(23):10966-10970.
146. Steindler C, Matteucci A, Sessa G, Weimar T, Ohgishi M, Aoyama T, Morelli G, Ruberti I: **Shade avoidance responses are mediated by the ATHB-2 HD-zip protein, a negative regulator of gene expression.** *Development* 1999, **126**(19):4235-4245.
147. Salter MG, Franklin KA, Whitelam GC: **Gating of the rapid shade-avoidance response by the circadian clock in plants.** *Nature* 2003, **426**(6967):680-683.
148. Li DM, Sun H: **TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta.** *Cancer research* 1997, **57**(11):2124-2129.
149. Li L, Ernsting BR, Wishart MJ, Lohse DL, Dixon JE: **A family of putative tumor suppressors is structurally and functionally conserved in humans and yeast.** *The Journal of biological chemistry* 1997, **272**(47):29403-29406.
150. Maehama T, Dixon JE: **The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.** *The Journal of biological chemistry* 1998, **273**(22):13375-13378.

APPENDICES

Appendix A

Gene	Forward Primer 5'→3'	RE	Reverse Primer 5'→3'	RE
<i>daf-18</i>	CGCCAAAATGCTTGCCTCAGAGC	AgeI	ACATCTGGAGGAGGTAACCAATTGGG	SaII
<i>pqn-95</i>	GCGCAGTAAAGACAGAAAGAAACGA	AgeI	GCTGCCATTGAAAACAATAAATGTGGG	Bam
<i>vha-1</i>	TGTGTTTTGATTAGTGGGTCTCGCC	SaLI	TGTTTAGTGTCCGGTCGACATAACCTG	SmaI
<i>ima-1</i>	CTGCTACTGGTCTGATGAAGGAGA	SacI	CTTCATGCGATGGCCTGAATTCGTCT	AgeI
<i>imp-2</i>	CGGTGAGGGAAGAAAGACGAAT	SacI	CCATGTCCGAAAGGTTTGAATTCC	AgeI
<i>akt-2</i>	CTGCAGTGTCCGGTTGTCTTATCTTG	PstI	GCCAACCGGTTGTAGATGGGCGTTTTCAG TAGACAT	AgeI
<i>ntl-4</i>	GGATCCCAACGATTTCGGAATGGTGG AGCACC	Bam	GCCCAACCGGTTTCATCGCTGCAGGTAGAC ATTTTGGCG	AgeI
<i>thoc-2</i>	GCCCTGCAGCTAGGGTAGTTCACAA AATGT	PstI	GCCACCGGTTGGGTTAATGTTTCGAAATCG TTCAT	AgeI
<i>ran-1</i>	TCTTCTTGGATGCAACCCGGTCCAAC	AgeI	GCCACCGGATGCCCCTCCACCAGACAT GACTGA	AgeI

Table 6 Primers

The Thermo Fisher Scientific guidelines were followed to design primers that. The Oligo Analyzer by Integrated DNA Technologies was used to analyze the primers and make sure they met the requirements. The National Center for Biotechnology Information (NCBI) nucleotide database and Wormbase was used to find the base upstream sequences of each of the selected genes.

Appendix B

Table 7 RTqPCR Probes

Gene	Probe	Gene	Probe	Gene	Probe
<i>cpg-2</i>	Ce02448570_gl	<i>acs-2</i>	Ce024861991_gl	<i>lin-37</i>	Ce02448521
<i>pos-1</i>	Ce02478511_gl	<i>mex-6</i>	Ce02437281	<i>lin-54</i>	Ce02466796
<i>ran-1</i>	Ce02452473_gl	<i>daf-18</i>	Ce024851888_ml	<i>spn-4</i>	Ce024716594gl
<i>pmp-3</i>	Ce02485188_ml	<i>mex-5</i>	Ce02466891	<i>fat-2</i>	Ce02466860_ml
<i>sca-1</i>	Ce02452547_gl	<i>mtl-1</i>	Ce0255141si		