The Role of daf-19 in Non-Ciliated Neurons: How is Neural Development Regulated by Different daf-19 isoforms?

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The Role of \textit{daf-19} in Non-Ciliated Neurons

How is Neural Development Regulated by Different \textit{daf-19} Isoforms?

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A Thesis Submitted in Candidacy for Honors at Graduation from Lawrence University

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ABSTRACT

A degenerative disease-like phenotype, specifically reduction in synaptic protein levels in adult worms, is correlated with loss-of-function of the only RFX transcription factor gene, \textit{daf-19}, in \textit{C. elegans}. This gene encodes four known transcription factor isoforms, two of which are correlated with particular functions. The DAF-19C isoform activates genes responsible for cilia development, while DAF-19M is needed for cilia specification in males. A comparison of the transcriptome of \textit{daf-19} null and isogenic wild type adult worms suggests both positive and negative regulation of gene expression is correlated with the presence of DAF-19 proteins. We have assessed DAF-19 regulation of gene expression using transcriptional fusion constructs of putative target genes in \textit{daf-19} wild type and mutant worms. We have found that at least three genes are repressed by the DAF-19 transcription factor in particular nerve cells. In one case, differential gene expression is seen in sensory neurons while differential expression of two other genes is limited to interneurons. Interestingly, none of these genes have been shown to contain an X-box, the \textit{cis}-acting sequence used to activate genes involved in cilia formation. These data suggest that DAF-19 has an additional role beyond that of sensory neuron development and specification. Because the \textit{daf-19} gene produces at least four related proteins, further experimentation is required to determine which DAF-19 isoform(s) are responsible for repression of gene expression. We are currently using new alleles of \textit{daf-19} to determine whether the largest DAF-19 proteins, DAF-19A/B have a role in repressing target gene expression.
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INTRODUCTION:

The development of the nervous system depends on setting aside cells to eventually become the nervous system. Presumptive nerve cells must then migrate to adult positions, differentiate, grow axons and dendrites, form functional synapses, and survive (Hobert, 2005; Schmitz, 2007; Middlelkoop, 2014). All cells, including neurons, have the same genetic information in their nucleus in the form of tightly wound DNA. However, not all cells express the same genes. It is the unique expression of different gene combinations that distinguishes a neuronal cell from other cell types. For example, nerve cells contain the same 19,735 genes that are found in all C. elegans cells but typically only express a subset of those genes at any given time (Hillier et al., 2005). Both expression and repression of genes are responsible for establishing a wide array of cell types with varying shapes, functions, and locations. Some neurons express greater or fewer genes than other neurons, depending on whether the neuron is specialized for sensation, movement, or it becomes an interneuron connecting sensory and motor neurons to one another.
The Nervous System of *C elegans*

![Figure 1. A partial depiction of the *C. elegans* neuronal network.](image)

In *C. elegans*, the cells of the nervous system are organized into clusters called ganglia. The majority of these clusters can be found in the heads and tails of worms. The majority of *C. elegans* neurons are located in the head around the pharynx. In addition, there are two small posterior lateral ganglia on the sides, as well as some scattered neurons along the lateral body. The processes from most neurons travel in either the ventral or dorsal nerve cord and project to the nerve ring (NR) in the head. The nerve ring constitutes the major neuropil in animals.
C. elegans is a powerful model organism that can be used as a developmental and genetic tool for neuronal studies. The transparency of this worm allows scientists to visualize its cells in vivo throughout its entire lifespan. Consequently, this worm is the only organism for which the entire cell lineage is known (Ward et al., 1975; Sulston, 1983). That is, every cell in its body can be traced to the beginning of the worm’s development and its path of differentiation is known (Huang & Sternberg, 2005). Similarly, it is the only animal for which each cell has been fully mapped, such that the developmental birth and death of every single cell, including neurons, is known (see Figure 1; Varshney, 2011). What is even more fascinating is that these pioneering studies also revealed how all of these neurons are connected to one another—the so-called connectome—which is allowing researchers to better link the particular function of single neurons to the entire neural circuit (Chalfie et al., 1985; Hart, 2006).
Neurons have two functional ends (Figure 2). This basic functional morphology of neurons in *C. elegans* parallels the functional organization of neurons in the human nervous system. This basic functional feature involves a simple “input-output” mechanism. In the input portion of this mechanism, sensory projections (dendrites) at one end of the neuronal cell body receive information from other neurons or directly from the environment via antennae-like devices named “cilia” that sense their chemical and physical environments. Information is then transmitted to the cell body. After being processed in the cell body, an outgoing neural signal will be sent to the other end of the cell—through the neuron’s axon, the outgoing projection of a neuron.

**Figure 2. The overall structure of a neuron.** Neural signals initiate at the dendrites or soma, then travel through the axon to the terminal buttons, and synapse onto other neurons, tissues, or glands. Image taken from: [http://neuropsychologysket-ches.com/Neurons.html](http://neuropsychologysket-ches.com/Neurons.html)
There are four main types of neuronal cell identities in *C. elegans* that share the aforementioned “input-output” mechanism: ciliated sensory neurons, non-ciliated sensory neurons, interneurons (or polymodal neurons) and motor neurons (Hobert, 2005; Varshney, 2011). Interneurons connect ciliated and non-ciliated neurons in different combinations, while motor neurons connect the nervous system to muscle cells (Varshney, 2011). Cilia are antennae-like sensory receptors that project from particular neuronal cells inside *C. elegans* to either the internal body of the worm or the outside environment, see Figure 4 (Ward, 1975; Blacque et al., 2005; Inglis et al., 2008). Cilia have different structures, and thus different functions (Figure 4). This allows ciliated neurons to directly collect and transmit a wide range of internal and environmental signals to other cells within the worm, including neurons and muscles. Non-ciliated sensory neurons, interneurons, and motor neurons play a large role in the conduction and coordination of internal and environmental signals to produce a variety of net behavioral outputs.

Both ciliated and non-ciliated neurons release neurotransmitters to communicate with one another. Therefore, it is important to understand the mechanisms that regulate the specification of neuronal cells into both ciliated and non-ciliated neurons. This developmental mechanism is especially important to understand, because neurodegenerative disease like Alzheimer’s, Huntington’s, and Parkinson’s are associated with the loss of synaptic proteins in neuronal cells (Arnaud et al., 2006).
Figure 3. Neurons in *C. elegans* have two functional ends: the sensory input, or dendritic end, and the signal output, or axonal projection. In a *C. elegans*, a neuron’s cell body can have two projections if it is a bipolar neuron (A), or one projection if it is a monopolar neuron (B). Any other branching (C & D) just extends from these two primary cell projections. Taken from WormAtlas.
Figure 4. Different *C. elegans* ciliary structures with descriptions of their functions. These dendrite-ends absorb Dil. Taken from WormAtlas.
Regulation of Genes in the *C. elegans* Nervous System

Gene expression is activated by proteins called transcription factors, which physically bind to DNA and initiate the transcription of nearby genes into mRNA. Following translation of mRNAs, the resulting proteins can function as enzymes, ion channels, receptors, structures, or other transcription factors. Gene expression also can be repressed by proteins whose expression is activated by transcription factors. In other words, the activation of one or multiple genes by a transcription factor may lead to the repression of other genes. Therefore, transcription factors can be indirectly involved in the repression of genes. The observation of both repression and activation of genetic expression is called differential gene expression.

Differential gene expression is driven in some cells of the *C. elegans* nervous system by the *daf-19* transcription factor gene, which encodes the RFX transcription factor protein, DAF-19. RFX transcription factor genes, like the *daf-19* gene, are important to study because they control the expression of genes involved in the maintenance and survival of a healthy nervous system (Bonnafe, 2004; Senti & Swoboda, 2008; El Zein et al., 2009; Segun-Estevez et al., 2009; Piasceki et al., 2010). A full deletion of the *daf-19* gene impedes the expression of genes that are dependent on DAF-19 for transcription. A complete deletion of the *daf-19* gene correlates with a reduction in synaptic protein levels in adult worms and neurodegenerative-disease-like symptoms as well as the abolition of all ciliary structures on sensory neurons (Swoboda, 2000; Chen et al., 2006; Inglis et al., 2008; Senti & Swoboda, 2008). Consequently, the absence of the *daf-19* gene can lead to diseases of the nervous system, like Alzheimer’s, Huntington’s, and Parkinson’s disease. This neurodegenerative mechanism is important to understand because
orthologs of the \textit{daf-19} transcription factor gene exist in the human genome (Aftab \textit{et al.}, 2008). Versions of the \textit{daf-19} gene, called RFX transcription factor genes, have also been found in \textit{Drosophila}, \textit{Saccharomyces cerevisiae}, \textit{Schizosaccharomyces pombe}, and mouse (Emery \textit{et al.}, 1996; Vandaele \textit{et al.}, 2001; Dubruille \textit{et al.}, 2002). Therefore, RFX transcription factors orthologous to DAF-19, are thought to be conserved across the entire eukaryotic kingdom (Emery \textit{et al.}, 1996). This makes \textit{C. elegans} a powerful model organism to understand the role of RFX transcription factors in neurodegeneration (Driscoll \textit{et al.}, 2003; Link, 2005; Murakami, 2007).

DAF-19 proteins (green ribbons in Figure 5) bind to DNA and control the expression of other genes. More specifically, once bound to a specific DNA sequence, a DAF-19 protein will recruit or activate the assembly of transcription machinery at nearby transcription start sites.

The \textit{daf-19} gene encodes four different versions, or isoforms, of related DAF-19 transcription factor proteins. The availability of different versions of the DAF-19 protein allows for differential gene regulation by different isoforms of the same gene. These \textit{daf-19} isoforms differentially regulate genes found in the nervous system. When the \textit{daf-19} gene is expressed, exon splicing sometimes creates longer or shorter versions of the DAF-19 transcription factor protein. The short versions of

\textbf{Figure 5. DAF-19 RFX transcription factor (green) binding to DNA.} Taken from RFX1 image on Wikipedia.
DAF-19 (DAF-19C and DAF-19M) have been found to bind to specific, evolutionarily conserved DNA sequences called x-boxes in the promoter regions of target genes (Zhang et al., 1993; Efimenko, 2005). Not only do the short versions of DAF-19 activate genes differentially themselves, they also work in conjunction with different x-boxes to regulate the location and intensity of gene expression (Chu et al., 2012; Henriksson et al., 2013). Therefore, the combination of both different DAF-19 transcription factor proteins and different DAF-19 DNA target sequences (x-boxes) contribute to the differential regulation of gene expression.

Only two of the DAF-19 protein isoforms have identified functions (Senti and Swoboda, 2008; Wang et al., 2010). The functions of the smaller proteins, DAF-19C and DAF-19M, are well known. The DAF-19C isoform is expressed exclusively in ciliated sensory neurons, where it activates genes responsible for cilia development (Swoboda, 2000; Dubruille et al., 2002; Chen et al., 2006; Senti & Swoboda, 2008). This process, called ciliogenesis, is responsible for the generation of cilia, which are antennae-like protrusions from the dendrite that permit neuronal cells to sense particular chemicals, for example, in their environment. The DAF-19M isoforms control neuronal gene expression in particular ciliated neurons in the male tail and can be said to specify the function of these neurons (Wang et al., 2010). Thus, DAF-19C specifies a neuron as a ciliated sensory neuron while DAF-19M specifies the identity of particular ciliated sensory neurons in males only.

The daf-19 gene produces two closely related longer proteins called DAF-19A/B whose functions are unknown (Swoboda, 2000; Senti & Swoboda, 2008). In collaboration with researchers at the Karolinska institute, we hypothesize that the DAF-19A/B protein isoforms are
responsible for controlling neuronal gene expression in non-ciliated neurons. Further, Senti & Swoboda (2008) showed that worms lacking all DAF-19 proteins showed marked reductions in synaptic proteins as adults (but corresponding RNA levels were normal) whereas daf-19 null worms expressing DAF-19A protein from an added cDNA clone had normal synaptic protein levels at the same ages. Thus, DAF-19A is said to rescue this synaptic phenotype. We therefore further hypothesize that DAF-19A is responsible for controlling the expression of genes that maintain synaptic protein levels, either directly or indirectly.

The DAF-19 Project

![Figure 6. daf-19 isoforms. daf-19 encodes four different proteins. DAF-19C directs cilia formation. DAF-19M confers some cilia identity in males. DAF-19A/B have unknown functions. Adapted from Wang et al. (2010).]
<table>
<thead>
<tr>
<th>Lab</th>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LU</td>
<td>628</td>
<td>daf-19(tm5562) six times back-crossed</td>
<td>865 bp deletion as follows: 583 bp from intron 1 + 189 bp exon 2 (entire exon) + 93 bases of intron 2. Only exon 2 in both DAF-19A and B is deleted.</td>
</tr>
<tr>
<td>LU</td>
<td>3063</td>
<td>daf-19(m86)II</td>
<td>Changes an arginine codon to a UGA stop codon (called an opal mutation) in exon 7. Lacks all DAF-19 proteins.</td>
</tr>
<tr>
<td>LU</td>
<td>3738</td>
<td>daf-19(WT); daf-12(sa204)X; him-5(e1490)V</td>
<td>Contains all DAF-19 proteins</td>
</tr>
<tr>
<td>LU</td>
<td>652</td>
<td>daf-19(af6); him-5(e1490)sEX:pC52D10.6</td>
<td>53 bp deletion of the 3' end of exon 4, including the splice site at the 3' end. Mutation affects only DAF-19B isoform</td>
</tr>
<tr>
<td>OF</td>
<td>5</td>
<td>daf-19(af5)II</td>
<td>A net deletion of 12 bases (16 bp deletion and a 4 bp insertion that includes an ATG in the +1 reading frame).</td>
</tr>
<tr>
<td>LU</td>
<td>664</td>
<td>daf-19(m86); daf-12(sa204); him-5 + daf-19C from pGG14 + elt-2::gfp</td>
<td>The daf-19(m86) allele was supplemented with a transgene containing a cDNA copy expressing DAF-19C.</td>
</tr>
<tr>
<td>LU</td>
<td>668</td>
<td>daf-19(m86); daf-12(sa204);him-5 + daf-19A unc-122::gfp and F57B10.9::gfp</td>
<td>The daf-19(m86) allele was supplemented with a transgene containing a cDNA copy expressing DAF-19A.</td>
</tr>
</tbody>
</table>

The long isoforms of daf-19, DAF-19A/B, are implicated in the regulation of genes found in non-ciliated neurons based on the observation that DAF-19A/B isoforms are expressed in all non-ciliated neurons and only in non-ciliated neurons (Senti & Swoboda, 2008). When DNA solely expressing DAF-19A is added to worms missing all other DAF-19 proteins, most behavioral and cellular phenotypes were rescued (Senti & Swoboda, 2008). Therefore, DAF-
DAF-19A/B might regulate genes involved in the maintenance and survival of a healthy nervous system.

DAF-19A/B transcription factor proteins may bind to DNA sequence motifs similar to x-box motifs bound by DAF-19C and M (see Figure 7; Senti & Swoboda, 2008). Alternatively, DAF-19A/B may use other partner proteins, via its dimerization domain, to bind to novel DNA sequence motifs. Once DAF-19A/B target genes have been characterized, we will be able to compare promoter regions of these genes to identify common DNA sequence motifs.

Differential regulation of genes in specific types of neurons by different daf-19 isoforms suggests an important role for daf-19 isoforms in neural development and behavior. In fact, the mutations to DAF-19A/B transcription factor proteins will provide evidence as to whether or not these longer daf-19 isoforms control gene expression necessary for protection against neurodegeneration. Overall, this information highlights the importance of the RFX transcription factor in both behavior and the development of the nervous system.
My research aims to (i) identify neural genes controlled by the DAF-19 RFX transcription factor, (ii) elucidate whether daf-19-target genes are controlled by the DAF-19A/B versions. Finally, (iii) I want to characterize the expression of genes that are DAF-19A/B-regulated, and (iv) identify the cells in which these DAF-19A/B-regulated genes are differentially expressed.

Approach: Biological Tools Used to Explore DAF-19 Gene Regulation:

Transcriptome Analysis

The transcriptome is a collection of mRNA expressed in an organism or tissue at the time that the cells were lysed and RNA collected. De Stasio and Phirke (unpublished) used a microarray containing probes for 18,000 genes to characterize the transcriptome of adult and larval worms (respectively). A comparison of the transcriptome of daf-19 null and isogenic wild
type adult worms suggests both positive and negative regulation of gene expression is correlated with the presence of DAF-19 proteins.

A transcriptome of \textit{daf-19} null animals is a record of all the genes that are activated or suppressed when all DAF-19 protein versions are gone. For example, if a gene is activated only when \textit{daf-19} is gone, this suggests that in a wild type animal, \textit{daf-19} normally represses activity of that gene either directly or indirectly. On the other hand, if a gene is repressed when \textit{daf-19} is gone, but is activated in the presence of \textit{daf-19}, one can conclude that \textit{daf-19} normally activates this gene in a wild type worm. The transcriptome of wild type worms of matched age serves as a control, or list of genes that are normally transcribed when all DAF-19 protein versions are present and functional in \textit{C. elegans}.

The transcriptome analysis using microarrays identified 170 potential DAF-19A/B transcription factor protein targets. Thirty-four of these genes were chosen for further analysis using a transcriptional fusion of the upstream putative control region of our target genes to DNA encoding GFP. These transcriptional fusions were expressed in isogenic strains to assess gene expression patterns in the presence and absence of DAF-19 proteins. Isogenic worms have the same genetic background with the exception of one mutant gene. In this study, \textit{daf-19} wild type (\textit{daf-19(WT)}) and worms lacking all known DAF-19 proteins, \textit{daf-19(m86)} worms, were used to study the regulation of neural genes when DAF-19A/B is mutated or completely non-functional. In addition, the De Stasio lab was able to procure DAF-19A/B-specific mutants and create additional isogenic strains by mating these mutants with worms expressing transcriptional fusions for our target genes (\textit{F57B10.9} and \textit{F52D2.2}). This means that we can
assess how daf-19 target neuronal genes are regulated by the DAF-19A/B protein-isoform. So, not only am I able to distinguish between the effects of different DAF-19 protein versions, I am also able to discriminate between different parts of a specific DAF-19 protein.

**Transcriptional Fusions: Using Fluorescent Proteins to Tag and Visualize a Gene of Interest**

How do we know which genes are and which genes are not controlled by certain DAF-19 protein versions? Many biological tools can be used to signal the presence and/or absence of a particular gene. In my research, I used transcriptional fusions to genetically tag a gene of interest with a gene that encodes a fluorescent protein (Figure 8). Once a transcriptional fusion is made, the fluorescent protein will be expressed whenever and wherever the gene of interest is expressed. Consequently, the fluorescent protein will mimic the expression of any gene we want by physically tagging that gene. These transcriptional fusion constructs are called “transgenes” when they are expressed in an animal or plant.

There are different colors of fluorescent proteins: green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), etc. When one of these fluorescent proteins is illuminated with a particular wavelength of light, that wavelength of light will excite electrons within the fluorescent protein that will absorb a portion of the light and emit a wavelength of light that corresponds to the designated color of the protein. As a result of this color-specific emission and the transparency of *C. elegans*, we are able to spatially and temporally localize the expression of any gene.
This construct is known as a transcriptional fusion because although GFP is produced via the transcriptional control elements of our different target genes, our target genes are not produced into proteins by the plasmid, though these genes are still being expressed from normal, chromosomal copies. GFP is, therefore, produced in the cells that express the transcription factors that bind to the control elements of our target genes, but the GFP protein does not localize to the specific cellular locations where our target gene functions. Therefore,
we are not able to draw inferences about the specific locations within a cell where our target genes (*F57B10.9* and *F52D2.2*) might function by visually localizing the GFP protein.

In order to obtain a temporal representation of reporter gene expression, transgenic worms of all ages were imaged, thus yielding a developmental representation of the expression patterns of any reporter gene. In order to identify the ages of individual worms, I compared the images I produced via confocal microscopy to WormAtlas schematics of the developmental stages of hermaphroditic worms (Appendix 1A). Similarly, to determine the precise cells in which the reporter gene is differentially expressed, I compared the images I produced via confocal microscopy to WormAtlas schematics of neuronal maps (Altun and Hall, 2012). It is important to note that the absence of fluorescence does not mean that neurons are not present. Rather, the absence of fluorescence merely means that the reporter gene is not expressed in the non-fluorescent neurons.

The ability to determine precise expression patterns of each fluorescent, transcriptional reporter gene has allowed us to characterize not only the location and stage of development at which genes are transcribed, but also the number of cells in which a reporter gene is transcribed. Therefore, I have also used transgenes to quantify the difference in the expression patterns of *daf-19 WT* worms and *daf-19* null mutant worms. Quantifying the expression of reporter genes is a measure of the penetrance of a gene.

Quantifying the expression of reporter genes is necessary because the expression patterns of transgenes expressed from extra-chromosomal arrays varies from animal to animal due to genetic mosaicism. The reporter gene is microinjected into the hermaphrodite gonad
along with a co-injection marker and carrier plasmid, known as an extra-chromosomal array. This array is not inherited reliably along with the genomic DNA during cell division. Consequently, an unstable number of transgene arrays will be present in the progeny of transgenic worms. In fact, there will also be slight variations in cell-to-cell expression between individuals of a single strain, thus resulting in mosaicism and an inconsistent pattern of transgene expression. It is therefore necessary to visualize various individual worms in order to deduce a representation of transgene expression patterns.

**Biological Replicates: “Second Lines” of C. elegans with the Same Transcriptional Fusions**

To test whether daf-19 isoforms consistently regulate the same genes, in the same neurons, at the same stages of development, I have used biological replicates of C. elegans with the same transcriptional fusions. These biological replicates are called “second lines” because they are C. elegans strains with transcriptional fusions injected into a different worm’s gonad. This approach has allowed me to deduce whether I see reproducible differences amongst the various daf-19 isogenic mutants. If I had not used second lines of C. elegans strains, the differences in fluorescent gene expression could be attributed to other phenomena.

**Obtaining Different daf-19 mutants:**

C. elegans mutants were obtained from our collaborators in Stockholm (m86, of5, of6) and from the Mitani lab in Japan (tm5562). The latter mutant strain was back-crossed in our lab by Savannah Vogel and Kristen Bischell. Back-crossing mutants reduces any other mutations that the worm strain might have. Back-crossing involves mating the tm5562 mutant 6
generations with the wild type strain, OE3738. This process insures that we are only seeing the effects of an exon 2 deletion to the DAF-19A and B isoforms.

**MATERIALS AND METHODS**

**Maintaining and Constructing C. elegans Strains**

*C. elegans* strains were maintained on 5mm agar plates containing a centered, standardized quantity of the OP50 *E. coli* strain, which *C. elegans* fed on *ad libitum*. These plates were kept at 20°C.

*C. elegans* strains were made with varying *daf-19* genetic backgrounds. Consequently, worms that contained a transgene only had genetic variability in regards to the integrity of the *daf-19* gene. Therefore, in a single strain of *C. elegans*, identical transgene arrays were transferred to worms with different *daf-19* alleles by mating. Consequently, this approach allowed us to visualize differential transcription of the fluorescent protein in varying *daf-19* genetic backgrounds *in vivo*.

**Dye-Fill Assays:**

Dil is commonly used as an anterograde dye. That is, Dil is first absorbed by the cilia on the dendritic ends of neurons (Figure 4), and it then travels to the axonal projection via the neuronal body. The ability to absorb Dil requires cilia, therefore, only animals in which DAF-19C is expressed to drive ciliogenesis—the generation of cilia—are able to absorb the Dil. This anterograde dye is, therefore, useful to distinguish *C. elegans* with cilia and therefore expressing DAF-19C from worms lacking this isoform and therefore also lacking cilia.
Worms were washed off agar plates with 1 mL of M9 buffer and placed into a 1 mL Eppendorf. This mix was centrifuged and most of the M9 buffer removed along with bacteria, leaving a loose pallet of worms. A sufficient volume of M9 was added to the tube to bring the total volume to 0.5 mL. 3 μL of Dil (2mg/mL) was added to the tube mixture. Worms were then incubated in the Dil-M9 mixture for one hour at 25°C. Worms were then washed in M9 buffer once and placed on streaked agar plates containing a standard dose of the OP50 E. coli strain. Worms were then allowed to eat and defecate for at least 12 hours to remove dye from the intestinal tract prior to visualization by fluorescent microscopy. This 12 hour recovery period also helped reduce the intensity of the fluorescent signal from Dil in neurons, thus providing clearer confocal images.

Confocal Microscopy Preparation and Image Acquisition

Healthy transgenic worms from non-starved NGM plates were collected into an Eppendorf tube by washing their plates with 1mL of M9 buffer. 1.5mL of 2% agarose in M9 buffer was then heated and mixed with 15μL of sodium azide, to temporarily paralyze the worms. A thin, circular gel pad was formed by pipetting 5μL of the agarose mixture onto the center of a glass slide. The circular pad was used to immobilize transgenic worms for confocal imaging. 3 to 5μL of M9 buffer containing transgenic worms was added onto the gel and a coverslip was placed on top to prevent it from drying and to facilitate imaging with the use of a thin piece of glass.

Transgenic worms were imaged with a Leica confocal microscope. Argon and HeNe 543 lasers at 10% power were used along with detectors (HyD) that were set to capture the
wavelengths of light emitted as GFP and dsRED or Dil (for dye-filled worms). Display colors were set as green and red, respectively.

RESULTS:

The data collected in the current study supports the hypothesis that F57B10.9 and F52D2.2 are differentially regulated in specific neurons by DAF-19A/B isoforms. To accomplish this goal, I examined daf-19 isogenic strains that carried either F57B10.9 or F52D2.2 chimeric transgenes. That is, I produced worm strains that had mutations in the daf-19 gene and then mated each of these strains, separately, with a single transgenic strain carrying the GFP reporter gene for either F57B10.9 or F52D2.2. Specifically, I produced transgenic strains with the of5, tm5562, and of6 variations of daf-19 to determine if expression of F57B10.9 and F52D2.2 transgenes is DAF-19A/B dependent. Finally, I compared the images I produced using confocal microscopy to neuronal schematics in WormAtlas, and thus identified the specific neurons in which the chimeric transgenes were differentially expressed. By identifying the neurons expressing the transgene and understanding what the typical functions of these neurons are, we may be able to gain novel insight into the function of the F57B10.9 and F52D2.2. Analysis of transcriptional GFP fusion expression patterns by confocal microscopy revealed reproducible expression of GFP in a subset of neurons in hermaphrodites. A description of each gene and a detailed analysis of the expression pattern of each transcriptional fusion is provided below.
The F57B10.9 Gene

F57B10.9 is orthologous to the human gene SPG20, or Spg-20 (Shaye and Greenwald, 2011; Truong et al., 2015). In fact, a Clustal Omega protein sequence alignment of these genes found them to have 65% similarity. Mutations in SPG20 which result in the complete loss of expression of the protein spartin lead to Troyer syndrome (Patel et al., 2002), which is characterized by spasticity of the leg muscles, progressive muscle weakness, paraplegia, muscle wasting in the hands and feet (distal amyotrophy), small stature, developmental delay, learning disorders, speech difficulties (dysarthria), and mood swings, exaggerated reflexes in the lower limbs (hyperreflexia), skeletal abnormalities, and a bending outward of the knees (valgus) (Bakowska et al., 2008). The name SPARTIN thus derives from these symptoms: Spastic Paraplegia Autosomal Recessive Troyer syndrome.

Spartin also shares sequence similarity with spastin (Ciccarelli et al., 2003). The sequence shared by spartin and spastin is similar to that of proteins involved in the morphology and membrane trafficking of endosomes—compartments of the endocytotic membrane transport pathway that originate in the Golgi membrane (Phillips et al., 2002). Most notably, spastin is thought to be involved in a particular mechanism of endosomal transportation that involves microtubules (Phillips et al., 2002). When overexpressed, spastin binds to microtubules and this action results in the redistribution the microtubule array. Spartin shares the N-terminal region of spastin that is capable of binding to microtubules (Ciccarelli et al., 2003; Renvoise et al., 2010). Therefore, spartin is predicted to also be involved with microtubule interaction and trafficking.
In the nervous system, modest \textit{SPG20} expression has been found in fetal and adult human brains, including the amygdala, cortex, and thalamus (Auer-Grumbach \textit{et al.}, 1999; Manzini \textit{et al.}, 2010). Lower levels of expression have been found in the hippocampus and cerebellum. In the mouse brain, \textit{SPG20} has similar expression levels to the human brain, except that higher levels of expression were found in the hippocampus and spinal cord (Manzini \textit{et al.}, 2010). Additionally, \textit{SPG20} has been found to be developmentally regulated, with maximum expression at midgestation and embryonic day 10 with a precipitous decline thereafter. Even though \textit{SPG20} is present in nervous tissue in fetal stages, only low levels of it are expressed in the adult mouse brain. Interestingly, \textit{SPG20} is present in only low levels of expression in neurons and glia of the hippocampus and forebrain, and throughout the cerebellum, brainstem, and spinal cord (Manzini \textit{et al.}, 2010). Due to the large differences in the fluctuation of \textit{SPG20} expression levels, it is thought that \textit{SPG20} plays a role in morphogenesis and differentiation at several sites, including the initial frontonasal mass/forebrain, craniofacial structures, aortic arch/heart primordium, and limb buds during morphogenesis in mice (Manzini \textit{et al.}, 2010). For these reasons, \textit{F57B10.9} was chosen from the list of putative \textit{daf-19} regulated genes as possibly having a role in neuronal function.

\textbf{Characterizing \textit{F57B10.9} Gene Expression}

Confocal microscopy was used to image \textit{F57B10.9::GFP} transgene expression in worms with different \textit{daf-19} genetic backgrounds. \textit{F57B10.9::GFP} expression was assessed in male and hermaphrodite worms of all larval stages of development including adulthood. Specifically, \textit{F57B10.9::GFP} expression was assessed and recorded in the IL2 neuronal pair, the pharyngeal
region near the nerve ring; ventral, dorsal, and lateral nerve cords; and in the intestine, body wall muscle, hypodermis, epidermis, vulva, and tail of each worm (Appendix 1B). Consequently, I was able to quantify the expression of the reporter transgene in both sexes and deduce a representation of $F57B10.9::GFP$ expression patterns throughout development.

Previous research by McKay and colleagues (2003) has shown that $F57B10.9::GFP$ expression occurs in the nerve ring; ventral, dorsal, and lateral nerve cords; and in amphid, mechanosensory, pharyngeal, and tail neurons of adult worms. Korzynski (2012), Zhang (2013), and Hurlburt (2014) found similar expression patterns in worms containing all DAF-19 proteins ($daf-19(WT)$) and in worms lacking all DAF-19 proteins ($daf-19(m86)$). Overall, they found that $F57B10.9::GFP$ was consistently expressed in nerve cords, ventral nerve cord motor neurons, body neurons, tail neurons, and in approximately 20 neurons in the pharyngeal region. In addition to its significant presence and role in the nervous system, a 1.58 fold up-regulation of $F5B10.9$ mRNA in adult $daf-19$ mutant worms, makes $F57B10.9$ a promising DAF-19A/B target gene. I used previous records of $F57B10.9::GFP$ tissue and neuronal expression to inform my own investigations. Furthermore, I searched for and recorded the expression of the $F57B10.9::GFP$ transgene in the tissues where the transgene was previously observed to be expressed by McKay et al. (2003), Korzynski (2012), Zhang (2013), and Hurlburt (2014).

$F57B10.9::GFP$ expression in $daf-19(WT)$ worms, which contain all DAF-19 isoforms

In worms expressing all DAF-19 proteins, $daf-19(WT)$ worms, $F57B10.9$ transgene expression was found to be consistently activated in the ciliated IL2 neurons (indicated by a dotted square in Figure 9A) and a small cluster of non-ciliated neurons found in the isthmus.
region (Appendix 1B) of the pharynx of hermaphrodite worms (indicated by a dotted circle in Figure 9A; and Hurlburt, 2014). One hundred percent of worms, 59 total, with a daf-19(WT) genetic background displayed F57B10.9::GFP expression in the ciliated IL2 sensory neurons. In addition, one hundred percent of worms displayed expression of the reporter gene in at least 20 neurons in the pharyngeal region, posterior to the IL2 neurons. Further F57B10.9::GFP expression was also observed in the nerve cords (92%), body neurons (93%), 3-5 tail neurons (95%), and body wall muscle (63%). These results were consistent across worms of all stages of larval development and adulthood. In summary, one hundred percent of daf-19(WT) worms, which contain all DAF-19 proteins, display F57B10.9::GFP expression in the ciliated IL2 neuronal pair.

F57B10.9::GFP expression in daf-19(m86) worms, which lack all DAF-19 isoforms

Recall that worms that have an m86 mutation in the daf-19 gene lack all DAF-19 proteins and, therefore, cannot produce cilia to absorb Dil. Consequently, these mutants were identified by their inability to dye-fill and their green transgene fluorescence.

In contrast to daf-19(WT) worms, F57B10.9::GFP transgene expression in worms (N = 31) lacking all DAF-19 proteins (daf-19(m86) mutants) was never found in the ciliated IL2 neuronal pair (indicated by the dotted square in Figure 9B). One hundred percent of all daf-19(m86) mutants showed transgene expression in at least 20 neurons in the pharyngeal region, posterior to the ciliated IL2 neurons (indicated by the dotted circle in Figure 9B). However, compared to daf-19(WT) worms, F57B10.9::GFP transgene expression in daf-19(m86) mutants was found in a larger number of neuronal bodies posterior to the anterior bulb of the pharynx.
Additional transgene expression was also found in the ventral nerve cords (97%), body neurons (84%), 3-5 tail neurons (97%), and body wall muscle (10%). These results were consistent across worms of all stages of larval development and adulthood. Conclusively, worms lacking all DAF-19 proteins do not express the F57B10.9::GFP transgene in IL2 neurons, but do express the transgene in a larger number of posterior neuronal bodies than daf-19(WT) worms.

The identity of IL2 neurons was previously determined by Alex Hurlburt (2014); he also found no age-related difference in transgene expression. The identity of IL2 neurons was confirmed through a modified calcium acetate dye-fill assay (Appendix 1D; and Tong and Burglin, 2010; Hurlburt, 2014) that specifically stains IL2 neurons (indicated by white arrows in Figure 20). Since IL1 neurons are also ciliated and they structurally resemble IL2 neurons, it was especially crucial to eliminate the IL1 neurons as a possible location for differential F57B10.9::GFP expression. The only noticeable morphological difference between IL1 neurons and IL2 neurons is the shape of their axonal projections. The axonal projections of IL2 neurons have the shape of hooks and they should not interlink (Figure 9). In contrast, the axonal projections of the IL1 neurons are barely separated from one another and they are much more dispersed (Figure 9). Notice how the morphology of these two different types of neurons is indistinguishable (Figure 9).

Thus far, this investigation has added to the findings of Korzynski (2012), Zhang (2013), and Hurlburt (2014) in that daf-19(WT) worms and worms lacking all DAF-19 proteins (daf-19(m86)) showed differences in F57B10.9::GFP expression pattern. Most notably, daf-19(WT) worms express F57B10.9::GFP in IL2 neurons while daf-19(m86) mutants did not. In addition,
**daf-19(m86)** mutants showed transgene expression in a larger number of neuronal bodies in the posterior region of the pharynx. Consequently, worms with a **daf-19(WT)** and **daf-19(m86)** genetic background have been pivotal in understanding the effects of DAF-19 proteins on **F57B10.9::GFP** expression. However, these genetic backgrounds are not sufficient to understand the isoform-specific roles of different DAF-19 isoforms on **F57B10.9::GFP** expression. Thus, the rest of my investigations involved characterizing the expression patterns of the **F57B10.9::GFP** transgene in different DAF-19A/B genetic backgrounds.
Figure 9. Axonal projections of IL1 neurons and IL2 (Fig 10) neurons are indistinguishable.
Ek-Vazquez, 30

**F57B10.9::GFP expression in daf-19(of5) worms, which lack DAF-19A and B isoforms**

*F57B10.9::GFP* transgene expression in worms lacking the DAF-19A/B proteins, that is, worms with a *daf-19(of5)* mutant background, revealed GFP expression in the ciliated IL2 neurons (indicated by dotted square in Figure 12D) of every worm analyzed (N = 41).

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Left-side image of IL2 neurons (6 total) taken from WormAtlas.

Calcium acetate staining to identify IL2 neurons (white arrow). Green: *F57B10.9::GFP*. Red: DiI. Image taken from Alex Hulrburt (2014). White scale bars are 20 µm.

*daf-19(WT)* worms display *F57B10.9::GFP* expression in IL2 neurons (white arrow). Green: *F57B10.9::GFP*. Red: DiI. Image taken from Alex Hurlburt (2014). White scale bars are 20 µm.

**Figure 10. Using calcium acetate staining to identify IL2 neurons.**
Interestingly, 20% of the worms analyzed showed *F57B10.9::GFP* expression in only two of the three IL2 neuronal pairs. The IL2 neuronal pair that did not exhibit *F57B10.9::GFP* expression varied tremendously; thus, *F57B10.9::GFP* expression was not consistently present or absent in a specific neuronal subset of IL2 pairs. On the other hand, *F57B10.9::GFP* expression was observed in all three IL2 neuronal pairs in 70% of the worms analyzed.

In addition, *F57B10.9::GFP* expression was always found in a cluster of neuronal bodies posterior to the IL2 neuronal pairs (indicated by dotted circle in Figure 12D). Even more specifically, neuronal bodies were always found both anterior and posterior to the nerve ring. The number of posterior neuronal bodies expressing *F57B10.9::GFP* is much larger in daf-19(*of5*) animals than that seen in daf-19(WT) animals. In fact, the larger number of posterior neurons expressing the transgene resembles the transgene expression levels present in daf-19(*m86*) mutants.

Additional expression of the transgene was observed in the nerve cords (100%), body wall muscle (93%), and tail neurons (100%). Transgene expression was not, however, observed in the intestine (0%) or vulva’s (0%) of worms. The expression of the transgene in all of these different tissues and neurons did not differ across different larval stages of development or adulthood. Transgene expression patterns were developmentally consistent from the first larval stage (L1) to old adult worms (OA).
**F57B10.9::GFP expression in daf-19(tm5562) worms, which lack exon two of DAF-19A/B**

Worms that have a tm5562 mutation in the daf-19 gene lack exon two of the DAF-19 A and B isoforms and, therefore, can still produce cilia to absorb Dil. Consequently, these mutants were identified by their green transgene fluorescence and their ability to dye-fill.

Similar to daf-19(WT) worms, F57B10.9::GFP transgene expression in daf-19(tm5562) worms was always found in ciliated IL2 neurons (indicated by dotted a square in Figure 12C). However, F57B10.9::GFP transgene expression in daf-19(tm5562) mutants was consistently found in a larger number of neuronal bodies posterior to the anterior bulb of the pharynx (indicated by dotted circle in Figure 12C). Transgene expression in daf-19(tm5562) mutants occurred in a larger number of posterior neurons than transgene expression in daf-19(WT) worms (indicated by the dotted circle in Figure 11A). Transgene expression in the posterior neurons of daf-19 (tm5562) mutants is, therefore, most like that of daf-19(m86) mutants. However, transgene expression in the ciliated IL2 neurons of daf-19(tm5562) mutants is most similar to transgene expression in the IL2 neurons of mutants which lack all DAF-19 proteins, daf-19(m86) mutants (indicated by dotted square in Figure 11B). Additional transgene expression was also found in the ventral nerve cords (95%), body neurons (100%), 3-5 tail neurons (92%), and body wall muscle (92%). However, transgene expression was not observed in the intestine (0%) or vulva’s of daf-19(tm5562) worms (0%).

While these transgene expression patterns were found to be consistent across all stages of larval development and adulthood, it is important to note that the high consistency of F57B10.9::GFP expression in body wall muscle was unique to daf-19(tm5562) mutants (92%)
and daf-19(WT) worms (63%). In contrast, only ten percent of daf-19(m86) worms displayed F57B10.9::GFP expression in the body wall muscle. Therefore, while F57B10.9::GFP expression is downregulated in response to the absence of all DAF-19 proteins (daf-19(m86) mutants), its expression is aberrantly upregulated when worms lack only the amino acids encoded by exon two of the DAF-19A/B isoforms—the daf-19(tm5562) mutation.

**F57B10.9::GFP expression in daf-19(of6) worms, a mutation to DAF-19B**

The of6 mutation only alters the DAF-19B isoform. By altering the DAF-19B isoform of a F57B10.9::GFP transgenic strain, I was able to visualize and understand how these alterations affect the F57B10.9::GFP expression. The following results are, therefore, a record of how F57B10.9::GFP expression varies in a genetic background where the DAF-19B isoform has been altered. It is important to recall that transgene expression was carefully documented in the specific tissues and cells that include the IL2 neurons; the dorsal, ventral, and lateral nerve cords; ventral nerve cord motor neurons, intestine, body wall muscle, hypodermis, epidermis, vulva, and tail of each worm (N = 24).

F57B10.9::GFP expression in a daf-19(of6) mutant background was always found in IL2 neurons (indicated by a dotted square in Figure 12E) and in the posterior cluster of neurons (indicated by a dotted circle in Figure 12E). Specifically, transgene expression always occurred in cells both anterior and posterior to the nerve ring. However, F57B10.9::GFP transgene expression did not occur in as many cell bodies as in the daf-19(m86) genotype. In fact, it seems to be expressed in few neuronal bodies—an expression pattern very similar to that of F57B10.9::GFP expression in a daf-19(WT) background (indicated by dotted circle in Fig. 11A).
Additional \textit{F57B10.9::GFP} expression was found in the ventral nerve cords (100%), body neurons (100%), body wall muscle (83%), hypodermis (96%), and tail neurons (100%). While the transgene was expressed in multiple tissues, the expression of the transgene remained consistent throughout all larval stages and adulthood. In summary, worms with an altered DAF-19B isoform express the \textit{F57B10.9::GFP} transgene in IL2 neurons, but they only express the transgene in a small number of neuronal bodies posterior to the IL2 neurons.

\textbf{\textit{F57B10.9::GFP} expression in worms that overexpress the DAF-19C isoform}

In a doubly transgenic strain in which the \textit{daf-19(m86)} allele was supplemented with a transgene containing a cDNA copy expressing DAF-19C, the \textit{F57B10.9::GFP} transgene was always expressed in all three IL2 neuronal pairs (indicated by a dotted square in Figure 13F). That is, when only DAF-19C is overexpressed in worms that previously lacked transgene expression in IL2 neurons, \textit{F57B10.9::GFP} expression is “rescued” in all three IL2 neurons with one hundred percent penetrance. That is, \textit{F57B10.9::GFP} expression goes back to “normal” when DAF-19C is overexpressed in worms that previously lacked all DAF-19 proteins. This transgene expression pattern in the IL2 neurons, therefore, resembles that seen in worms containing all DAF-19 proteins (\textit{daf-19(WT)} worms). Overall, transgene expression in IL2 neurons is similar in worms that exclusively overexpress DAF-19C, \textit{daf-19(WT)} worms, \textit{daf-19(of6)} mutants, \textit{daf-19(of5)} mutants, and \textit{daf-19(tm5562)} mutants.

In worms that overexpress only the \textit{DAF-19C} isoform, \textit{F57B10.9::GFP} expression always occurred in a small cluster of neurons posterior to the IL2 neurons (indicated by a dotted circle in Figure 13F). More specifically, the transgene expression that occurred in neurons posterior to
the IL2 neurons occurred in both anterior and posterior locations relative to the nerve ring. Low levels of transgene expression in the posterior neurons were also seen in \textit{daf-19(WT)} worms. Additional \textit{F57B10.9::GFP} expression was found in the ventral nerve cords (83%) and tail neurons (100%). The expression patterns that I observed in this DAF-19C rescue were consistent in at all stages of development and adulthood. I can thus conclude that DAF-19C is not responsible for WT expression of \textit{F57B10.9} in neurons posterior of the nerve ring.

\textbf{\textit{F57B10.9::GFP} expression in worms that overexpress the DAF-19A isoform}

In a doubly transgenic strain in which the \textit{daf-19(m86)} allele was supplemented with a transgene containing a cDNA copy expressing DAF-19A, the \textit{F57B10.9::GFP} transgene was never expressed in IL2 neurons (indicated by dotted square in Figure 13G). That is, transgene expression in the IL2 neurons of worms that previously lacked all DAF-19 proteins, was not rescued with the overexpression of DAF-19A. Therefore, DAF-19A overexpression was not sufficient to activate transgene expression in the ciliated IL2 neurons and produce a genotype and phenotype that resembles transgene expression in \textit{daf-19(WT)} worms.

Worms overexpressing DAF-19A always show \textit{F57B10.9::GFP} expression in the same 3 cell bodies (indicated by dotted circles in Figure 13G), which reside in the general pharyngeal region. Two of these three cell bodies were identified as neurons. To identify which specific neurons these were, I compared the confocal images that I produced to WormAtlas schematics of neurons in the pharyngeal region (Appendix 1B). The morphology of their axonal projections, cell bodies, and dendritic branching resembles that of the I5 and either the OLQ or OLL neuron.
### Fig 14. Summary of \textit{F57B10.9::GFP} Expression in Different \textit{daf-19} Backgrounds

<table>
<thead>
<tr>
<th>\textit{daf-19} mutations</th>
<th>\textit{F57B10.9::GFP} Expression in IL2s</th>
<th>Posterior Cluster of Neurons Expressing \textit{F57B10.9::GFP}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{daf-19(WT)}</td>
<td>+</td>
<td>Few (at least 20)</td>
</tr>
<tr>
<td>\textit{daf-19(m86)}</td>
<td>-</td>
<td>Many (at least 20)</td>
</tr>
<tr>
<td>\textit{daf-19(tm5562)}</td>
<td>+</td>
<td>Many (at least 20)</td>
</tr>
<tr>
<td>\textit{daf-19(of5)}</td>
<td>+</td>
<td>Many (more than 20)</td>
</tr>
<tr>
<td>\textit{daf-19(of6)}</td>
<td>+</td>
<td>Few (at least 15)</td>
</tr>
<tr>
<td>\textit{DAF-19C Overexpression}</td>
<td>+</td>
<td>Few (4-6 neurons)</td>
</tr>
<tr>
<td>\textit{DAF-19A overexpression}</td>
<td>-</td>
<td>Few (3 neurons)</td>
</tr>
</tbody>
</table>
**F52D2.2 (rgs-8.1) Gene Background**

The F52D2.2 gene was chosen for analysis based on its orthology to the human gene, RGSL1 (regulator of G-protein signaling like 1) and members of the RGS (regulators of G-protein signaling) family including RGS13. F52D2.2 gene has five exons and it encodes a protein that regulates other proteins. More specifically, F52D2.2, or rgs-8.1, is a regulator of G-protein signaling. G proteins—which belong to a group of enzymes called GTPases—are a family of proteins inside cells that transmit stimuli outside of a cell to the cell’s interior. The activity of G proteins is regulated by factors that control their ability to bind to and hydrolyze GTP and GDP. When G proteins are bound to GTP, they are activated. However, when they are bound to GDP, they are deactivated.

Evidence of differential F52D2.2::GFP expression in worms of different developmental stages with different daf-19 genetic backgrounds made the F52D2.2 gene an especially interesting DAF-19 target gene to explore. The transcriptome microarray of three-fold stage embryos (indicated by a red circle in Appendix 1C) revealed that, when compared to F52D2.2::GFP expression levels in daf-19 wild type embryos, daf-19(m86) embryos demonstrated a 0.66 fold decrease in the levels of transgene expression (Phirke, 2011). Meanwhile, the expression levels of the transgene was less severely impacted in daf-19 mutant worms at the L1 larval stage of development. In contrast, De Stasio’s analysis of adult worms revealed that in daf-19(m86) mutants, transgene expression levels were reduced (0.36 fold) when compared to the expression levels of the transgene in daf-19 wild type worms (De Stasio, unpublished).
Due to its reduced levels of expression across different stages of development in the presence and absence of daf-19, F52D2.2 was investigated as a possible DAF-19A/B target gene. Bonnie Arbuckle (unpublished) from the De Stasio lab previously characterized the expression of the F52D2.2::GFP transgene in both a daf-19(WT) and daf-19(m86) genetic background.

Characterizing F52D2.2 Gene Expression

A transcriptional fusion consisting of 2677 base pairs of DNA upstream of the ATG start codon of F52D2.2 was fused to the coding region of GFP, thus yielding the F52D2.2::GFP transgene that spatially and temporally mimics the expression of the F52D2.2 gene. Confocal microscopy was used to image and analyze F52D2.2::GFP expression in male and hermaphrodite worms with different daf-19 genetic backgrounds at all stages of larval development and adulthood. Consequently, I was able to quantify the expression of the reporter gene in both sexes and deduce a representation of F52D2.2::GFP expression patterns throughout development. Finally, I used previous records of F52D2.2::GFP cell and tissue expression to inform my own investigations. Furthermore, I searched for and recorded the expression of the F52D2.2::GFP transgene in the tissues where the transgene was previously observed to be expressed by Bonnie Arbuckle (unpublished).

Bonnie Arbuckle (unpublished), from the De Stasio lab, noted F52D2.2::GFP expression in worms with a daf-19+ and a daf-19− genetic background. While Arbuckle recorded the expression of the transgene in various cells and tissues, she eventually determined that the only transgene expression differences amongst daf-19+ and a daf-19− worms existed in the ventral in
the I2 neuronal pair (Figure 15). I have confirmed the identities of these neurons by comparing Arbuckle’s confocal images to neuronal schematics on WormAtlas (Figure 15).

In *daf-19(WT)* animals (N = 37), Arbuckle noticed that 25% of worms analyzed expressed the transgene in the ventral nerve cord (indicated by white arrows in Figure 16A). However, none of these worms displayed transgene expression in I2 neurons (indicated by dotted circle in Figure 16A). Arbuckle’s findings revealed additional transgene expression in tail neurons (100%). However, transgene expression was never observed in the body wall muscle, hypodermis, or epidermis of *daf-19(WT)* worms. The expression patterns observed both by me and by Bonnie Arbuckle are representative of all stages of larval development and adulthood. Patterns of F52D2.2::GFP expression are, therefore, consistent throughout the development of *C. elegans*.

**F52D2.2::GFP expression in daf-19(m86) worms, which lack all DAF-19 isoforms**

Next, the F52D2.2::GFP extrachromosomal array was transferred to *daf-19(m86)* worms (N = 36), which lack all DAF-19 isoforms. F52D2.2::GFP expression was imaged and analyzed in the I2 neurons and in the VNC of worms with a *daf-19(m86)* genetic background. As a result, Arbuckle (unpublished) noticed that the transgene was expressed in the VNC (indicated by white arrows in Figure 16B) with 60% penetrance. Thus, transgene expression in the VNC was preserved in the absence of all DAF-19 proteins. It is important to note that the penetrance of the transgene’s expression in the VNCs of worms analyzed was higher in worms with a *daf-19(m86)* background, 60%, than worms with a *daf-19(WT)* background, 25%.
Arbuckle also noticed that, in 70% of worms, the transgene was expressed in an additional pair of neurons—the I2 neurons (indicated by the dotted circle in Figure 16B). Therefore, in daf-19(m86) mutants, the transgene is consistently expressed in an extra pair of neuronal bodies when compared to transgene expression in daf-19(WT) worms. My own investigations confirmed Arbuckle’s findings and revealed additional transgene expression in tail neurons (100%). However, I did not find transgene expression in the body wall muscle, hypodermis, or epidermis of daf-19(WT) worms. The expression of the transgene was variable, but overall, it was consistent across all stages of larval development and adulthood.
Figure 15. *F52D2.2::GFP* expression in I2 neurons is only seen in worms with a *daf-19(m86)* and a *daf-19(of5)* genetic background. Notice the similarity in the unique anterior process (green arrows) of I2 neurons. I2 neurons were identified by comparing the images I produced via confocal microscopy to I2 neuronal schematics on WormAtlas. White bars: 20 µm.
**F52D2.2::GFP expression in daf-19(of5) worms, which lack the DAF-19A and B isoforms**

While transgene expression was characterized in the presence and absence of all DAF-19 isoforms, analysis of *daf-19(WT)* and *daf-19(m86)* worms is not sufficient to understand the isoform-specific roles of DAF-19A/B. The chimeric transgene was, therefore, transferred to *daf-19(of5)* mutants (N = 30), which possess a mutation that renders the DAF-19A and B isoforms non-functional. Consequently, the following results characterize the expression patterns of *F52D2.2::GFP* in worms lacking solely the DAF-19A/B isoforms.

Seventy percent of *daf-19(of5)* worms analyzed displayed *F52D2.2::GFP* expression in the I2 neuronal pair (indicated by a dotted circle in Figure 17D). Transgene expression was also found in the VNCs of all worms analyzed (indicated by white arrow in Figure 17D). This transgene expression pattern is similar to that seen in *daf-19(m86)* mutants. Additional transgene expression was found in other body neurons (13%), cells near the vulva (< 1%), hypodermis (< 1%), and tail neurons (100%). While transgene expression in *daf-19(of5)* mutants was variable, especially in body neurons, it is important to note that these results are a collective representation of mosaic, extra-chromosomal *F52D2.2::GFP* expression across all developmental stages including adulthood. The expression patterns provided are, therefore, developmentally consistent.

**F52D2.2::GFP expression in daf-19(tm5562) worms, which lack exon two of DAF-19A/B**

The previous investigation of *F52D2.2::GFP* expression in mutants lacking solely DAF-19A/B was further narrowed by exploring the role of a specific amino acid sequence in the DAF-19A/B isoforms. The *daf-19(tm5562)* mutant provides the opportunity for such an analysis. The
tm5562 mutation in daf-19 is a deletion to solely the second exon of both DAF-19A and B isoforms. By transferring the F52D2.2::GFP transgene to a daf-19(tm5562) genetic background, I was able to visualize and record the expression patterns of the transgene in worms that lack the amino acid sequence encoded by exon 2 of the DAF-19A and B isoforms.

Confocal imaging of several daf-19(tm5562) worms (N = 36) revealed the complete absence of F52D2.2::GFP expression in I2 neurons (indicated by a dotted circle in Figure 17C). Therefore, a complete loss of the amino acids encoded by exon 2 of the DAF-19A/B isoforms does not result in transgene expression in the I2 neurons. It is important to note, however, that F52D2.2::GFP expression still occurs in the ventral nerve cord (25%, white arrow in Figure 17C). The expression patterns collected via confocal microscopy for daf-19(tm5562) mutants expressing F52D2.2::GFP are consistent throughout all developmental stages.

**F52D2.2::GFP expression in daf-19(of6) worms, a mutation to DAF-19B**

Worms (N = 36) with a mutation to DAF-19B never showed F52D2.2::GFP expression in the I2 neurons (indicated by dotted circle in Figure 17E). However, transgene expression in daf-19(of6) mutants was always observed in the VNC of these animals. The overall transgene expression pattern in daf-19(of6) mutants resembles that seen in wild type animals, which contain all DAF-19 proteins. Since transgene expression was not upregulated in the absence of DAF-19B, it clear that the suppression of F52D2.2::GFP expression is not dependent on DAF-19B.
**F52D2.2::GFP expression in worms that overexpress the DAF-19C isoform**

In a doubly transgenic strain in which the *daf-19(m86)* allele was supplemented with a transgene containing a cDNA copy expressing DAF-19C, the *F52D2.2::GFP* transgene was never expressed in I2 neurons (indicated by dotted circle in Figure 18F) or in the ventral nerve cords of animals (indicated by white arrows in Figure 18F). That is, when only DAF-19C is overexpressed in worms that previously displayed transgene expression in I2 neurons, *F52D2.2::GFP* expression is completely lost in all dye-filling worms overexpressing DAF-19C. This complete loss of transgene expression in the I2 neurons and in the VNC is, therefore, a unique expression pattern that is exclusive to worms overexpressing DAF-19C.

**F52D2.2::GFP expression in worms that overexpress the DAF-19A isoform**

In a doubly transgenic strain in which the *daf-19(m86)* allele was supplemented with a transgene containing a cDNA copy expressing DAF-19A, the *F52D2.2::GFP* transgene was never expressed in I2 neurons (indicated by dotted circle in Figure 18G).

That is, transgene expression in the I2 neurons of worms that previously lacked all DAF-19 proteins, was not rescued with the overexpression of DAF-19A (indicated by dotted circle in Figure 18G). Therefore, DAF-19A overexpression was not sufficient to activate transgene expression in the ciliated IL2 neurons and produce a genotype and phenotype that resembles transgene expression in *daf-19(WT)* worms.
Worms overexpressing DAF-19A consistently show $F52D2.2::GFP$ expression in 2-5 previously unseen neuronal bodies (indicated by dotted circles in Figure 18G), which reside in the general pharyngeal region. I identified one of these cell bodies as the I5 neuron (blue arrow in Figure 19). To identify which specific neurons these were, I compared the confocal images that I produced to WormAtlas schematics of neurons in the pharyngeal region (Figure 19).
Confocal image shows I5 neuron. Green: F52D2.2::GFP expression in a worm overexpressing DAF-19A is present in several cell bodies, including the I5 neuron (blue arrow).

WormAtlas neuronal schematic of an I5 neuron.

Figure 19. The morphology of the neuron (blue arrow) present in the confocal image (top) matches the unique morphology of the I5 neuronal schematic provided by Worm Atlas.
**Fig. 20 Summary of F52D2.2::GFP Expression in Various daf-19 Backgrounds**

<table>
<thead>
<tr>
<th>daf-19 mutations</th>
<th>F52D2.2::GFP Expression in I2s</th>
<th>F52D2.2::GFP Expression in the VNC</th>
</tr>
</thead>
<tbody>
<tr>
<td>daf-19(WT)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>daf-19(m86)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>daf-19(of6)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>daf-19(of5)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>daf-19(tm5562)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DAF-19C Overexpression</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAF-19A overexpression</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION:

F57B10.9 Expression is both activated and repressed by DAF-19 isoforms

F57B10.9::GFP expression was consistently found in nervous tissue that includes nerve cords, neurons in the pharyngeal region of the head, tail neurons, and along the ventral body wall. Previous work by Alex Hurlburt (2014) revealed the identity of some neuronal bodies where transgene expression was DAF-19 dependent. Most notable, is the difference in the expression of the transgene in the IL2 neurons. Hurlburt (2014) not only identified these neuronal cell bodies as IL2 neurons via a modified calcium acetate dye-filling, but he also determined that only daf-19 worms expressed the transgene in IL2 neurons.

By knocking down and overexpressing different DAF-19 isoforms, I have determined that F57B10.9::GFP expression in IL2 neurons was only observed in worms in which the DAF-19C isoform was expressed. Transgene expression in IL2 neurons was first observed in daf-19 wild type worms, which contain all DAF-19 isoforms. However, when all DAF-19 proteins were knocked out, transgene expression in IL2 neurons was also lost. At this point in the investigation we knew that transgene expression in the IL2 neurons was activated by the daf-19 gene. However, this was not sufficient information to deduce which specific DAF-19 isoform was responsible for activating the expression of the transgene in IL2 neurons. Therefore, the of5, tm5562, of5, and of6 mutations in the daf-19 gene were instrumental to discovering the isoform-specific functions of the different DAF-19 isoforms.

In the De Stasio lab, we specifically seek to understand how the DAF-19A and B isoforms regulate neuronal genes. To understand the functions of the DAF-19A and B isoforms, we
decided to knock down these two DAF-19 isoforms and observe transgene expression in this genetic background in vivo; thus, allowing the intact organism to demonstrate the effects of non-functional DAF-19A/B on F57B10.9::GFP expression. The consistent expression of the transgene in the IL2 neurons of worms lacking both DAF-19A and B isoforms suggests that transgene expression in IL2 neurons is DAF-19A/B independent. DAF-19A/B independence in IL2 neurons is further supported by the consistent expression of the transgene in the IL2 neurons of daf-19(of5), daf-19(tm5562), and daf-19(of6) mutants—all of which contain mutations in only the DAF-19A and/or B isoforms. Moreover, the loss of transgene expression in the IL2 neurons in daf-19(m86) mutants was rescued by solely overexpressing DAF-19C. This suggests that transgene expression in IL2 neurons is DAF-19C dependent. We can, therefore, infer that F57B10.9 expression is also DAF-19C dependent in IL2 neurons. In conclusion, DAF-19C is necessary for the expression of F57B10.9::GFP expression in IL2 neurons. My investigations, however, do not answer whether DAF-19C is sufficient for the transgene expression in IL2 neurons. There are many other proteins that regulate the control elements of gene transcription and mRNA translation.

Compared to daf-19(WT) worms, animals with mutations specific to the DAF-19A and B isoforms consistently showed differential transgene expression in a cluster of neurons posterior to the IL2 neurons. When all DAF-19 proteins were completely abolished, transgene expression was consistently found in a larger number of neuronal bodies located posteriorly to the IL2 neurons (Hurlburt, 2014). At this point in time, the data collected suggested that transgene expression in those posterior neuronal bodies was DAF-19 dependent. More specifically, since transgene expression was upregulated in the absence of all DAF-19 isoforms, it was evident that
F57B10.9::GFP expression was normally repressed by daf-19. However, since we are particularly interested in the isoform specific functions of the DAF-19A and B isoforms, I transferred the transgene into several daf-19a/b mutants.

As hypothesized, F5210.9::GFP expression in the posterior neurons of daf-19(of5) mutants was indeed upregulated. Therefore, completely non-functional DAF-19A and B isoforms are associated with the upregulation of F57B10.9::GFP in neurons posterior to the IL2 neurons. Therefore, it is clear that transgene expression in the cluster of posterior neuronal bodies is normally suppressed by DAF-19A and B isoforms. That is, transgene expression in the aforementioned posterior neurons is dependent on DAF-19A/B for suppression. Interestingly, I have associated this transgene suppression with the specific amino acid sequences encoded by exon two of the DAF-19A and B isoforms.

Transgene expression in daf-19(tm5562) mutants is upregulated, suggesting that, in wild type worms the amino acids encoded by exon two largely contribute to the suppression of transgene expression in the aforementioned posterior neuronal cell bodies. Since a similar transgene expression was seen in daf-19(of5) mutants, I decided to rescue the DAF-19A isoform by overexpressing it. The purpose of overexpressing DAF-19A is to determine whether suppression of the transgene in the posterior neuronal bodies would be re-established to resemble wild type worms. As a result, transgene suppression in the posterior neurons was indeed re-established but to an even greater degree than in wild type worms. The overexpression of DAF-19A, therefore, over-suppresses transgene expression in neuronal
bodies posterior to the IL2 neurons. Another study may, instead, only rescue the amino acids encoded by exon 2 of the DAF-19 A and B isoforms.

**F52D2.2 Expression**

*F52D2.2::GFP* expression was consistently found in the nervous system. With exception to the DAF-19C rescue, transgene expression was always found in the ventral nerve cord of worms. Wild type worms showed consistent transgene expression in the ventral nerve cord. However, in the absence of all DAF-19 proteins, transgene expression was seen in an additional pair of neurons, which I hypothesize to be the I2 neuronal pair. Since transgene expression is upregulated in the absence of DAF-19, this suggests that transgene suppression is dependent of DAF-19. My investigations with the other *daf-19* genetic mutants revealed novel isoform-specific regulatory roles for DAF-19 A and B.

Only *daf-19*(m86) mutants and worms lacking both DAF-19A and B isoforms (*daf-19*(of5) mutants) displayed transgene expression in I2 neurons. Consequently, our hypothesis that DAF-19A/B may regulate *F52D2.2* expression is supported by the data collected from the *daf-19*(of5) mutant. However, further investigation led me to exclude transgene suppression in the I2 neurons as solely DAF-19B dependent. When only DAF-19B is altered, as is the case in the *daf-19*(of6) mutation, transgene expression is not seen in I2 neurons, but only seen in the ventral nerve cord. This suggests DAF-19B is either not involved in the suppression of *F52D2.2* expression, or it is not sufficient to repress the expression of the transgene in I2 neurons.

Since the DAF-19B isoform is either not necessary or sufficient to suppress *F52D2.2::GFP* expression, I continued to investigate the regulatory role of the DAF-19A/B isoforms on
*F52D2.2* expression. The extrachromosomal array was transferred to a *daf-19(tm5562)* genetic background. Consequently, I was able to see the effects of losing exon 2 of the DAF-19 and B isoforms on *F52D2.2* expression. In *daf-19(tm5562)* animals, transgene expression was consistently observed in the I2 neurons, thus suggesting that the amino acids encoded by exon 2 of the DAF-19A and B isoforms is either necessary or sufficient (or both) to suppress the expression of the transgene in I2 neurons.

Overexpressing DAF-19C resulted in the complete suppression of the transgene. Therefore, overexpression of DAF-19C is associated with downregulation of *F52D2.2* expression. It is important to note that wild type levels of DAF-19C expression are associated with transgene expression in the ventral nerve cord. However, once over expressed, DAF-19C represses the transgene in the same location. This study, therefore, has revealed that *F52D2.2* expression and repression is dose dependent on the levels of DAF-19C expression.

By adding back DAF-19A to *daf-19(m86)* mutants, I hoped to determine whether the suppression of the transgene would be re-established. Interestingly, since DAF-19A was not expressed in wild type levels, but instead overexpressed, the transgene was consistently expressed in previously unseen neurons. One of these neurons was identified as the I5 neuron. The effects of DAF-19A overexpression on *F52D2.2* expression has, therefore, proven to be unpredictable.

**CONCLUSIONS AND FUTURE RESEARCH**

This study demonstrated that *F57B10.9* expression is both activated and repressed by DAF-19 proteins. More specifically, I showed that in the IL2 neurons, DAF-19C is responsible for
the activation of *F57B10.9::GFP* expression. Simultaneously, I found that DAF-19 normally represses *F57B10.9::GFP* expression in neuronal bodies posterior to the nerve ring. More specifically, I was able to associate loss of transgene repression with the loss of the amino acids encoded by exon two of the DAF-19A and B isoforms. Next, I determined which DAF-19 isoform, or combination thereof, is necessary and/or sufficient to maintain *F57B10.9::GFP* repression in the posterior neuronal bodies. I found that the *of6* mutation to DAF-19B was not sufficient to inhibit transgene repression in the posterior cluster of neuronal bodies. In these *daf-19(of6)* mutants, DAF-19A was still normally expressed, which suggests that the integrity of DAF-19A expression is sufficient to maintain repression in the posterior cluster of neurons. Moreover, this suggests that the amino acids rendered non-functional in *daf-19(of6)* mutants are not necessary for transgene repression in the posterior cluster of neurons. This study also demonstrated that *F52D2.2::GFP* suppression is maintained by the amino acid sequences encoded by exon 2 of the DAF-19A and B isoforms. Further investigations could focus on identifying the specific neurons in which *F57B10.9* and *F52D2.2* are differentially expressed. Understanding the role of these specific neurons in the nervous system can guide future investigations into how the DAF-19 isoforms might affect the function of specific neural circuits.

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References:


Appendix 1A. *C. elegans* larval stages of development.
Appendix 1B. Morphology of C. elegans Hermaphrodite.
Appendix 1C. Life Cycle of *C. elegans*, taken from “Introduction to *C. elegans* Anatomy” (Altun et al., 2002). Circled (red) embryonic stage represents the 3-fold embryonic stage.