Regulators of VC Motor Neuron Differentiation and Specification in *C. elegans*

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THESIS

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Dedication

La Familia Perez

"Toda descripción, por objetiva e ingenua que parezca, constituye interpretación personal, punto de vista propio del autor. Sabido es que el hombre mezcla a todo su personalidad, y cuando cree fotografiar el mundo exterior, a menudo se contempla y se retrata a sí mismo."

Santiago Ramón y Cajal

Reglas y Consejos Sobre Investigación Científica

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List of Abbreviations

ACh	Acetylcholine
bHLH	basic helix loop helix
bp	base pairs
cDNA	complementary DNA
CRISPR	clustered regularly interspaced palindromic repeats
Dpy	Dumpy
Egl	Egg laying defective
GABA	gamma-Aminobutyric acid
GMC	ganglion mother cell
HSN	hermaphrodite specific neurons
Kb	kilobases
MCM	mystery cell of the male
N2	C. elegans WT Bristol strain
NB	neuroblast
PCR	polymerase chain reaction
VC	ventral cord type C motor neuron
VNC	ventral nerve cord

Summary

Nervous system development is in part regulated by the coordinated action of proneural genes. Classical proneural gene function occurs in the early stages of neurogenesis to promote neural precursor fate from a stem cell. Subsequently, neurodevelopmental stages include the differentiation, maturation, axon branching, and terminal differentiation of a specific neuronal subtype. All neurons pass through these developmental stages to mature and acquire their unique identity in a network of other neurons. This work investigated the role of conserved bHLH genes in nervous system development.

The model organism, *C. elegans*, contains a sex-specific motor neuron class, the VCs, that are not well characterized. They are the only sex-specific class of motor neurons in the ventral nerve cord and make up 6 out of 302 neurons found in the hermaphrodite. Their subclassification is position-dependent, but the mechanisms governing their class and subclass identities are not well understood.

Herein this work addresses how the VC class acquires their differentiation features, and whether proneural gene function of the family of basic helix-loop-helix (bHLH) DNA-binding proteins affects their development. Does the gene *hlh-3* have a role in VCs? Does the gene *hlh-10* have a role in VCs? To characterize the function of the bHLH family genes *hlh-3* encoding HLH-3 and *hlh-10* encoding HLH-10, this investigation examined the expression of these genes in VCs throughout development and demonstrated that *hlh-3* expression is present from birth through adulthood, whereas *hlh-10* is only expressed in later developmental stages. Next, VC differentiation was monitored with a suite of molecular markers in the presence and absence of *hlh-3* and *hlh-10* function. The primary findings

highlight new evidence that VC differentiation is dynamic in time and space, that *hlh-3* functions cell-autonomously in VC class specification and differentiation (Chapter II), and that *hlh-10* has a repressive role in the regulation of VC class identity genes (Chapter III).

Finally, this work concludes with the examination of a potential mechanism for the regulation of expression of a homeodomain gene, *lin-11*, shown to function in VCs and downstream of *hlh-3*. By site-directed mutagenesis of a predicted binding site for bHLH-containing proteins, the function of an E-box in the regulatory region of the *lin-11* promoter was assayed. This work sought to address: do bHLHs regulate the VC homeodomain gene *lin-11* via an E-box in the VC enhancer? This work found that the E-box is not required for the expression of *lin-11* in VCs, but instead, is required for the repression of *lin-11*.

In summary, this work identifies a novel function for the bHLH gene, *hlh-3*, in nervous system development of *C. elegans*, functioning in VC specification and terminal differentiation, instead of a classical proneural role. Additionally, this work provides evidence of a novel function for *hlh-10* as a repressor, shown to repress the expression of VC identity genes.

1 Chapter I: Introduction

CHAPTER I:

Introduction

1.1 Background

The nervous system has evolved into intricate, coordinated circuitry providing an organism's ability to sense, integrate, and respond to environmental cues (i.e., chemical, thermal, and visual, to name a few). Neurons are superficially classified as sensory, motor, or interneurons within a circuit but, in fact, their complex molecular profiles and morphologies result in an even larger number of classifications and subtypes (Zeng & Sanes, 2017).

The characterization of the diverse morphology of neurons and anatomy of vertebrate nervous systems can be traced back to the findings of Santiago Ramón y Cajal over a century ago. He observed the fine neuronal branching patterns from multiple cortical preparations of various living organisms (Garcia-Lopez, 2010; Sotelo, 2003). Deeply observant of the neuronal structure, Santiago Ramón y Cajal produced exquisitely detailed hand drawings that illustrated the processes of neurons as finite extensions connecting neurons to each other (Figure 1.1). These observations compelled him to propose the neuron doctrine, that the nervous system is composed of cells, individual entities. This transformed our understanding of neuronal structures and pioneered modern neuroscience.

Today's characterization of neuronal diversity has been achieved by advanced molecular methods including lineage tracing, single-cell transcriptional profiling, and fluorescent activated cell sorting (Poulin et al., 2016). However, such approaches overshadow the power of characterizing neuronal diversity with molecular markers, which demonstrate subclass specific differences within a neuronal class.

A B

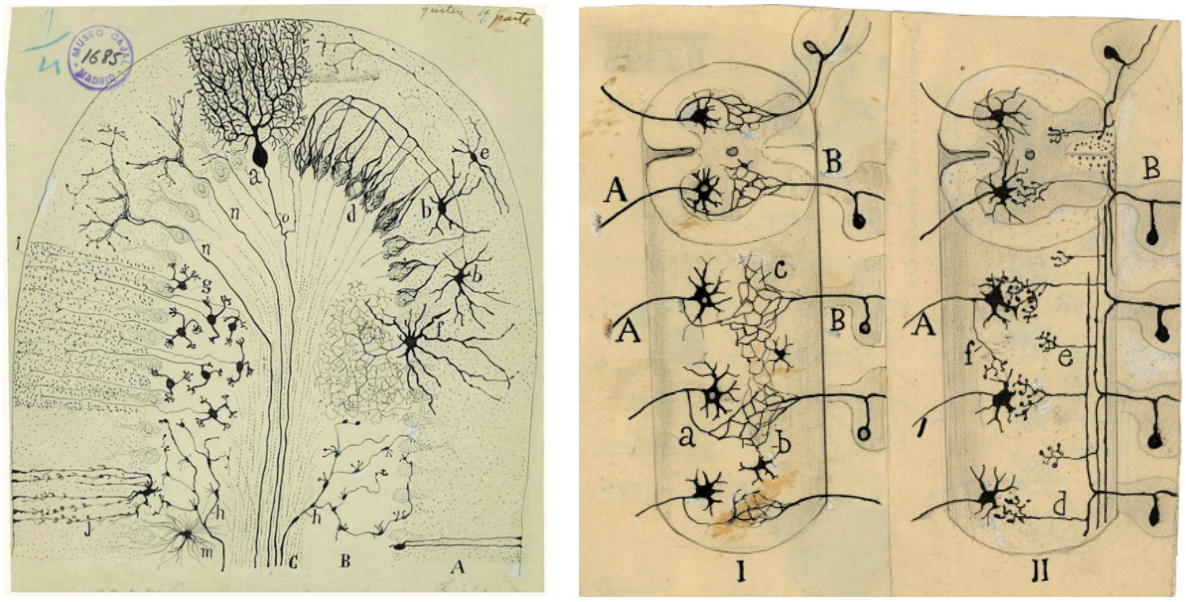


Figure 1.1. Hand drawings of neurons by Santiago Ramón y Cajal.

A) Distinct cerebellar neuron types (Adapted from Sotelo, 2003). B) Spinal cord neuron arrangements distinguishing reticular theory (I) from neuron theory (II) Adapted from (DeFelipe, 2009).

1.2 *C. elegans* as a model organism in neurodevelopment

Neurobiology research in *C. elegans* exemplifies simple and robust approaches to study neuron diversity through fluorescent gene reporter assays and standard genetic analyses. Investigations in this field have illuminated the wealth of neuronal classes and types as defined by their unique molecular profiles. These studies have also provided an insight into the conserved mechanisms promoting neuronal identity and diversity (Hobert et al., 2016; Hobert & Kratsios, 2019).

In our laboratory, we use the invertebrate *Caenorhabditis elegans (C. elegans)* to address questions related to the formation and function of the nervous system. Since the pioneering work of Sydney Brenner and his associates, and up to date, this roundworm has been developed as a model system in neuroscience. The entire cell lineage, from the fertilized egg to the adult has been described; the entire genome has been sequenced and thoroughly annotated, and tools to conduct classical and molecular genetic approaches are widely available. Moreover, *C. elegans* was the first multicellular organism to have a fully mapped nervous system, resulting in a wiring diagram generated from electron microscopic images (White et al., 1986). This wealth of information has revealed that the nervous system of *C. elegans* is relatively simple, harboring 302 neurons in hermaphrodites, yet complex as it can include 118 or more neuronal classes (Hobert et al., 2016; White et al., 1986).

1.2.1 Primary contributions to scientific advances

C. elegans is an invaluable model that has led to the discovery of many conserved genes and elucidated their role in key developmental pathways (Corsi et al., 2015). For example, *C. elegans* research has pioneered groundbreaking discoveries including RNA interference and the mechanisms of programmed cell death or apoptosis (Fire et al., 1998;

Hedgecock et al., 1983; Horvitz, 1986). Moreover, it was the first model organism to lead scientific advances such as (1) a fully mapped cell lineage (2) a nervous system wiring diagram (3) a sequenced genome (4) demonstrated utility for using GFP tagged reporter as fluorescent markers (Chalfie et al., 1994; The *C. elegans* Sequencing Consortium, 1998; Sulston & Horvitz, 1977; White et al., 1986).

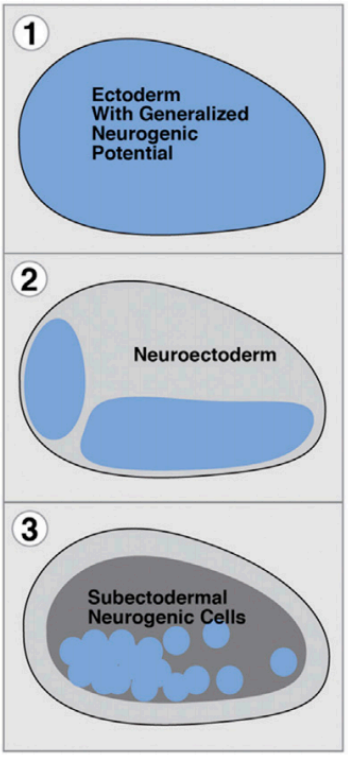
1.2.2 Advantages to working with *C. elegans* for laboratory research

Key features that make *C. elegans* easily adaptable for laboratory research include (1) self-fertilization whereby hermaphrodites produce clonal progeny but they can also mate with males and give rise to genetic variability (2) a short life cycle [3 days at 20°C] (3) transparent bodies, which facilitate the characterization of fluorescent reporters (4) and invariant cell lineages among *C. elegans* descendants (5) and they are relatively inexpensive to grow and maintain (Brenner, 1974; Stiernagle, 2006). Lastly, the *C. elegans* community is known for the shared resource repositories including the Caenorhabditis Genetics Center, National BioResource Project and Addgene.

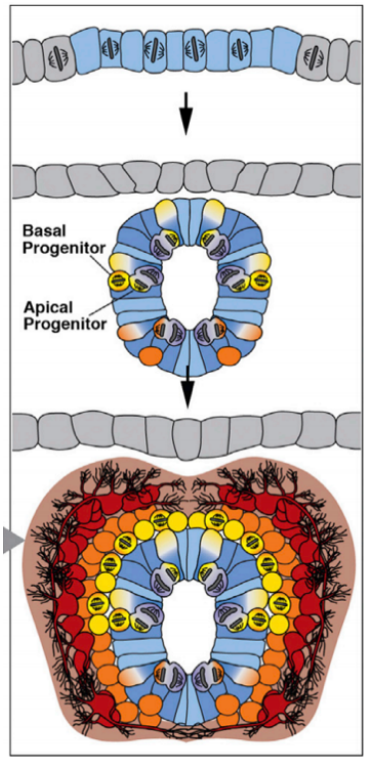
1.3 An overview of early neurogenesis

The nervous system in both vertebrates and invertebrates arises from ectodermal and ectodermal-like cells (Figure 1.2) (Hartenstein & Stollewerk, 2015). These ectodermal cells give rise to neural precursors which differentiate into neurons. Below I discuss how the ectodermal cells give rise to neural precursors within vertebrates and invertebrates.

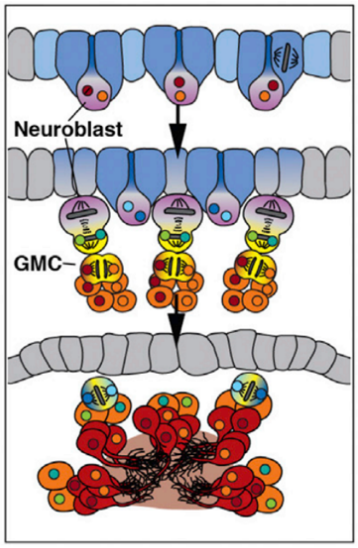
A


1
Ectoderm
With Generalized
Neurogenic
Potential
2
Neuroectoderm
3
Subectodermal
Neurogenic Cells

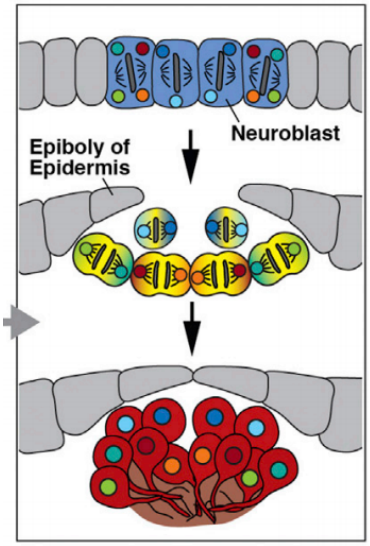
B


Basal
Progenitor
Apical
Progenitor

C


Neuroblast
GMC

D


Epiboly of
Epidermis
Neuroblast

E

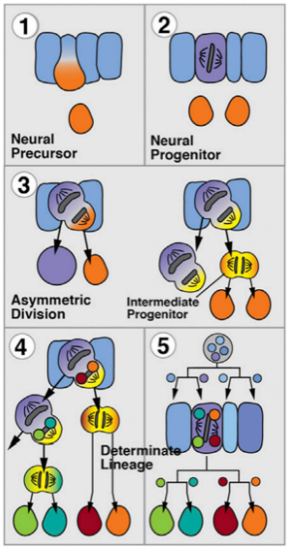

1
Neural
Precursor
2
Neural
Progenitor
3
Asymmetric
Division
Intermediate
Progenitor
4
5
Determinate
Lineage

Figure 1.2. Neural progenitors arise from neuroepithelium.

A) The ectoderm gives rise to neuroectoderm. Neural progenitor movement and differentiation is illustrated in B) Vertebrata; neural progenitors give rise to neurons or glia. C) Hexapoda; neuroblasts generate ganglion mother cells which divide multiple times to give rise to neurons and glia. D) Nematoda; neuroblasts asymmetrically divide to give rise to neurons. E) patterns of proliferation: (1) direct conversion from neuroectoderm to neural precursor (2) neuroectoderm produces proliferating neural progenitors (3) neural progenitors undergo self-renewing mitosis by asymmetric division generating an intermediate progenitor (4) invariant pattern of asymmetric division (5) fixed lineage mechanism. B-C, E) Colored circles and cells represent the unique distribution of "hypothetical" neural determinants. Neuroepithelium is represented in blue; dividing progenitors are represented in purple, intermediate progenitors are represented in yellow, undifferentiated precursors in orange, and mature neurons are represented in red. Adapted from Hartenstein & Stollewerk, 2015.

1.3.1 Vertebrate neurogenesis

In vertebrates the ectoderm gives rise to surface ectoderm as well as neuroectoderm. The neuroectoderm is generated on the dorsal side of the embryo by inhibition of BMP signaling a process known as neural induction (Leclerc et al., 2012; Ozair et al., 2013). Neuroectoderm gives rise to the nervous system through the generation of two structures (1) the neural tube, which gives rise to the central nervous system (CNS) and (2) the neural crest cells, which give rise to the peripheral nervous system (PNS). In the vertebrate nervous system, the neuroectoderm, also known as undifferentiated neuroepithelial cells, or neural progenitors (Hartenstein & Stollewerk, 2015; Leclerc et al., 2012) divide asymmetrically to give rise to a neural stem cell and a neural or glial precursor. Subsequently, each neural precursor differentiates into one or more neurons.

1.3.2 Invertebrate neurogenesis

As an example of invertebrate neurogenesis, the Drosophila nervous system is generated on the ventral side of the embryo. In the first wave of neurogenesis, during embryonic development, neuroepithelial cells give rise to neuroblasts from a ventrolateral region. The ventrolateral region is neurogenic, forming equivalence clusters, from which neuroblasts (NBs) are then selected through Notch-Delta signaling and lateral inhibition (Homem & Knoblich, 2012; Simpson, 1997). The NBs produce distinct descendants dependent on whether they are Type I NBs or Type II NBs. The Type I NBs (progenitor) divide asymmetrically to generate a neuroblast and a ganglion mother cell (GMC), which can divide once to produce neurons or glia. The Type II NBs divide asymmetrically to self-renew and generate an immature precursor- this precursor can divide asymmetrical to self-renew

and generate a GMC (Figure 1.2 C, E-3) (Hartenstein & Stollewerk, 2015; Homem & Knoblich, 2012).

1.3.3 *C. elegans* neurogenesis

The majority of the *C. elegans* nervous system is generated from embryonic cell divisions derived from the AB blastomere, which is one of the six founder cells within the *C. elegans* embryo. Descendants of these founder cells give rise to distinct tissues (ie., muscle, nervous system, intestine, and pharynx). While the AB cell generates the majority of the nervous system and hypodermis (the skin equivalent for worms), some neurons are also generated from other blastomere divisions (Sulston et al., 1983; Sulston & Horvitz, 1977). Similar to vertebrates and Drosophila, neurons are generated from ectodermal-like cells. Unusually however, the ectodermal-like cells asymmetrically divide into neuroblasts within fixed lineages, which directly differentiate into neurons (Figure 1.2 D, E-5) (Hartenstein & Stollewerk, 2015); these asymmetric patterns of division are identical across each individual.

1.4 Conservation and function of proneural genes in neurodevelopment

In both vertebrates and invertebrates, proneural proteins are critical in cementing the commitment of the neuroepithelial cells to the neuronal path or fate (Bertrand et al., 2002; Jimenez & Campos-Ortega, 1990). Simplified steps that illustrate when proneural gene activity is required for the selection of neural precursors are shown in Figure 1.3.

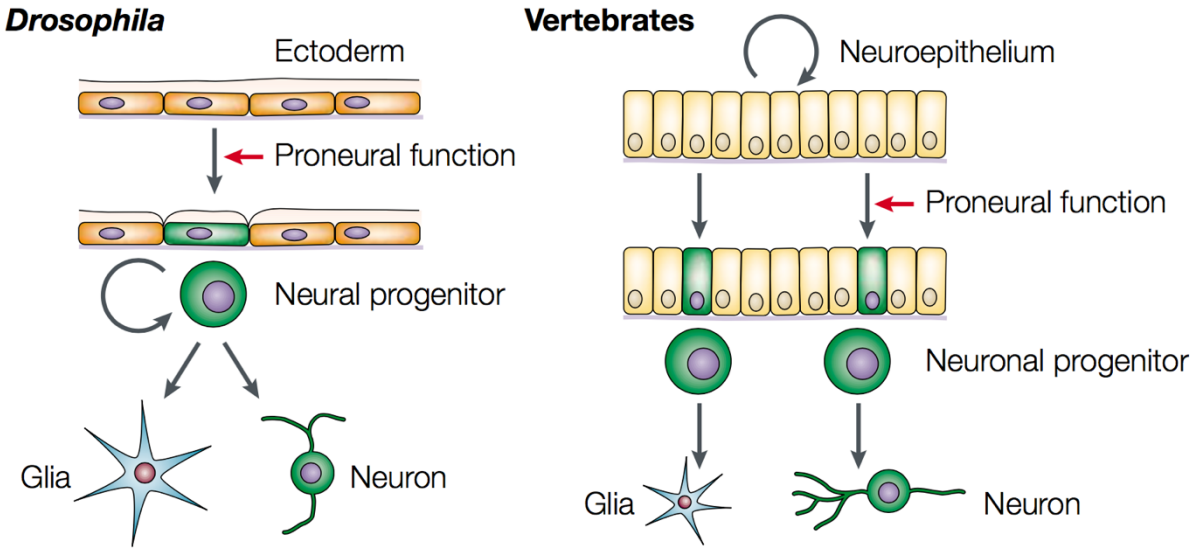


Figure 1.3. Proneural gene activity in vertebrates and invertebrates

An illustration of the steps in neurogenesis. Vertebrates are distinct from Drosophila in that they require a commitment to neuronal fate, by acquiring a neuronal progenitor fate before differentiating into a neuron. Drosophila does not have neuronal progenitors; instead, the neural progenitor can give rise to both neurons and glia. Adapted from Bertrand, 2002.

1.4.1 The Achaete-Scute proneural gene family

The proneural genes comprise four protein families: Neurogenin, NeuroD, Atonal, and Achaete-Scute (Brown and Baker, 2018). These proneural genes encode Class II bHLH domain proteins which heterodimerize with the ubiquitously expressed Class I bHLH E proteins and have been shown to function as transcriptional master regulators in neurodevelopment (Bertrand, 2002). Proneural proteins have documented early roles in specifying neuroblast identity, as well as a later role in promoting differentiation of distinct neuronal classes. These early and later roles are evidenced by their expression throughout multiple steps in neurogenesis, shown as early as in neural progenitors and as late as in terminally differentiated neurons (Figure 1.4) (Guillemot & Hassan, 2017).

The Achaete-Scute family was first identified and characterized in Drosophila. There are four genes in Drosophila, which make up the Achaete-Scute Complex (AS-C): achaete (ac), scute (sc), l'scute (lsc) and asense (ase) (Alonso & Cabrera, 1988; Villares & Cabrera, 1987). Among them asense is different from the others as it is classified as a neural precursor gene (Brand et al., 1993). Phylogenetic analyses indicate that the three proneural genes (ac, sc, and lsc) are an "evolutionary invention" within Drosophila; this is because other insects just have one representative AS-C gene in addition to asense (Baker & Brown, 2018). The AS-C genes are essential in the formation of the Drosophila external sensory organs in larvae and adults. Moreover, the expression onset of these proneural genes is distinct from that of *C. elegans* development which has a fixed lineage with predetermined expression of proneural genes. In contrast, in the embryonic CNS of Drosophila, proneural genes are expressed in subsets of cells within the neuroectoderm, and this defines the equivalence groups from which the neuroblasts are selected via lateral inhibition. Proneural genes are required for the

commitment of neuroectoderm to neuroblast, specification of neural precursors, and generation of mostly all neurons (Campos-Ortega and Knust, 1990; Skeath and Doe, 1996; Garcia-Bellido and Celis, 2009).

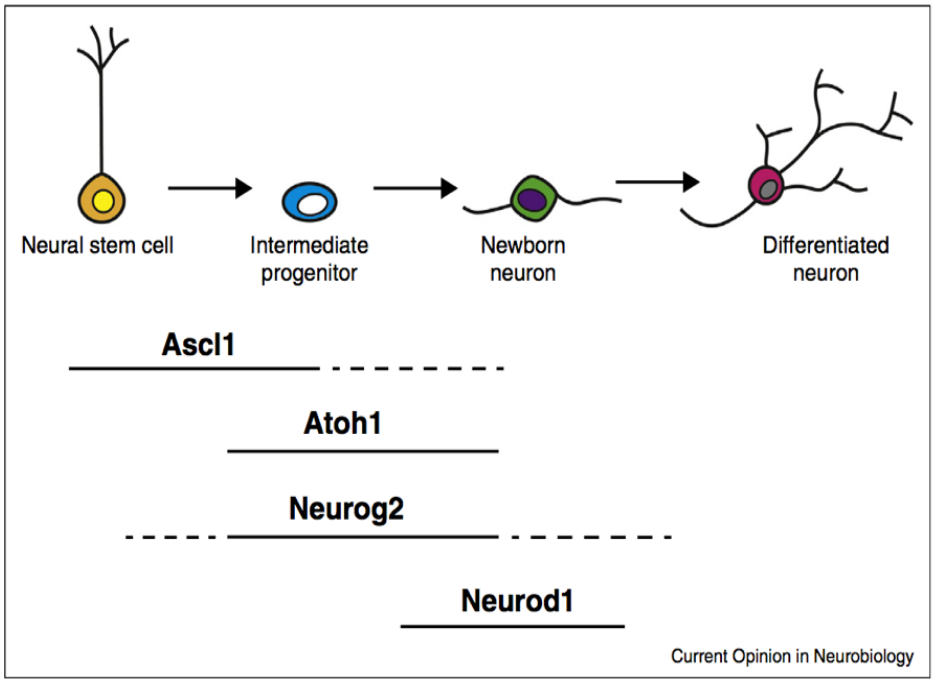


Figure 1.4. Classical proneural gene expression through neurodevelopmental stages.

The expression of representative vertebrate proneural gene family members Ascl1, Atoh1, Neurog2, and Neurod1 are illustrated. Ascl1 is the predominant proneural gene expressed in neural stem cells of the vertebrate nervous system. Neurogenin2 expression is broader between neural stem and newborn neuronal stages. Continuous lines represent consistent expression whereas dashed lines indicate restricted expression to certain lineages. Adapted from Guillemot & Hassan, 2017.

1.4.3 HLH-3 is the ortholog of human ASCL1

As mentioned before, we use *C. elegans* as a model in which to study nervous system generation and function. In particular, and in this study, we have been characterizing the function of the conserved bHLH transcription factor HLH-3 in the generation and differentiation of the sex specific motor neurons VCs (discussed in the next section 1.6). HLH-3 is a Class II bHLH transcription factor, ortholog of human ASCL1(Kim et al., 2018). The field's understanding of ASCL1 is primarily drawn from investigations of the mammalian achaete-scute like homolog-1 (MASH1) (Johnson et al., 1990). Herein, I illustrate the sequence conservation identity of the bHLH domain for both proteins (Figure 1.5A). The sequence of HLH-3 resembles human ASCL1 more than the mouse protein MASH1 (also reported MASH-1) (Figure 1.5B) (Krause, 1997; Grove et al., 2009). In the literature, "Ascl1" gene function is reported in mostly mouse models, and both Ascl and Mash1 are used interchangeably. Therefore, I describe the role of this conserved factor throughout the rest of this introduction in reference to the mouse model as "Ascl1/Mash1," unless specifically stated otherwise.

1.4.4 The proneural roles of Ascl1/Mash1

Ascl1/ Mash1 has early roles as a proneural factor that induces neural progenitor fate and neurogenesis. However, these classical proneural factors, Ascl1/Mash1, also have functions in neuronal subtype specification.

The expression of Ascl1/Mash1 was first established in neural crest cells (Sommer et al., 1995; Guillemot et al., 1993). Consistent with expression in neural crest cells, recent work has detected Ascl1/Mash1 expression as transient in all enteric neural stem cells, derivatives of neural crest cells (Memic et a., 2016). Subsequently, expression of

Ascl1/Mash1 has been documented in the progenitors for structures of the forebrain, midbrain, and hindbrain of mice (Kim et al., 2008). Its presence and function early on in embryonic neurodevelopment is critical as loss of function Mash1 mice die upon birth (Guillemot et al., 1993). Ascl1/Mash1 is, therefore, a key proneural factor required for

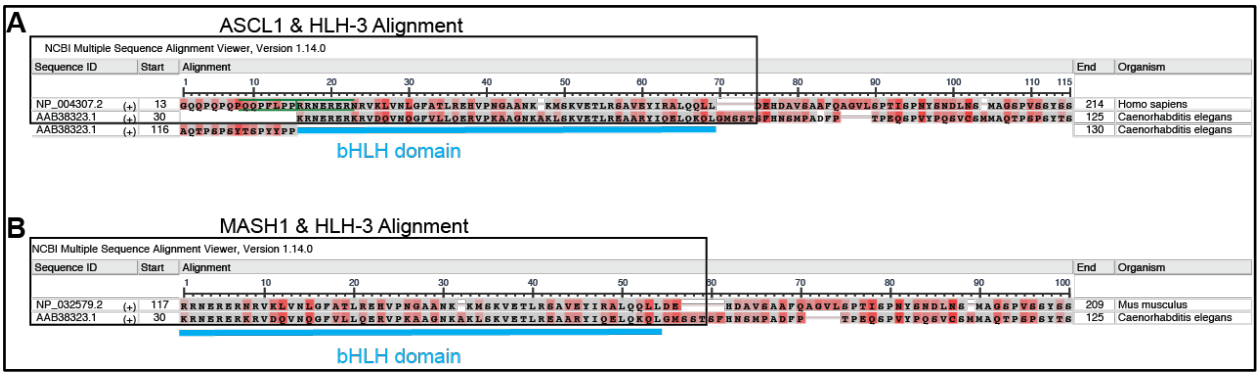


Figure 1.5. Amino acid alignment of conserved bHLH proneural proteins.

A) ASCL1(human) & HLH-3 Alignment identity 61% identity within the bHLH domain. The full length sequence for HLH-3 extends into a third line; ASCL1 does not. (B) MASH1 & HLH-3 Alignment identity 59% identity within the bHLH domain. The bHLH domain is indicated in blue. Identical amino acids are highlighted in gray and mismatches are highlighted in red. Gaps are indicated by empty spaces (-). Sequences were aligned with NCBI: blastp (Altschul et al., 1990).

neurogenesis in the central nervous system and the peripheral nervous system, acting as a key neuronal fate determinant (Guillemot et al., 1993; Summer et al., 1995; Hirsch et al., 1998; Casarosa, et al., 1999; Tomita et al., 2000; Borromeo et al., 2017; Memic et al., 2016)

In addition to the expression pattern and loss of function analyses discussed above, ChIP-ChIP analyses of Ascl1 transcriptional targets also support the role of Ascl1 as a proneural gene. From mouse embryonic ventral telencephalon and neural cell culture, ASCL1 transcriptional targets were identified. Gene ontology analysis for biological processes confirms that these genes induce cell fate specification, neurite morphogenesis, and neuronal differentiation; moreover, validating its critical role in neurogenesis (Castro et al., 2011).

1.4.5 Ascl1/Mash1 has roles in differentiation and specification of neuronal subtypes

In addition to its early acting proneural role (described above in 1.4.4), Ascl1/Mash1 function is also critical for neuronal subtype specification. Ascl1/Mash1 null animals show a lack of- or reduction- in neuronal subtype markers such as in neuroendocrine neurons in the ventral hypothalamus (McNay et al., 2006), central serotonergic neurons (Pattyn et al., 2004), enteric serotonergic neurons (Memic et al., 2016), and GABAergic forebrain and spinal cord neurons (Fode et al., 2000; Parras et al., 2002). ChIP-ChIP gene ontology analysis has also provided clues of the key biological processes associated with ASCL1 function other than neurogenesis. These processes include neuronal subtype specification, neurotransmitter synthesis, the transmission of nerve impulse, and axon guidance (Castro et al., 2011). Its ability to function in neuronal specification is dictated by specific chromatin-binding preferences (to CAGCTG and CAGGTG) distinguishing it from other proneural factors that also regulate neuron subtype specification (i.e. Neurogenin) and maintain the accessibility of

chromatin to bind by downstream factors associated with ASCL1 binding (Aydin et al., 2019). In summary, Ascl1 expression in unique neuronal lineages and the transcriptional targets within those lineages demonstrates it has non-proneural roles linked to specifying certain neuronal subtypes.

Furthermore, because of its inherently coupled functions in promoting neural fate as well as neuronal specification, Ascl1 has been widely used for cellular reprogramming experiments (Castro et al., 2011; Guillemot & Hassan, 2017; Liu et al., 2015). For example, by forced expression, Ascl1 can reprogram dorsal midbrain astrocytes into neurons (Liu et al., 2015). Additionally, Ascl1 can reprogram fibroblasts from an induced pluripotent stem cell (iPSC) to neuron and specify either GABAergic or glutamatergic function depending on the mixture of other factors (Imayoshi & Kageyama, 2014).

1.5 Proneural genes in *C. elegans*

1.5.1 Conserved proneural genes of Atonal and NeuroD and Neurogenin

Proneural genes in *C. elegans* have also been shown to exhibit early and late roles. *C. elegans* has several conserved Class II bHLH genes from the Neurogenin, NeuroD, Atonal, and Achaete-Scute families. To discuss a few, the NeuroD ortholog, *cnd-1*, functions in progenitor specification of the embryonic motor neurons (Hallam et al., 2000). The Neurogenin 1 ortholog, *ngn-1*, is expressed in the AB lineages (AB is one of the six founder cells in the embryo); consistent with its roles as a proneural factor it plays a role in specifying AIY cell fate and generally activating known neurodevelopmental terminal selectors (Christensen et al., 2020). Terminal selectors are factors that initiate and maintain the

terminal differentiation program for subtype identity (Allan & Thor, 2015). The ortholog of Atonal, *lin-32* (Ledent & Vervoort, 2001), has a proneural role in the Q neuroblast (Zhu et al., 2014); but has also been shown to function in the development of male specific sensory structures of the tail, which have large defects but development of these structures is not absent (ie., rays) (Miller & Portman, 2011; Portman & Emmons, 2004; Zhao & Emmons, 1995). However, its role in proper ray development in the male tail is not essential, suggesting that other factor(s), such as HLH-2, can promote ray development (Miller & Portman, 2011).

1.5.2 Conserved Achaete-Scute genes

The Achaete-Scute family of genes in *C. elegans* include *hlh-4*, *hlh-14*, and *hlh-3* (Grove et al., 2009; Kim et al., 2018). *hlh-4* does not display proneural roles but has been reported to act as a terminal selector of the chemosensory identity of the ADL neuron (Masoudi et al., 2018). The proneural gene *hlh-14* is expressed in the neuroblast that gives rise to the PVQ, HSN, and PHB neurons. It functions to promote neurogenesis of all three neurons (Frank et al., 2003). Additionally, *hlh-14* has been shown to have a proneural function in the neuroblasts giving rise to AFD, OLL, and ASE neurons; as well as the neuronal specification of the ASE neurons (Poole et al., 2011). Finally, the role of the other Achaete-Scute ortholog, *hlh-3* appears to be proneural as well as later acting as discussed below.

1.5.3 HLH-3 has proneural and non-proneural roles

The functions of *hlh-3* have been elucidated with loss of function assays and reporter analysis. A reporter of the *hlh-3* gene, *hlh-3::gfp* is expressed in many neural precursors and

overlaps with the localization of its heterodimeric binding partner HLH-2 in embryogenesis (Krause et al., 1997). Consistent with its classification as an ectoderm expressed gene (Hashimshony et al., 2015), another promoter fusion of *hlh-3::gfp* identifies temporal expression beginning mid-embryogenesis and with spatial expression in the head and body neurons and P cells (Grove et al., 2009).

To date the function of *hlh-3* has been implicated in the differentiation of the HSNs (axon pathfinding and serotonergic fate) (Doonan et al., 2008; Lloret-Fernández et al., 2018). Absence of *hlh-3* function results in abnormal axon pathfinding and reduced expression of serotonergic expression, among terminal identity features. These defects impair HSN function producing Egg-laying defective (Egl) hermaphrodites (Doonan et al., 2008). Within this pair of neurons, *hlh-3* function is required even before maturation stages, specifically shown to act as a proneural factor as these neurons lack expression of the pan-neuronal gene *rab-3* (Lloret-Fernandez et al., 2018). Additionally, it has been shown to have a role in promoting the neural transformation of the I4 neuron from mesoderm (Luo & Horvitz, 2017). As classically categorized, *hlh-3* can function as a proneural gene and can function past neural induction steps and in non-neural progenitors.

1.6 Neurodevelopment of the sex-specific ventral cord type C motor neurons

1.6.1 VCs are descendants from the post-embryonic P blast cells

The ventral nerve cord in the nematode is composed of neurons born in the embryo as well as neurons generated post-embryonically (Sulston, 1976; Sulston et al., 1983). In either case the precursors of these neurons are epidermal-like, in that they give rise to hypodermal

and neuronal precursors. This is most easily described in the lineages generated post-embryonically by the ectodermal like P cells.

The P lineage blast cells P0-P12 give rise to cells of the nervous system and cells of the hypodermis along the ventral cord axis (Sulston & Horvitz, 1977). The P lineage neuronal descendants include sex-shared motor neurons of the classes VA, VB, VD, and AS (according to their stereotypic anterior-posterior divisions), and it also generates sex-unique neurons in hermaphrodites and males. Midbody blast cells P3.a-P8.a give rise to the hermaphrodite VC neurons and P2.a-P12.a give rise to the male-specific CAs and CPs neurons. A distinct difference in the generation of these sex-unique neurons is the developmental timing of cell division: the VCs are born in the first larval stage (L1) and represent the differentiated versions of P3-P8.aap; the CAs and the CPs are born later, in the L3 larval stage and represent the differentiated versions of P2-P12.aapa and P2-P12.aapp, respectively (Figure 1.6) (Kalis et al., 2014). However, they both know their sex at the L1 stage (Kalis et al., 2014).

The position of the P lineage descendants along the anterior-posterior axis determines their survival. In hermaphrodites, VC cell survival depends on the function of *lin-39*, a Hox gene, and the TALE-class cofactor encoded by *ceh-20* which together repress *egl-1* (Potts et al., 2009). Loss of function of *lin-39* results in programmed cell death (PCD) of the VC neurons (Clark et al., 1993; Potts et al., 2009) as well as some of the anterior CA/CP neurons (Salser et al., 1993). *lin-39* function in hermaphrodites is suggested to primarily act in VC neuronal survival: *LIN-39 (RNAi)* treated animals are viable and the lineages giving rise to VA and VB sex-shared neurons of the P lineage are unaffected, while P3-8.aap lineages (VC neurons) undergo PCD (Potts et al., 2009).

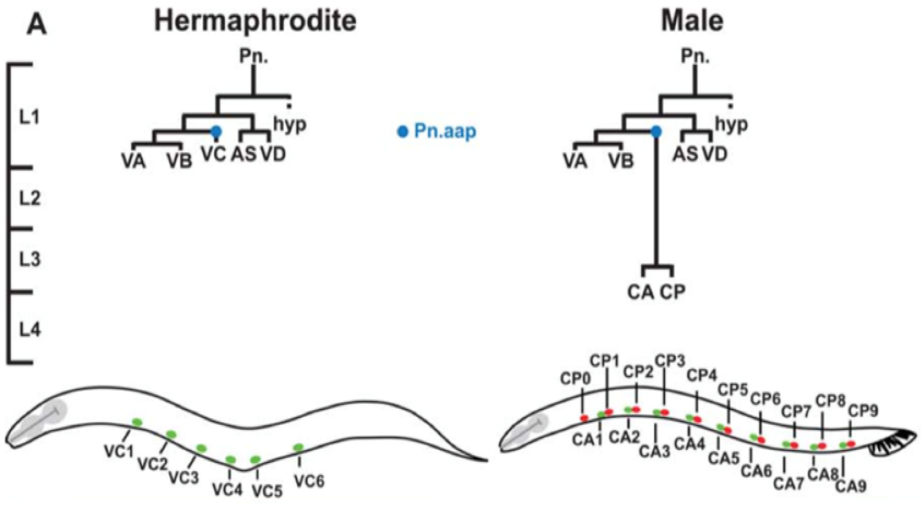


Figure 1.6. The sex-specific ventral cord motor neurons

Lineages that give rise to the sexually dimorphic ventral nerve cord neurons. The Pn.aap cells are the only P lineage descendants that give rise to sex-specific neurons in the ventral cord at different periods during development. The VCs are generated in hermaphrodites and the CA/CPs are generated in males. Adapted from Kalis et al., 2014.

1.6.2 VCs function in egg-laying behavior

The six VC neurons are found in the midbody of hermaphrodites. They are distinguished from each other based on their proximity to the vulva as well as function. The VCs that flank the vulval opening, vulva-proximal VC4 and VC5, are known to function in the egg laying circuitry as stimulators through neuromuscular junctions with vulval muscles, vm2s. The VCs also function as inhibitors of egg laying by inhibiting the function of the HSNs (which stimulate egg laying) (Schafer, 2006). The presence of reciprocal synapses with the HSNs is a feature unique to the vulva-proximal VCs, further adding to their sub-type specificity from the vulva-distal VCs 1-3 and VC6. The distal VCs do not make connections with the HSNs, instead they make synaptic connections to the inhibitory, embryonically born neurons the DDs, and they also are distinct in that they extend axons along the ventral nerve cord (Schafer, 2006).

The hermaphrodite VC neurons are distinct among themselves in their molecular profiles as well. The vulval-proximal VCs are distinct from the vulval-distal VCs. All six VCs are cholinergic, evident at the adult stage by the presence of immunoreactivity to the vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT): the vulval-distal VCs 1-2 and 6 are less immunoreactive (VAChT) than the vulval-proximal VCs 4-5 and the vulval-distal VC neurons express *cho-1*, encoding a choline transporter (Duerr et al., 2008; Lickteig et al., 2001). Finally, although all VCs express *lin-11*, encoding a LIM homeodomain transcription factor, LIN-11 has a specific role in the vulva-proximal VCs 4 and 5, acting in an EGF-dependent pathway during L4 larval development to ensure *unc-4* gene expression in these two VC neurons (Zheng et al., 2013). In summary, the terminal fates

of the P lineage derived sex-unique neurons are therefore complexly regulated and dependent on their position relative to the anterior-posterior axis and proximity to the vulva.

1.7 Overall aim and chapter goals

Regulators and mechanisms that direct VC specification and differentiation are not well characterized. This thesis work aimed to determine whether the bHLH proneural-like gene *hlh-3* has a role in the generation and/or differentiation of this motor neuron class, that is whether the *hlh-3* gene has a function as a classical proneural gene or only works at later stages of neurodevelopment.

Chapter II contains an unpublished preprint (Perez & Alfonso, 2020). Here I characterized the differentiation of VCs through development by analyzing the expression pattern of VC class-specific markers in a WT background. Then I engineered an endogenously tagged reporter line with CRISPR-Cas9 producing *hlh-3::gfp* to characterize its expression in VCs over development. This expression analysis revealed *hlh-3* is expressed in VC neural precursors and in the VCs throughout development. Next, I characterized these VC markers in the absence of *hlh-3* function. I demonstrated that *hlh-3* has a role in the expression of these markers and its function is required for a subclass of VCs. Furthermore, I determined that the *hlh-3* function is cell-autonomous. This chapter appears as a preprint on BioRxiv (https://doi.org/10.1101/2020.06.04.134767) and is awaiting Review at the time this thesis was written.

In Chapter III, I characterized the expression pattern of another bHLH gene, *hlh-10*, in the ventral cord throughout development. Then, to determine whether it too functions in VC differentiation, I examined VC class-specific markers in the absence of its function.

Additionally, I examined VC class-specific markers in a double mutant loss of function background of *hlh-3* and *hlh-10*. This work demonstrated that *hlh-10* has a negative role in the regulation of VC specific transcriptional reporter genes.

In Chapter IV, I created two constructs containing a VC enhancer fragment of *lin-11* with and without an E-box site, presumably a candidate binding motif for the Class bHLH factors HLH-3 and HLH-10. I characterized the expression of these two constructs (integrated transcriptional reporters) in several genetic backgrounds. This work demonstrated that the E-box site is a repressor site for expression of *lin-11*.

2 Chapter II: The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in C. elegans

The work within this chapter "The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in *C. elegans*" was submitted as a preprint on BioRxiv on June 05, 2020 (Perez & Alfonso, 2020).

Chapter II:

The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in *C. elegans*

2.1 Abstract

Neural specification is regulated by one or many transcription factors that control expression of effector genes that mediate function and determine neuronal type. Here we identify a novel role for one conserved proneural factor, the bHLH protein HLH-3, implicated in the specification of sex-specific ventral cord motor neurons in *C. elegans*. Proneural genes act at in early stages of neurogenesis in early progenitors, but here, we demonstrate a later role for *hlh-3*. First, we document that differentiation of the ventral cord class C neurons (VC) within their motor neuron class, is dynamic in time and space. Expression of VC class-specific and subclass-specific identity genes is distinct through development and dependent on where they are along the A-P axis (and their position in proximity to the vulva). Our characterization of the expression of VC class and VC subclass-specific differentiation markers in the absence of *hlh-3* function reveals that VC fate specification, differentiation, and morphology requires *hlh-3* function. Finally, we conclude that *hlh-3* cell-autonomously specifies VC cell fate.

2.2 Introduction

Cells in the nervous system are extremely diverse in shape, function, and the mechanisms by which they connect to other cells. Generation of neurons and their acquisition of unique features require the commitment to neural fate by an ectodermal descendant, the

specification of neural class within the neuronal precursor, and the differentiation into unique transcriptomic and morphological states of the postmitotic cell. Importantly, the acquisition of unique neuronal class identity features, compared to pan-neuronal identity, is seen to be regulated differently. Redundant regulators with multiple cis-regulatory inputs induce pan-neuronal features whereas terminal differentiation of neurons is induced by single inputs, encoded by so-called terminal selectors, and results in the expression of a unique repertoire of genes that promote neural class diversity (Hobert, 2016b; Stefanakis et al., 2015). Thus, the neural diversity displayed by the nervous system is possible by the concerted action of terminal selector factors that function spatiotemporally with precision (Allan & Thor, 2015; Hobert, 2016a; Hobert & Kratsios, 2019; Kratsios et al., 2017).

In *C. elegans*, the mechanisms that regulate neural specification can be studied thoroughly in time and in space, at single-cell resolution. This is a powerful model system that harbors a fully mapped body plan and nervous system, with continuously updated genomic and transcriptomic annotations, supporting studies in developmental biology, evolutionary conserved genes and networks, and beyond (Baker & Woollard, 2019; Cooper et al., 2018; Corsi et al., 2015; Emmons, 2016; Hammarlund et al., 2018; Sulston & Horvitz, 1997). Moreover, the classes of motor neurons in the ventral cord of *C. elegans* are diverse and contain both sex-shared neurons and sex-specific neurons, with known positions, connectivity, and neurotransmitter fate. However, the mechanisms regulating the differentiation of the sex-specific motor neurons are not well understood. Our work implicates the proneural gene *hlh-3* in the development of the hermarphrodite sex-specific ventral cord motor neurons.

Here we characterize the role of a conserved proneural-like protein, HLH-3, the ortholog of ASCL1, the homolog of mammalian achaete-scute homolog-1 (MASH-1), in *C. elegans* nervous system development. HLH-3 contains a conserved basic helix-loop-helix (bHLH) domain, which is 59% (31/54) identical to MASH-1 and 61% identical to ASCL1 (33/54). HLH-3 heterodimerizes with the Class I bHLH transcription factor HLH-2, predicted ortholog of TCF3/TCF4/TCF12 (Kim et al., 2018; Krause et al., 1997). Our previous work has implicated HLH-3 in the terminal differentiation of the hermaphrodite-specific motor neurons, HSNs, a bilateral pair of neurons that function in the egg-laying circuitry (Doonan et al., 2008; Raut, 2017, Schafer, 2006). Work by others has shown that the gene *hlh-3* has diverse functions in the nervous system: it is necessary for the appropriate death of the sisters of the NSMs (Thellmann et al., 2003); it works in combination with other transcription factors to induce the serotonergic program in HSNs, and moreover, its ortholog, ASCL1, can be a functional substitute (Lloret-Fernández et al., 2018); it promotes neurogenesis of I4 (Luo & Horvitz, 2017); it co-regulates the initiation of expression of the terminal selector gene *ttx-3* (Murgan et al., 2015); and it regulates the chemoreceptor gene *srh-234* (Gruner et al., 2016). The above studies have implicated *hlh-3* in the development of sex-shared neurons, but its role in sex-specific neuron development is not fully understood.

We were the first to report that *hlh-3* is expressed in the embryonically generated P cells, ectodermal-like precursors of all post-embryonically generated ventral cord motor neurons. We also showed that by the third larval stage (L3) expression of a truncated translational fusion *hlh-3* reporter was restricted to the VCs, a hermaphrodite sex-specific type of neuron (Doonan et al., 2008). This expression pattern is consistent with a role in neuroblast specification, a function of canonical proneural proteins. However, it remained to

be determined whether *hlh-3* had a function in the specification of their lineage descendants (including sex-shared as well as sex-specific). Here we report on the role of *hlh-3* in the development of sex-specific postembryonic ventral cord motor neurons, VCs. We show it is necessary for the acquisition and maturation of the hermaphrodite sex-specific VC class only.

The postembryonic ventral cord motor neurons are made up of both sex-specific and sex-shared neurons arising from the anterior descendants of ectodermal-like P blast cells (Pn.a) (Sulston & Horvitz, 1977). After two additional cell divisions, the Pn.aap cells give rise to the sex-specific neurons of the ventral cord. In hermaphrodites, the P3-P8.aap cells give rise to the ventral cord neuron type C (VC) (Figure 2.1A, B), whereas in males Pn.aapa and Pn.aapp (where n = descendant of P3 to P11), give rise to the ventral cord neuron type CA and CP, respectively (Sulston et al., 1980). Their fate acquisition (generation) is influenced by positional cues (Hox genes), differential survival (programmed cell death), and sexual identity (VC vs. CA/CP). The VCs of the hermaphrodite are positioned in the midbody and make up six of the total eight sex-specific neurons. Equivalent lineage descendants (Pn.aap) of P1, P2, and P9-12 cells in hermaphrodites undergo programmed cell death (Clark et al., 1993). These neuron classes (VC, CA and CP) provide an opportunity to study the molecular mechanisms that drive sex-specific neuron differentiation.

Little is known about how VCs are generated and differentiate. Survival of VCs requires the function of the HOX gene *lin-39* and the HOX cofactors encoded by *unc-62* and *ceh-20* (Clark et al., 1993; Salser et al., 1993). UNC-62, along with LIN-39, promotes survival of the VCs by ensuring CEH-20 localizes to the nucleus; the LIN-39/CEH-20 complex then represses *egl-1* transcription (Liu et al., 2006; Potts et al., 2009). Sexual determination of the Pn.aap cells is established by the first larval (L1) stage (as VCs in

hermaphrodites and the precursors of CAs and CPs in males) (Kalis et al., 2014). It was also shown that LIN-39 is not required for the expression of the VC terminal differentiation feature *ida-1*. Moreover, since the surviving descendants from P1, P2, and P9-12 still express *ida-1::gfp* in *lin-39(lf); ced-3(lf)* double mutants, it was concluded that the role of LIN-39 is most likely restricted to VC survival, not differentiation (Kalis et al., 2014). However, recent evidence has implicated a role for LIN-39 in the expression of a VC marker *srb-16* (Feng et al., 2020). Nevertheless, the mechanisms underlying VC class specification and differentiation are not well understood. To date, it is not known which factor(s) initiate the differentiation program of VCs to establish a class-wide identity.

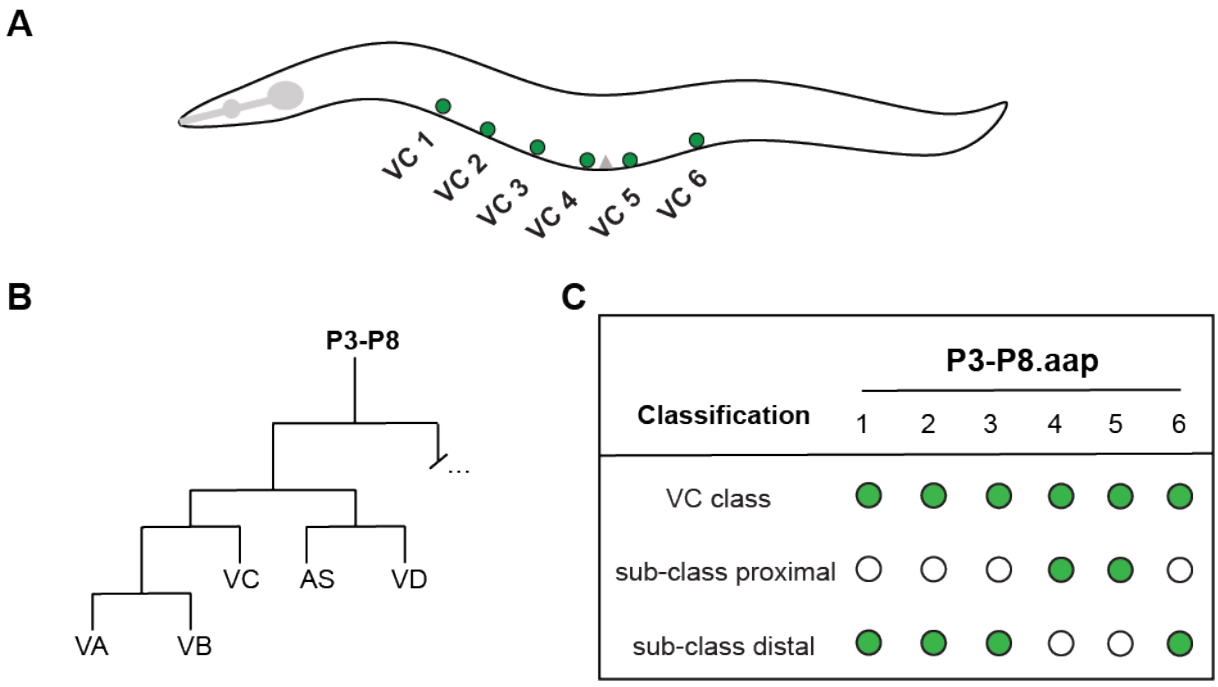


Figure 2.1. The ventral cord type C motor neuron class

A) Illustration of the position of the six VCs along the ventral nerve cord in the midbody region of an adult hermaphrodite. Anterior is to the left, ventral is down, gray triangle on the ventral surface indicates the location of the vulva. B) Diagram of the reiterative post-embryonic cell divisions produced by the P3.a to P8.a neuroblasts and give rise to the VCs (adapted from Sulston and Horvitz, 1977). C) Diagram for VC classification includes two sub-classes: proximal (VC 4 and VC 5) and distal (VCs 1-3, 6). This classification format will be used throughout the rest of the figures.

While the mechanisms that regulate VC class specification have yet to be determined, the mechanisms that regulate VC subclass identity are better understood. Within the VC class, two VC subclasses are distinguished spatially by their proximity to the vulva, categorized as proximal VCs or distal VCs (Schafer, 2006). The two VC neurons that flank the vulva are categorized as "proximal" (VC4 and VC5), whereas the other four VCs are "distal" to the vulva (VC 1-3, and VC6) (Figure 2.1C). Genetic analysis of *unc-4* has revealed that VC subclass is determined by spatial cues. Specifically, the expression of *unc-4* as a VC proximal subclass identity gene requires the secretion of EGF from vulval tissue (vulF cells) (Zheng et al., 2013). EGF signaling promotes proximal VC subclass fate by de-repression of *unc-4* in the proximal VCs only. Thus, a non-cell autonomous mechanism mediates one aspect of VC differentiation, specifically in proximal VCs.

Here we build on the current knowledge of neural specification in *C. elegans* and discover that the proneural-like bHLH factor, HLH-3, mediates specification and differentiation of the VC sex-specific motor neurons, that is, it is needed early and late in development. By using molecular markers to assess VC differentiation in the absence of *hlh-3* function, we find that VC class and subclass identity, as well as morphology, is compromised. Our work identifies a new role function for the Achaete-Scute homolog, HLH-3, in the ventral cord of *C.* elegans, that is, the control of sex-specific motor neuron development. We conclude that HLH-3 is necessary for the expression of the earliest VC class-specific transcriptional regulator *(lin-11)* and is required for the expression of later acting VC class-specific genes.

2.3 Materials and Methods

2.3.1 Strain maintenance

All strains were maintained at 22°C on nematode growth media using standard conditions (Brenner, 1974). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *hlh-3 (tm1688)* was isolated by the National Bioresource Project of Japan. *cccIs1* was kindly shared by Dr. Jennifer Ross Wolf, *uIs45* was kindly shared by Dr. Martin Chalfie, *otIs456* was kindly shared by Dr. Oliver Hobert, and *otIs564* was kindly shared by Dr. Paschalis Kratsios. See Table 1 for a complete list of strains used in this study.

2.3.2 Construction of transgenic strains

The transgenic strain harboring *icIs270* was generated by the integration of *akEx31 [pglr-5::gfp + lin-15(+)]* using UV-TMP treatment followed by outcrossing (see below). The VC rescue array *icEx274 [VC::hlh-3cDNA::gfp; pmyo-2::mCherry]* was generated by co-injection of the constructs pCFJ90 *(pmyo-2::mCherry)* and pRD2 *(VC::hlh-3cDNA::gfp)* into the mutant strain harboring *hlh-3(tm1688); otIs45* at 20 ng/microliter and 2 ng/microliter, respectively. pRD2 was generated by Dr. Ryan Doonan to address whether *hlh-3* could rescue the egg-laying defective phenotype in *hlh-3(tm1688)* (Doonan, 2006). The pRD2 construct contains a VC specific promoter obtained from the vector pDM4, kindly provided by Dr. Michael Koelle driving expression of a *hlh-3* cDNA (Doonan, 2006).

Strain	Genotype
AL166	*inIs179 [pida-1prom::gfp] II ; him-8(e1489) IV ; hlh-3(tm1688) II*
AL184	*vsIs48 [punc-17::gfp; him(e1490) V*
AL195	*vsIs48 [unc-17::gfp; him-5(e1490) V; hlh-3(tm1688) II*
AL270	*icIs270 [pglr-5::gfp + lin-15(+)]*
AL273	*hlh-3(tm1688) II ; icIs270 [pglr-5::gfp + lin-15(+)]*
AL281	*uIs45 [punc-4::MDM2::GFP + rol-4(+)]; hlh-3(tm1688) II*
AL284	*icIs270 [pglr-5::gfp]; ced-3(n717), unc-26(e205) IV; hlh-3(tm1688) II*
AL287	*icIs270 [pglr-5::gfp]; ced-3(n717), unc-26(e205) IV*
AL303	*otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+); him-5(e1490); him-5(e1490) V hlh-3(tm1688) II*
AL325	*hlh-3(tm1688) II; mjIs27 [mir-124p::gfp + lin-15(+)]*
AL331	*ic271 [hlh-3::gfp] II*
AL338	*hlh-3(tm1688) II; otIs456 [plin-11::mCherry; pmyo-2::GFP]*
AL341	*otIs456 [plin-11::mCherry; pmyo-2::GFP]*
AL346	*hlh-3(tm1688) II ; otIs456 [plin-11::mCherry; pmyo-2::GFP]; icIs274 [VC::hlh-3cDNA::gfp]*
AL348	*ic271 [hlh-3::gfp] II; otIs456 [plin-11::mCherry; pmyo-2::GFP]*
BL5717	*inIs179 [pida-1prom::gfp] II ; him-8(e1489) IV*
OH11954	*otIs456 [plin-11::mCherry; pmyo-2::GFP]*

OH13105	*otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+); him-5(e1490) V*
SX621	*lin-15B&lin-15A(n765) X; mjIs27 [mir-124p::gfp + lin-15(+)]*
Tu3067	*uIs45 [punc-4::MDM2::GFP + rol-4(+)]*
JWR29	*tph-1::mCherry; lin-39fosmid::gfp*
AL262	*tph-1::mCherry; lin-39fosmid::gfp; hlh-3(tm1688)*

Table 1. List of Strains

2.3.3 Integration of extrachromosomal arrays

The transgenic strain harboring *icIs270* was generated by exposing L4 hermaphrodites to UV-TMP (350microJoules x 100 on Stratagene UV Stratalinker; 0.03microgram/microliter TMP. Irradiated animals were placed onto seeded NGM plates and transferred the next day to fresh seeded NGM plates (3 Po/plate). These were followed to clone F1s (~150) and subsequently to clone three F2s per F1.

2.3.4 Construction of HLH-3::GFP CRISPR-Cas9 engineered line [*ic272*]

Construction of the CRISPR line required modification of two plasmids: the single guide RNA or sgRNA plasmid, pDD162 (Addgene #47549), and the repair template plasmid, pDD282 (Addgene #66823) (Dickinson et al., 2015). The target sequence GCTATGATGATCACCAGAAG was selected using the CRISPR design tool on Flybase consisting of a high optimal quality score (96). The sgRNA was cloned into pDD162 to create pLP1. The 5'arm homology arm was designed as a gBlock containing a silent mutation at the PAM site to prevent Cas9 off-targeting. The gBlock was PCR amplified with primers acgttgtaaaacgacggccagtcgccggca and CATCGATGCTCCTGAGGCTCCCGATGCTCC and cloned into pDD282. The 3' homology arm was designed via PCR using the primers CGTGATTACAAGGATGACGATGACAAGAGATAATCTGTTAAGTTGTACC and ggaaacagctatgaccatgttatcgatttccaaggagctggtgcacaag. The PCR product was purified and cloned into pDD282 to create pLP2. The modified constructs pLP1and pLP2, as well as the co-injection plasmid pGH8 (Addgene #19359) were co-injected into an N2 strain: sg-RNA plasmid (pLP1) at 50ng/uL; *hlh-3* repair template plasmid (pLP2) at 10ng/uL, and pGH8 at

2.5ng/uL. Screening was carried out according to the published protocol (Dickinson et al., 2015).

2.3.5 Microscopy

Animals were mounted on 3% agarose pads containing droplets of 10mM levamisole. Fluorescent images were acquired with AxioVision on Zeiss Axioskop 2 microscope. Following the collection of images, some conversions were made with FIJI version 2.0.0 (grayscale images were converted with Lookup tables: Red or Green) and processed into Adobe Illustrator for formatting. Fluorescent reporters were observed under confocal microscopy for the detection of a fluorescent protein signal (presence or absence) in transgenic lines. This study does not report quantification of intensity for any fluorescent reporter observed.

2.4 Results

2.4.1 The Class II bHLH protein HLH-3 is expressed and localized to the nuclei of VCs from L1 through adulthood

We have previously shown that in hermaphrodites, *hlh-3* is expressed in the postembryonic descendants of the ectodermal-like P cells as well as the HSNs (Doonan et al., 2008). We also have shown that *hlh-3* function is cell-autonomously required for normal axon pathfinding and terminal differentiation of the HSNs (Doonan, 2006; Doonan et al., 2008; Raut, 2017). In those studies, analysis of the expression of a translational fusion reporter with only the first eight amino acids of HLH-3 fused to GFP revealed that expression was widespread in the Pn.a descendants, dynamic, and with time, restricted to the

VCs (Pn.aap) and HSNs. To confirm the endogenous spatiotemporal expression pattern of *hlh-3* we created *ic271 [hlh-3::gfp]*, a CRISPR-Cas9 fluorescent tag at the C terminus of the *hlh-3* genomic locus (Figure 2.2A) following established genome-engineering protocols (Dickinson et al., 2015), and characterized its expression pattern. Our analysis supports our initial findings (Doonan, 2006; Doonan et al., 2008), the recently reported observation that *hlh-3* expression reappears in the HSNs at the L4 developmental stage (Lloret-Fernández et al., 2018), and expands our understanding of its role in the VCs (Doonan, 2006; Raut, 2017). We confirmed that *hlh-3* is expressed post-embryonically in the P cells and their descendants and becomes restricted to the terminally differentiated VCs present in adults (Figure 2.2C, D, and E). After hatching, animals show the expression of *hlh-3* throughout the ventral nerve cord (VNC). We highlight the expression of *hlh-3* in an early L1 animal wherein Pn.p expression extinguishes faster than that in Pn.a and its descendants (Figure 2.2C, left panel). As development proceeds, expression is extinguished from other descendants of the Pn.a cells and restricted to the VCs (Figure 2.2C middle and right panels). While fluorescent reporter intensity was not quantified, *hlh-3* expression appears to be down-regulated in a window of the fourth larval stage (L4) development ranging from mid L4 to late L4, before increasing in adulthood (Figure 2.2D, middle and right panels). To ensure that the detected nuclei in adults are those of VCs, we characterized whether there was co-expression of *hlh-3::gfp* with *plin-11::mCherry*, a known VC marker (Figure 2.2E, bottom left). We find that the *hlh-3::gfp* positive nuclei are also *plin-11::mCherry* positive (Figure 2.2E, top right panel). Interestingly, low levels of *hlh-3::gfp* expression is also observed in a pair of vulval cells during mid-late substages of L4 development, suggesting a role for *hlh-3* in these lineages. Expression of *hlh-3* in VCs from their birth in L1 through their terminally

differentiated stage in adulthood prompted us to investigate the role of *hlh-3*, as a factor required for an early role in promoting VC fate and required for maintenance of VC fate throughout development. Throughout we will use the allele *hlh-3(tm1688)*, which eliminates the majority of the bHLH domain and transcription start site rendering this a null allele and further referred to in this paper as *hlh-3(lf)* (Figure 2.2B, Doonan et al., 2008).

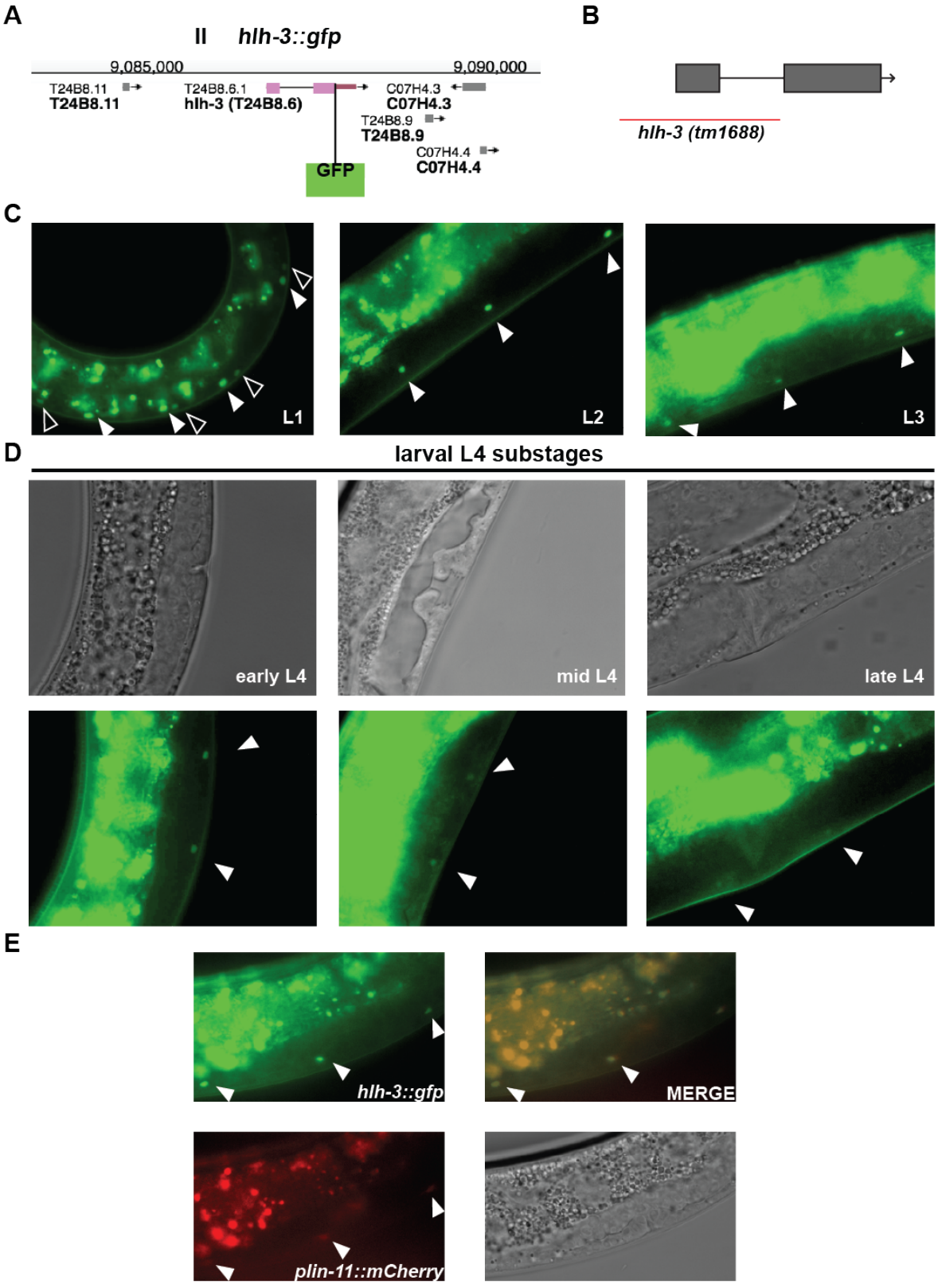

A
II hlh-3::gfp
9,085,000
9,090,000
T24B8.11
T24B8.11
T24B8.6.1
hlh-3 (T24B8.6)
C07H4.3
C07H4.3
T24B8.9
T24B8.9
C07H4.4
C07H4.4
GFP
B
hlh-3 (tm1688)
C
L1
L2
L3
D
larval L4 substages
early L4
mid L4
late L4
E
hlh-3::gfp
MERGE
plin-11::mCherry

Figure 2.2. HLH-3 is first detected in nuclei of the Pn descendants (Pn.a and Pn.p) and becomes restricted to the nuclei of VCs as development proceeds

A) Diagram of the CRISPR-Cas9 engineered C-terminal GFP insertion at the *hlh-3* locus (*ic271*). B) The *hlh-3(tm1688)* allele represents a 1242 bp deletion that spans chromosome II from 35,589 to 36,831 and removes exon 1. Removal of this region, including most of the bHLH domain, results in a null allele (Doonan et al., 2008). C: Representative images of the midbody ventral cord of hermaphrodites harboring HLH-3::GFP (*ic271*) at different larval developmental stages (L1, L2, and L3). At L1, filled arrowheads point to larger, more intense nuclei, presumably the Pn.a blast cells; whereas the outlined arrowheads point to the diminishing expression in Pn.p blast cells (left panel). Filled arrowheads in L2 and L3 represent expression in VC nuclei (middle and right panels). D) Representative images of the midbody ventral cord of hermaphrodites harboring HLH-3::GFP (*ic271*) over distinct L4 developmental stages (early, mid, and late). Larval substages (top panels) are classified by vulva morphology (Mok et al., 2015). Filled arrowheads point to the proximal VCs (bottom panels). E) Overlapping expression (merge, top right) of the VC marker, *plin-11::mCherry (otIs456)* (bottom left) and HLH-3::GFP *(ic271)* (top left), in an animal at the L4 molt (bottom right). Filled arrowheads point to co-labeled proximal VCs.

2.4.2 The differentiation of VC class and VC subclass motor neurons is dynamic

Before we analyzed the role *hlh-3* in VCs, we first characterized differentiation features of VCs by defining VC class versus VC subclass-specific terminal identity. Here we took advantage of fluorescent reporter genes that serve as markers of VC fate. We examined the expression of the known VC class-specific markers *lin-11*, *ida-1*, and *glr-5* encoding a LIM homeodomain transcription factor (Freyd et al., 1990), a protein tyrosine phosphatase-like receptor protein homolog of IA2 (Cai et al., 2001, 2004; Zahn et al., 2001), and a glutamate receptor subunit (Brockie et al., 2001), respectively. We confirmed that expression of *lin-11* in VCs is observed as early as the second larval stage (L2), and through adulthood (Hobert et al., 1998; Zheng et al., 2013).

Unlike *lin-11*, a transcriptional regulator, the other VC class terminal identity genes *ida-1* and *glr-5* are expressed later in development, arising at the L4 developmental stage (Figure 2.3A, B). Analysis of these VC class differentiation markers throughout L4 substages revealed distinct spatiotemporal patterns suggesting different pathways regulate them. Expression of *ida-1* and *glr-5* is not equivalent across all 6 VCs during L4 development (Figure 2.3A, B). Classification of the L4 substages (early, mid, and late) is based on the vulval L4 morphology as previously described (Mok et al., 2015). We noted that *glr-5* expression is first detected in the early L4 substages and only in the proximal VC subclass, whereas expression can be detected in the distal VCs by late L4 substages (Figure 2.3B). In contrast to *glr-5*, *ida-1* expression is nearly equivalent in all VCs since the beginning of L4, but its expression is always detectable in the posterior VCs (Figure 2.3A). Thus, while the six VCs terminally express their class-specific terminal differentiation genes *ida-1* and *glr-5*, the initiation of transcription is distinct across the sub-stages of L4 development (Figure 2.4).

Next, we characterized the expression pattern of the VC subclass-specific terminal identity genes *unc-4* and *unc-17*. Others have shown that *unc-4* expression requires *lin-11* and vulval EGF signaling (Zheng et al., 2013). We corroborate that *unc-4* expression is detected after the mid-L4 stages and is maintained throughout adulthood only in VC 4 and VC 5 (Figure 2.3C). The expression of UNC-17, in turn, is known to require a posttranscriptional step mediated by UNC-4 (Lickteig et al., 2001). Therefore, we analyzed the expression of two transcriptional *unc-17* reporters. To our surprise, and in contrast to work by others, we only detect the expression of *unc-17* in VC 4 and VC 5 at the adult stage regardless of which reporter we characterized (Figure 2.5) (Pereira et al., 2015). However, our work is different from others in that we did not assess a translational reporter. Instead, we looked at two transcriptional reporters *vsIs48 (punc-17::gfp)* and *mdEx865 [unc-17p::NLS::mCherry + pha-1(+)]* and did not observe *unc-17* expression in the distal VCs 1-3 and 6 with either reporter (*vsIs48* expression is shown in Figure 2.5B top panel; *mdEx865* expression is not shown). Although we do not see the *unc-17* reporters in the distal VCs we still detect a VC marker (*lin-11*) in these cells (Figure 2.5B middle and bottom panels). Our observations are also consistent with previous reports that anti-UNC-17 immunoreactivity is robust in VC 4 and VC 5, but rarely detectable in distal VCs (Duerr et al., 2008; Lickteig et al., 2001) and possibly only in the second larval (L2) stage (Alfonso et al., 1993).

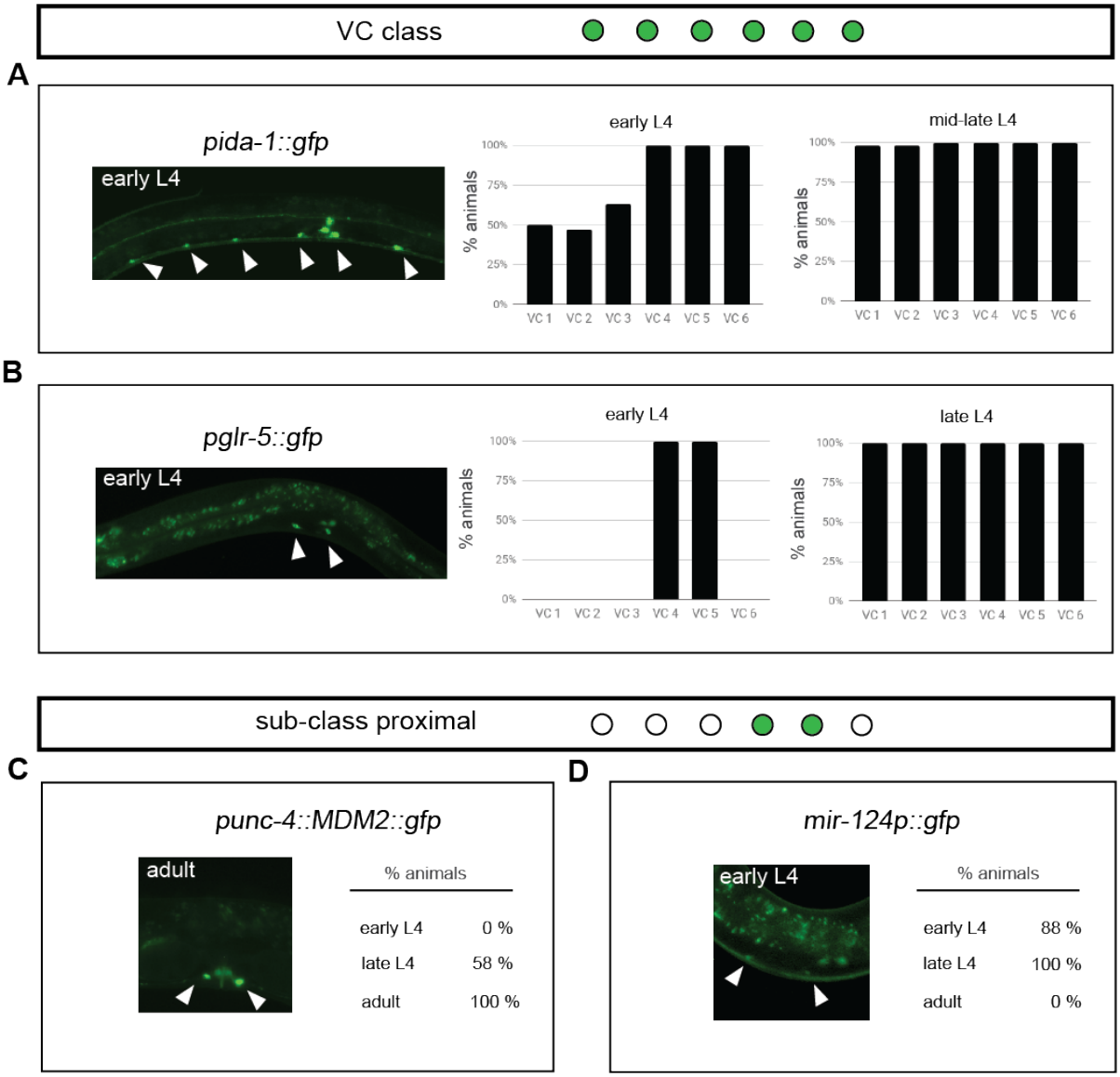


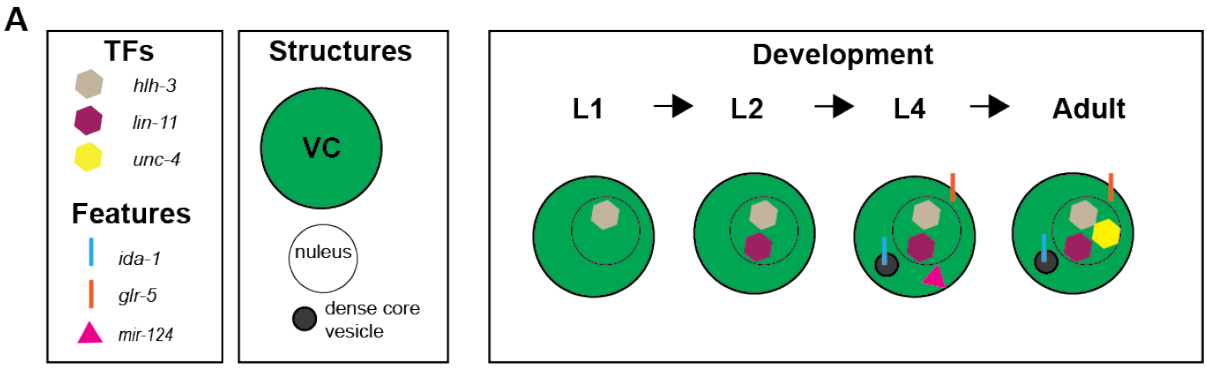
Figure 2.3. The spatiotemporal expression of VC class and subclass-specific identity genes is dynamic

A) Expression pattern of the VC class differentiation feature *ida-1* in early and mid-late L4 developmental stages. Image shows an early L4 hermaphrodite expressing *inIs179 [pida-1::gfp]* in all VCs (indicated by arrowheads). Graphs report the percent of animals expressing *inIs179 [pida-1::gfp]* (early L4, n = 20; mid-late L4, n = 40) in each VC. Since all VCs

express *pida-1::gfp* by mid-L4, the sub-stages in late L4 were grouped together with mid-L4 substages. B) Expression pattern of the VC class differentiation feature *glr-5* in early and late L4 development. Image shows an early L4 hermaphrodite expressing *icIs270 [pglr-5::gfp]* in the proximal VCs (indicated by arrowheads). Graphs report the percent of animals expressing *icIs270* in early L4 (n = 10), mid L4 (n = 15), and late L4 (n = 15) developmental stages in each VC. C) Quantification of expression of the VC subclass feature, *unc-4,* from L4 development through adulthood. Image shows the expression of *uIs45 [punc-4::MDM2::GFP]* in an adult (indicated by arrowheads). The percent of animals expressing the VC subclass marker in both cells (VC 4 and VC 5) of early L4 (n = 8), late L4 (n = 12), and adults (n = 19). D) Quantification of expression of VC subclass feature, *mir-124*, from L4 development to adulthood. Image shows expression of *mjIs27 [mir-124p::gfp + lin-15(+)]* in the proximal VCs of an early L4 hermaphrodite. Percent of animals expressing the VC subclass marker in both cells during these substages is listed adjacent to the image in early L4 (n = 8), late L4 (n = 13), and adults (n = 10).

2.4.3 *mir-124* is a novel VC subclass-specific identity feature

In our search for VC subclass identity genes, we found *mir-124*, the highly conserved non-coding microRNA, as a novel VC subclass-specific differentiation feature. In *C. elegans* it has been documented to be expressed in a variety of sensory neurons and the HSNs (Clark et al., 2010). Here we characterized *mir-124* expression across postembryonic development; we only see it in a restricted window. We find *mir-124* is expressed from early L4 larval substages through early adulthood, but not in mature gravid egg-laying hermaphrodites (Figure 2.3D), which suggests it is required for the maturation of the VCs but not for maintenance of VC fate. This expression pattern is unlike that of other proximal VC identity features *unc-4* and *unc-17*, which are expressed throughout adulthood (Figure 2.3C, Figure 2.5). Therefore, we classify *mir-124* as a novel VC subclass-specific feature expressed during early differentiation. In summary, we conclude that *mir-124* can be added to the list of VC identity features belonging to the proximal class (Figure 2.4).



B

VC identity	VNC Midbody Expression Pattern	Feature	VC 1	VC 2	VC 3	VC 4	VC 5	VC 6
	class	*hlh-3*						
	class	*lin-11*						
	class	*ida-1*						
	class	*glr-5*						
	subclass	*mir-124**						
	subclass	*unc-4*						
cholinergic VNC identity		*unc-17*						

Figure 2.4. Summary of VC class and subclass identity

A) Diagram of genes encoding transcription factors (TFs) and class- or subclass-specific genes and structures expressed in VCs throughout post-embryonic development. B) Summary of the expression pattern of distinct VC class and VC subclass identity genes in the midbody of the ventral cord of the hermaphrodite. While *unc-17* is expressed in the subclass proximal VCs, it is also expressed in all VNC cholinergic motor neurons, therefore not VC specific. Our analysis is based on the expression of integrated transcriptional reporters with the exception of the endogenous GFP tag to *hlh-3* (See Table 1 for the list of strains containing these markers). With the exception of *mir-124*, all reported genes are maintained through adulthood (denoted by an asterisk).

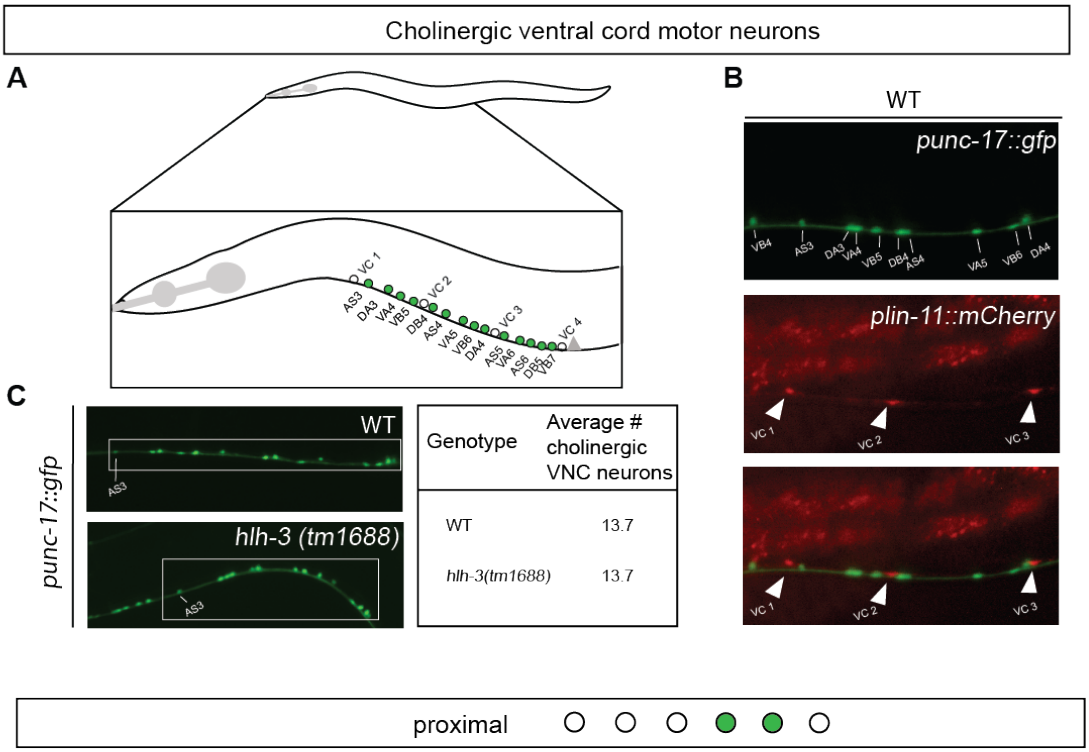

Cholinergic ventral cord motor neurons
A
B
WT
punc-17::gfp
plin-11::mCherry
C
punc-17::gfp
WT
hlh-3 (tm1688)
Genotype
Average # cholinergic VNC neurons
WT 13.7
hlh-3(tm1688) 13.7
proximal
D
punc-17::gfp
Adult
Adult
E
WT
hlh-3(lf)
punc-17::gfp
% animals
OFF
ON
VC 4
VC 5

Figure 2.5. Cholinergic, sex-shared ventral cord motor neurons differentiate normally in *hlh-3(lf)*.

A) Schematic of the number and position of cholinergic, sex-shared VNC neurons in the anterior body region, and between VC 1 and VC 4 (n = 14). B) An annotated image of adult WT hermaphrodite expressing *punc-17::gfp* in non-VC neurons (top panel), *plin-11::mCherry* in VCs (middle panel, filled arrowheads), and a merge of both images (bottom image). Anterior is left, ventral is down. C) Quantification of number of *punc-17::gfp* positive nuclei in the anterior region of the vulva in WT (n = 10) and *hlh-3(lf)* (n = 10) hermaphrodites. Representative images are shown on the left. The average number of positive nuclei is reported on the right for each genotype. D) Representative images of L4 and adult WT hermaphrodites harboring the *punc-17::gfp* (*vsIs48*) reporter. There is no detectable expression in mid-L4 development (top panel), but the expression is detected in adults (middle and bottom panels). E) Quantification of reporter expression in proximal VCs of WT (n = 16) and *hlh-3(lf)* (n = 15) in adulthood. On = detectable, Off = undetectable.

2.4.4 Classification of VC identity

Thus far, we have shown that the VC class of neurons acquire class-specific features via mechanisms that differ in time and space. We have also shown that not all six VCs are identical in their repertoire of transcriptional activity. In Figure 2.4, we summarize the spatiotemporal expression pattern of VC identity features. Two genes encoding presumptive transcription factors, *hlh-3* and *lin-11*, are VC class-specific and *hlh-3* expression precedes that of *lin-11* (Figure 2.4A-B). We classify the *ida-1* and *glr-5* genes as VC class-specific as well, as they are observed in all six VCs from L4 through adulthood. In contrast, *mir-124* is not expressed in adulthood, as the rest of the VC terminal identity features are. Finally, *unc-17* was observed in the proximal VCs only. It is worth emphasizing that aside from the analysis of the CRISPR-Cas9 engineered *hlh-3::gfp* line, our analysis is based on the characterization of transcriptional reporters (Table 1).

2.4.5 *hlh-3* function is required for the acquisition of VC class and VC subclass identity genes

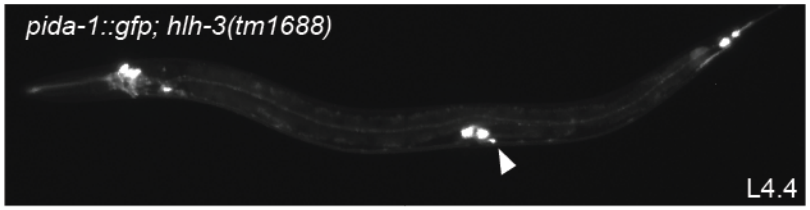
Previously, *hlh-3* has been shown to be required for HSN terminal differentiation (Doonan et al., 2008; Lloret-Fernández et al., 2018; Raut, 2017). To address whether *hlh-3* has a role in VC differentiation we first examined reporters of VC terminal identity the genes *lin-11*, *ida-1* and *glr-5*, in a strain harboring a total loss of *hlh-3* function allele, *hlh-3(tm1688)* (Doonan et al., 2008, Figure 2.2B). In fact, expression of the differentiation feature *ida-1* in the earlier stages of L4 development is completely absent in *hlh-3(lf)* hermaphrodites (Figure 2.6A, B). Moreover, we find that expression of the terminal VC class markers *lin-11*, *ida-1* and *glr-5* is eliminated specifically in the distal VCs of one day old *hlh-3(lf)* adult hermaphrodites (Figure 2.7A-B).

Next, we examined the expression of VC subclass-specific identity features, *mir-124*, *unc-4*, and *unc-17* (Figure 2.7). We find that the early differentiation subclass-specific feature *mir-124 (mjIs27: mir-124p::gfp + lin-15(+))* is completely absent in *hlh-3(lf)* (Figure 2.7D). We followed up with an analysis of *unc-4*. Others have shown that the expression of this VC subclass-specific terminal identity gene is de-repressed in WT animals after EGF signaling in mid-L4 development (Zheng et al., 2013). Here, we find that the absence of *hlh-3* function reduces expression frequency of *unc-4* (Figure 2.7D). Since *unc-4* expression is required for *unc-17* expression (Lickteig et al., 2001), not surprisingly we find that expression of *unc-17*, is missing the proximal VCs in *hlh-3(lf)* individuals (Figure 2.5E).

2.4.6 *hlh-3* is required for normal axon branching of proximal VCs

Consistent with the reduced expression of VC terminal identity molecular markers, proximal VCs have abnormal axonal branching in the vulval ring (Figure 2.7E). This defect suggests that proximal VC function may be impaired in *hlh-3(lf)*, as axonal branching is required for synaptic connections to the egg-laying circuitry. Thus, growth and maturation of VC axons require *hlh-3* function, as it is the case for the HSNs (Doonan, 2006; Doonan et al., 2008; Raut, 2017).

A

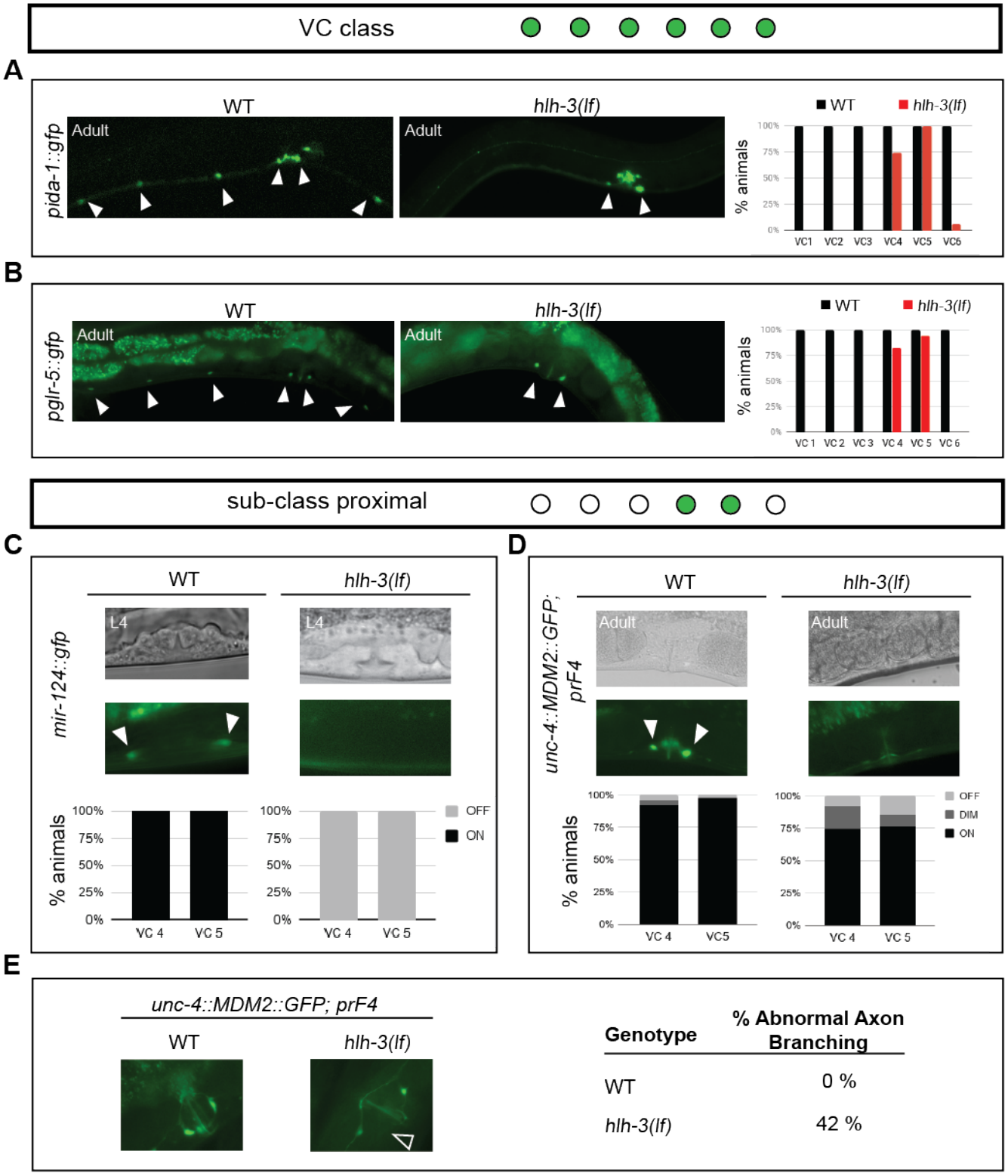


B

N	Genotype	L4 substages	VC 1	VC 2	VC 3	VC 4	VC 5	VC 6
14	hlh-3(tm1688)	L4.0-L4.3	0%	0%	0%	0%	0%	0%
48	hlh-3(tm1688)	L4.4-L4.9	5%	2%	7%	40%	67%	10%

Figure 2.6. *hlh-3* acts prior to early larval L4 substages.

A) Image of an early L4 *hlh-3(lf)* hermaphrodite expressing *pida-1::gfp* only in VC 5 (white arrowhead). In WT individuals this reporter is detectable in all VCs (Figure 2.3) as well as the round-shaped bodies near the vulva, a pair of uv1 cells. Expression in uv1 cells is not affected in *hlh-3(lf)* individuals. B) Quantification analysis of *pida-1::gfp* detection in each VC of *hlh-3(lf)* individuals during early L4 substages (L4.0-L4.3) or mid-late substages (L4.4-L4.9).



Genotype	% Abnormal Axon Branching
WT	0 %
hlh-3(lf)	42 %

Figure 2.7. VCs require HLH-3 to acquire class-specific and subclass-specific differentiation features and normal axon morphology.

A-B) Representative images of WT or *hlh-3(lf)* individuals harboring the indicated reporters. Filled arrowheads point to detectable VCs in either genotype. Graphs report the percent of animals expressing each reporter in each VC of WT (gray bars) and *hlh-3(lf)* (red bars). The expression of the *ida-1* marker (*inIs179*) was quantified in WT (n = 15) and *hlh-3(lf)* (n = 35) (panel A). The expression of the *glr-5* marker (*icIs270*) was quantified in WT (n = 15) and *hlh-3(lf)* (n = 30) (panel B). C-D) Representative images of WT and mutant hermaphrodites at different stages of development and harboring the indicated reporters of VC subclass features *mir-124 (mjIs27)* and *unc-4 (uIs45)*. Fluorescent images of the vulval region of DIC imaged hermaphrodites (top panels) only revealed expression in the proximal VCs of WT individuals (indicated by filled arrowheads). Quantification of the percent of animals with detectable reporter expression of *mir-124* (*mjIs27*) in VC 4 or VC 5 is reported in the graph below the images. Fluorescence was either detectable (on) or not detectable (off) for expression of *mjIs27* in WT mid L4s (n = 17) and *hlh-3(lf)* mid L4s (n = 14). Quantification of the percent of animals with detectable reporter expression of *uIs45* in VC 4 or VC 5 is reported in the graph below the images. Fluorescence was either bright (on), dim, or not detectable (off) for expression of *uIs45* in WT (n = 66) and *hlh-3(lf)* (n = 61) adults. E) Quantification of proximal VC axon branching in WT and *hlh-3(lf)* individuals. Normal axons branch into a vulval ring, as observed with *uIs45* in the WT genotype (top panel). In contrast, *hlh-3(lf)* hermaphrodites display abnormal axon branching (bottom panel). The

numbers to the right represent the percent of individuals with abnormal branching in adult WT (n = 15) and *hlh-3(lf)* (n = 24) adult hermaphrodites.

2.4.7 VCs survive in the absence of *hlh-3* function

Our analysis of VC class and VC subclass markers indicate that the expression of VC differentiation markers is compromised in *hlh-3(lf)* individuals. To ensure VC survival occurs we next sought to eliminate the possibility that VCs inappropriately undergo programmed cell death in *hlh-3(lf)*. Programmed cell death (PCD) is a conserved pathway executed by CED-3, a caspase that functions as the final determinant in the cell death pathway (Conradt et al., 2016). Inhibition of this pathway, by impairment of *ced-3* function, results in the survival of cells destined to die. In the context of the ventral nerve cord, the cells P1-P2.aap and P9-12.aap will survive (Figure 2.8A). Therefore, we introduced a *ced-3*(*n717*) null mutation into *hlh-3(lf)* mutants and analyzed the expression of a VC differentiation marker, *grl-5,* in *ced-3(lf)* and *ced-3(lf); hlh-3(lf)* individuals. Unlike *ced-3(lf)* hermaphrodites, which express *glr-5* in all VCs including the surviving P2.aap cell, we find that *ced-3(lf); hlh-3(lf)* mutants do not express *glr-5* in VCs or the surviving VC-like cell P2.aap (Figure 2.8B, C, and D). Therefore, we conclude that the reason VC neurons do not express *glr-5* in the absence of *hlh-3* function is that they need HLH-3 to fully differentiate and not because they undergo inappropriate PCD.

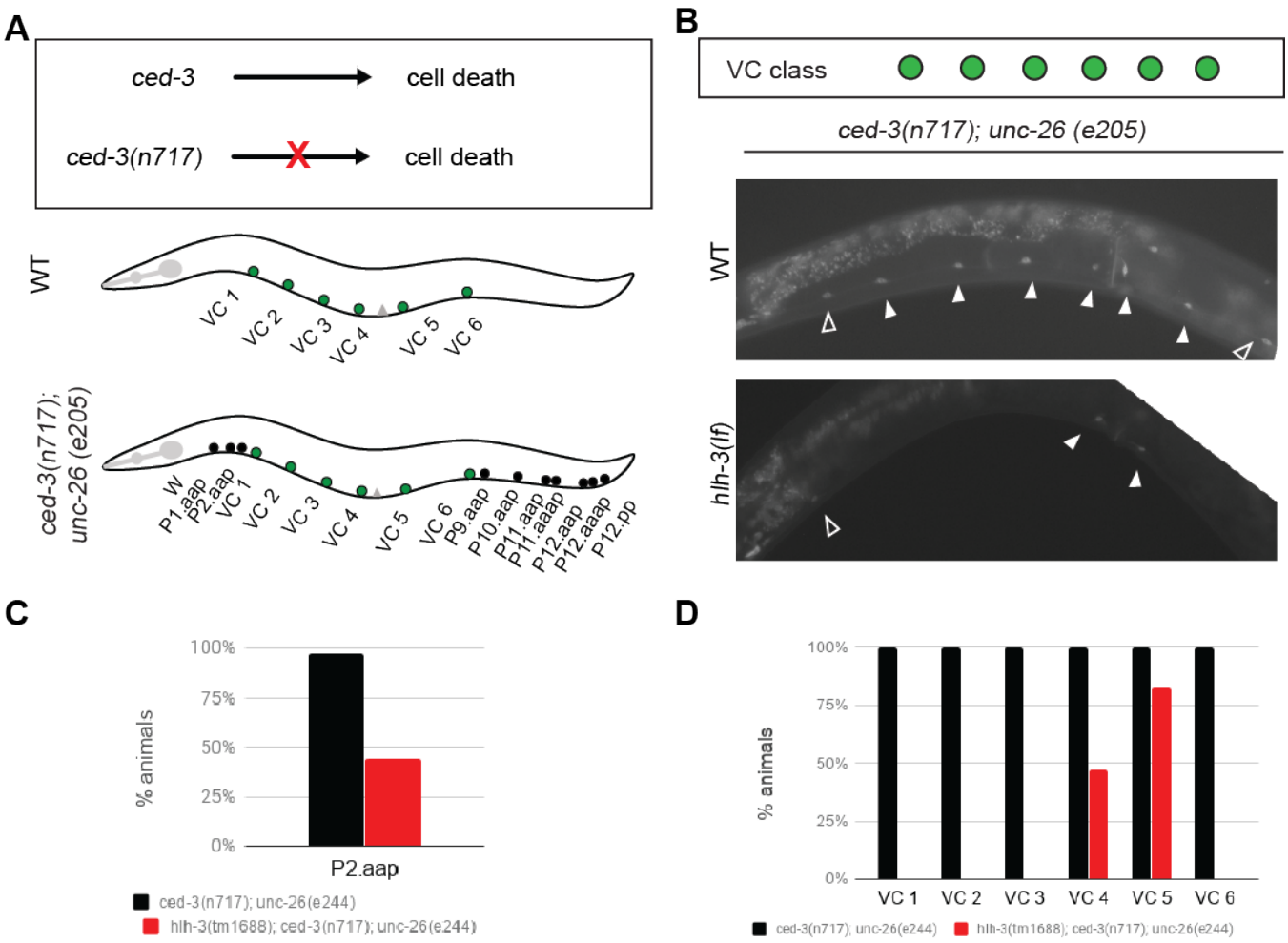


Figure 2.8. VCs do not inappropriately undergo programmed cell death in the absence of *hlh-3* function.

A) Diagram of outcome in the presence and absence of *ced-3* function. The presence of *ced-3* function in WT individuals results in PCD, the absence of *ced-3* function in the null allele *ced-3*(*n717*) prevents PCD. In the ventral nerve cord of WT animals, only the descendants of P3.aap to P8.aap or VCs report the expression of VC markers. However, in *ced-3(n717)* nulls, the VC-equivalent descendants of P1 and P9-12, that normally undergo PCD, do not undergo PCD and report expression of VC markers. B) Representative images of *ced-*

3(n717); unc-26(e244) individuals with (WT), or without (*hlh-3(lf))* function. The *glr-5* VC marker (*icIs270*) was utilized to monitor the presence of VCs (filled arrowheads) and VC-like surviving cells (outlined arrowheads; specifically, P2.aap and P9.aap). The reporter *icIs270* is only detected in the proximal VCs (filled arrowheads) and a VC-like cell (outlined arrowhead; P2.aap) in the double mutant *hlh-3(lf)*; *ced-3(lf)*. C) Quantification of the percent of one day old adults expressing *icIs270* in P2.aap in *ced-3(n717); unc-26(e244); pglr-5::gfp* (n = 35), and *hlh-3(tm1668); ced-3(n717); unc-26(e244); pglr-5::gfp* (n = 34).
D) Quantification of the percent of one day old adults expressing *icIs270* in each VC of *ced-3 (n717); unc-26(e244); pglr-5::gfp* (n = 35) and *hlh-3(tm1668); ced-3(n717); unc-26(e244); pglr-5::gfp* (n = 34).

2.4.8 *hlh-3* functions cell-autonomously in the VC class

To address whether *hlh-3* functions cell-autonomously, we assayed expression of a VC differentiation marker *plin-11::mCherry* in *hlh-3(lf)* mutants with a rescuing copy of *hlh-3*. The rescuing extrachromosomal array *[icEx274 (plin-11::pes-10::hlh-3cDNA::gfp; pmyo-2::mCherry)]* was made by introducing a *hlh-3* cDNA into pDM4 (previously shared by Michael Koelle) harboring a VC-specific regulatory region of *lin-11* fused to the basal *pes-10* promoter (Doonan, 2006). We find that whereas *hlh-3(lf)* mutants fail to express the VC differentiation marker *plin-11::mCherry* in most VCs, *hlh-3(lf)* mutants that contain the rescuing extrachromosomal array *icEx274* express *plin-11::mCherry* in almost all VCs (Figure 2.9A, B). These findings demonstrate that *hlh-3* function is cell-autonomous.

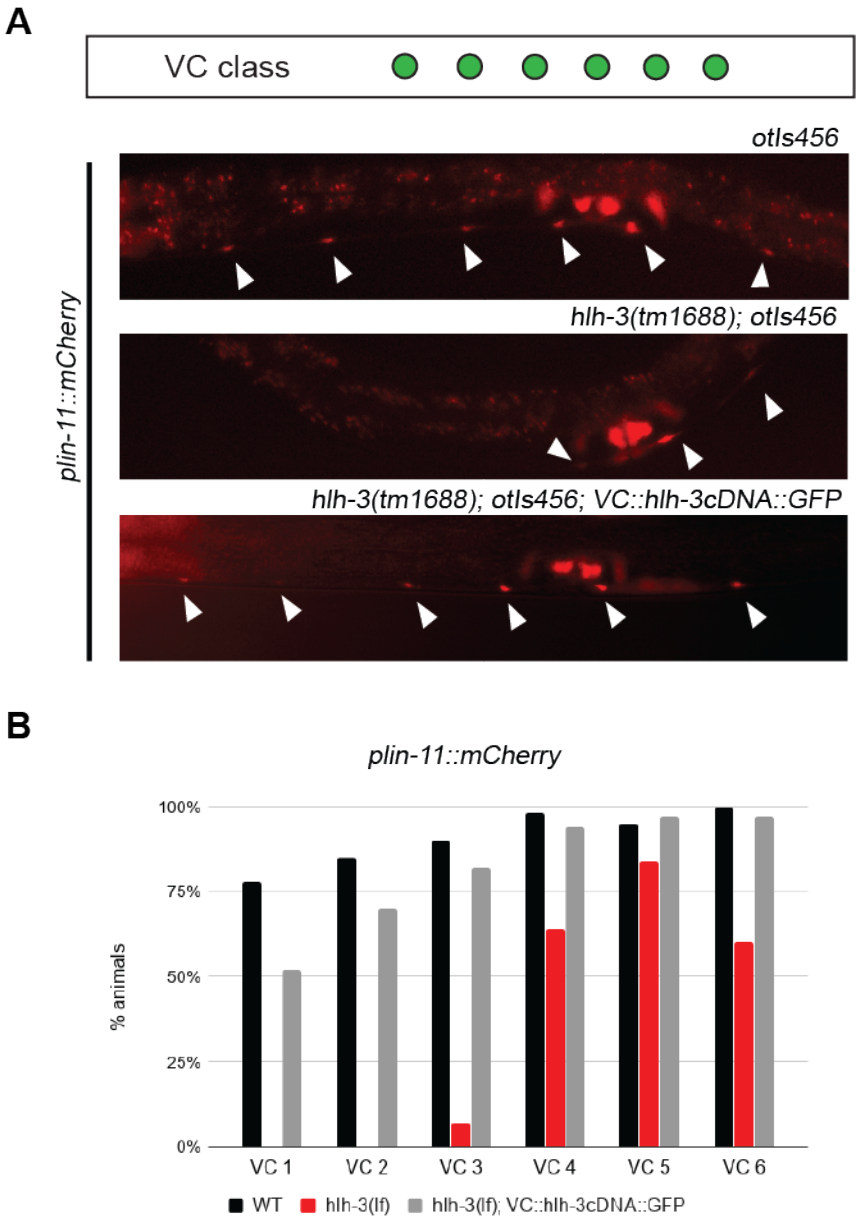


Figure 2.9. The function of *hlh-3* in VCs is cell-autonomous

A) Representative images of individuals harboring the *lin-11* marker (*plin-11::mCherry*) in WT (top panel), *hlh-3(lf)* (middle panel), and VC-specific rescued lines (bottom panel). The reporter *otIs456* is normally expressed in all VCs (top panel, filled arrowheads). B) Quantification of the percent of mid-late L4 animals expressing *plin-11::mCherry* in each VC of WT (n = 41), *hlh-3(lf)* (n = 48), and *hlh-3(lf);VC::hlh-3cDNA::gfp* (n = 39).

2.4.9 *hlh-3* does not affect the differentiation of other sex-shared neurons in the ventral cord

Given that expression of the *hlh-3* CRISPR-edited reporter is detectable in the P cells and its descendants we wished to address whether the absence of *hlh-3* function resulted in defects in the sex-shared neurons. To address this question, we analyzed the expression of cholinergic and GABAergic markers in *hlh-3(lf)* mutant hermaphrodites. The transcriptional reporter *vsIs48 [punc-17::gfp]* gene marks all cholinergic neurons expressing a vesicular acetylcholine transporter (within the VNC this includes VA, VB, AS, DA, DB, Figure 2.5A) (Wormbase: Curatorial remark). The transcriptional reporter *otIs564 [punc-47::mChOpti]* marks all GABAergic neurons expressing a vesicular GABA transporter (within the VNC this includes DD and VD neurons, Figure 2.10A) (Gendrel et al., 2016). We find that the total number of cholinergic neurons anterior to the vulva is equivalent between WT and *hlh-3(lf)* individuals (Figure 2.5C). Likewise, the total number of GABAergic neurons is equivalent between WT and *hlh-3(lf)* hermaphrodites (Figure 2.10B). These analyses demonstrate that the cholinergic and GABAergic sex-shared ventral cord motor neurons acquire their terminal neurotransmitter fate. Thus, *hlh-3* function is not necessary for the acquisition of the terminal fates in sex-shared neurons, rendering its function specific to the terminal differentiation of sex-specific ventral cord VC neurons.

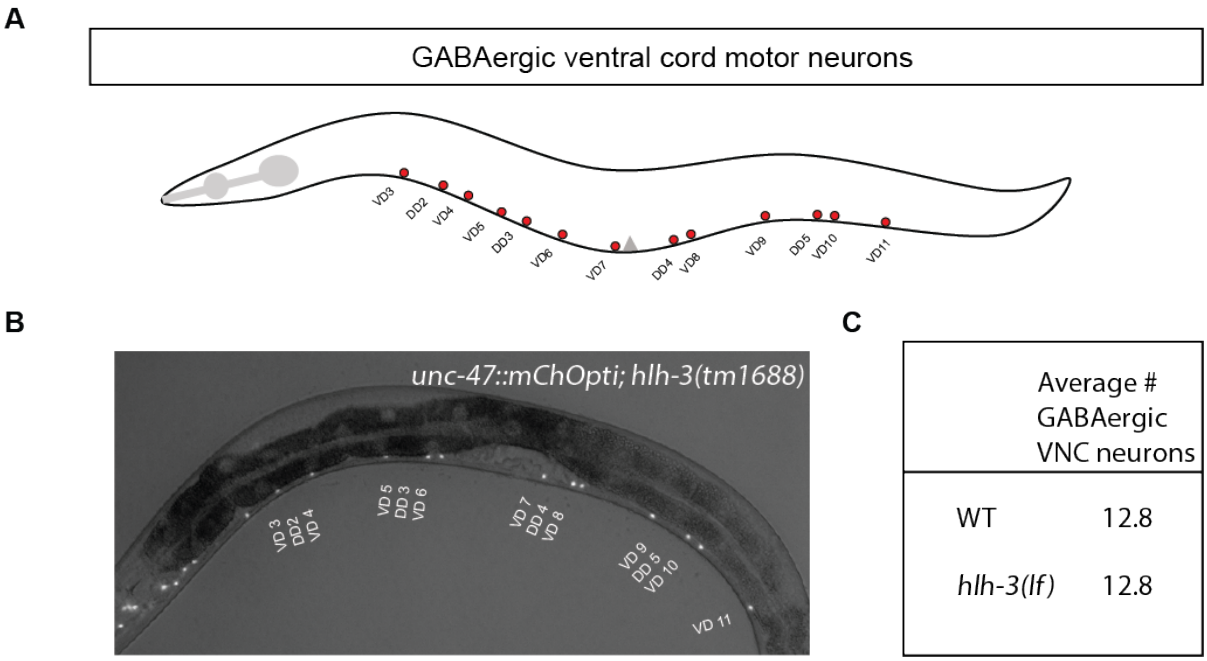


Figure 2.10. GABAergic ventral cord motor neurons differentiate normally in *hlh-3(lf)*.

A) Illustration of the positions of the GABAergic VNC motor neurons scored (only VD 3 through VD 11 were scored, n = 13). B) Representative image of *unc-47* reporter expression (*otIs564 [unc-47fosmid::SL2::mChOpti::H2B; pha-1(+)]*) in a *hlh-3(lf)* mutant individual in L4 development. The gene *unc-47* encodes a vesicular GABA transporter; it marks GABAergic neurons in the VNC. Both WT and *hlh-3(lf)* individuals express the *unc-47* marker (WT not shown). C) Quantification of VNC neurons expressing *otIs564* reported as averages per genotype in one day old WT (n = 14) and *hlh-3(lf)* (n = 14) hermaphrodites.

2.4.10 The male-specific ventral cord motor neurons do not require *hlh-3* function

We wondered whether the male-specific ventral cord motor neuron differentiation was also dependent on *hlh-3* function. The CA and CP pairs of male motor neurons arise from the division of the Pn.aap neuroblast, anteriorly (type CA) and posteriorly (type CP) (Sulston et al., 1980; Figure 2.11A). We tracked differentiation of the CAs 1-9 and the CPs 1-6 with the differentiation markers for *ida-1 and tph-1*, respectively (Figure 2.11B). We find that the *hlh-3(lf)* males when compared to WT males show expression of differentiation markers in all CA and CP neurons, nearly at equivalent proportions (Figure 2.11C, D). This suggests that *hlh-3* does not have a role in promoting the differentiation of these neurons.

2.5 Discussion

2.5.1 *hlh-3* specifies VC fate

Our work identified a new role for the proneural gene *hlh-3* as a regulator of sex-specific motor neuron differentiation in the postembryonic VNC of the hermaphrodite. Both terminal and non-terminal identity features associated with the sex-specific motor neurons, VCs, are reduced or absent in animals that lack *hlh-3* function. While most of our analysis measures transcriptional gene activity of VC identity genes, we also demonstrate that the morphology of the VC subclass is affected. In summary, we implicate *hlh-3* in the specification of the VC motor neuron class.

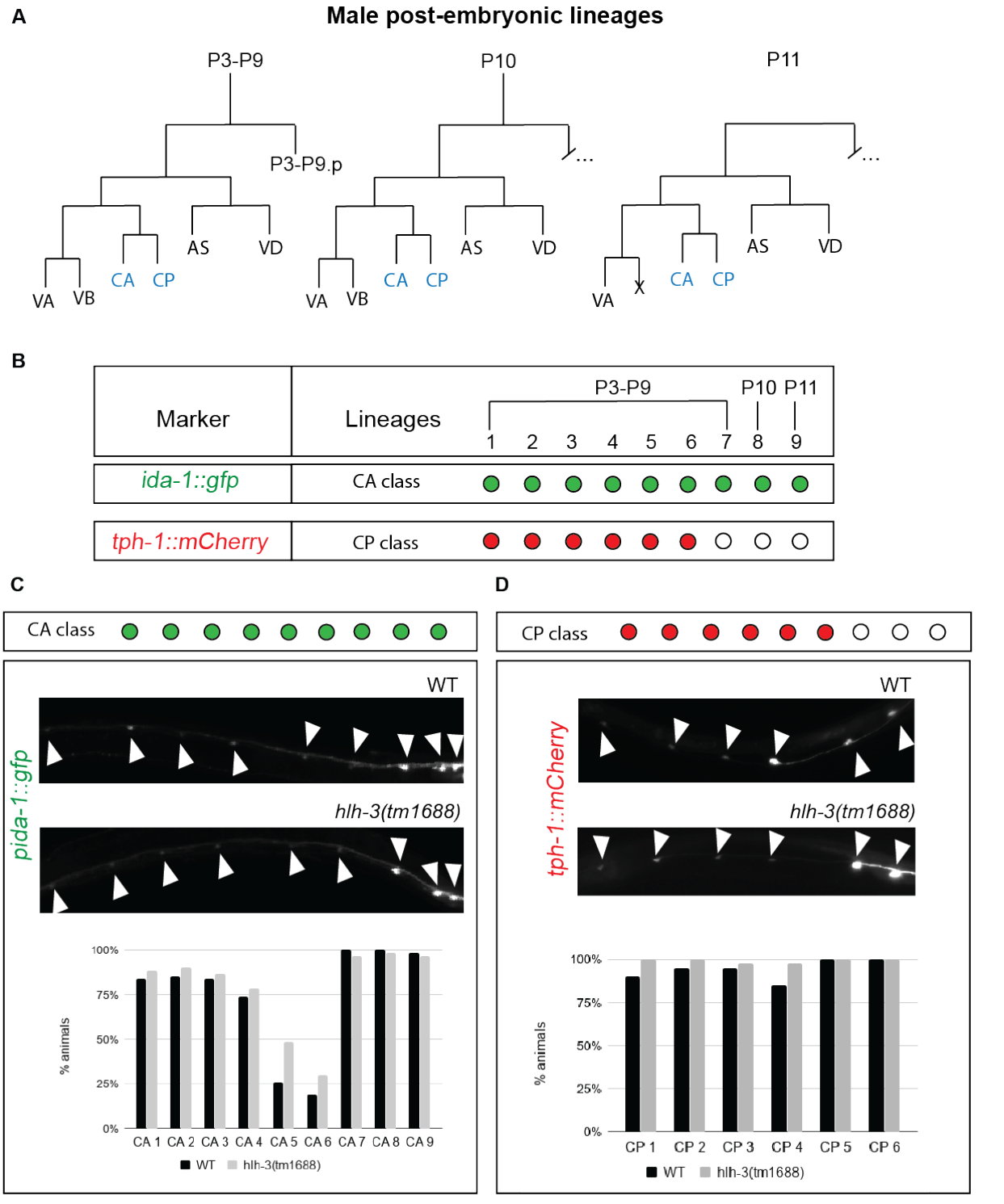

A
Male post-embryonic lineages
P3-P9
P10
P11
P3-P9.p
AS
VD
CA
CP
VA
VB
X
B
Marker
Lineages
P3-P9
P10 P11
1 2 3 4 5 6 7 8 9
ida-1::gfp
CA class
tph-1::mCherry
CP class
C
CA class
WT
pida-1::gfp
hlh-3(tm1688)
% animals
100%
75%
50%
25%
0%
CA 1 CA 2 CA 3 CA 4 CA 5 CA 6 CA 7 CA 8 CA 9
WT
hlh-3(tm1688)
D
CP class
WT
tph-1::mCherry
hlh-3(tm1688)
% animals
100%
75%
50%
25%
0%
CP 1 CP 2 CP 3 CP 4 CP 5 CP 6
WT
hlh-3(tm1688)

Figure 2.11. The differentiation of the male-specific ventral cord motor neurons derived from P cells is not affected by the absence of *hlh-3* function

A) Diagram of post-embryonic lineages in the ventral nerve cord that gives rise to CA and CP male-specific neurons. Notably, P2.a divisions give rise to CP0 but are not shown (adapted from Sulston et al., 1980). B) Summary of the expression pattern of *ida-1::gfp* and *tph-1::mCherry* in CAs and CPs, respectively (based on data from Kalis et al., 2014; Loer & Kenyon, 1993). C) Quantification of expression of *pida-1::gfp* in the adult male ventral cord of wild type and mutant individuals. Representative fluorescent images for each genotype (top). Graph reports the percent of animals with detectable expression in each cell of WT (n = 71) and *hlh-3(lf)* (n = 61) males. D) Quantification of expression of *ptph-1::mCherry* expression in the adult male ventral cord of wild type and mutant individuals. Representative fluorescent images for each genotype (top). Graph reports the percent of animals with detectable expression in each cell of WT (n = 20) and *hlh-3(lf)* (n = 41) males.

2.5.2 Differentiation of the proximal VCs involve *hlh-3* dependent and *hlh-3*-independent mechanisms

Our work demonstrates that in the absence of *hlh-3* function, the differentiation of proximal VCs is less affected than that of distal VCs. We have gained some insight into these differences with the analysis of markers that are expressed in early L4 versus later L4 substages (Figure 2.3). Expression of VC class and VC subclass-specific identity features *ida-1*, *glr-5*, and *mir-124*, is seen in VCs in early L4 substages in a WT context, yet, are completely absent from these early substages through adulthood in animals that lack *hlh-3* function (Figure 2.12A). This indicates *hlh-3* function is required before L4 development (Figure 2.6). We also learned that in the mutant context, and during later stages of L4 development, expression of these VC identity features appeared in just a few VCs, the proximal ones. This suggests that there may be a parallel pathway, which can promote VC differentiation. Since the proximal VCs are less affected in their expression of the terminal identity genes that arise after mid-L4 development (*unc-4* and *unc-17*), we propose that this alternative pathway acts by mid L4 but not sooner. We infer that the *hlh-3* independent parallel pathway is mediated by EGF, a cue secreted as early as mid L4, already shown to be required for expression of *unc-4* in proximal VCs (Figure 2.12; Zheng et al., 2013). The presence of this parallel pathway could ensure that at least proximal VCs retain some function, as they are primary contributors to egg-laying by providing feedback to HSNs and vulva muscles (Schafer, 2006).

In summary, we have found that the acquisition of VC class features (shown herein) is impaired in *hlh-3(lf)* individuals. Of the features we have analyzed, only one subclass differentiation feature, expression of *mir-124*, is fully dependent on *hlh-3* function (Figure

2.7, Figure 2.12). Since *mir-124* expression is restricted to the VC proximal subclass, it may have a role in promoting VC subclass diversity. However, since the expression of *mir-124*, is seen prior to the EGF cue, and is completely absent in *hlh-3(lf)*, we believe that it is regulated by *hlh-3* and not by the EGF-dependent pathway. Further work has to address whether *mir-124* functions as an intrinsic, cell-autonomous mechanism to promote VC class diversity.

With this work, we propose that: (1) *hlh-3* functions cell-autonomously to specify VC class fate early in development (from L1 to L4) and (2) during L4 development an EGF-dependent cue promotes proximal VC subclass fate diversity for function in egg-laying. Our proposal is consistent with the observation that expression of *lin-11*, *glr-5*, *ida-1*, and *unc-4*, in the proximal VCs, is not significantly altered in the absence of *hlh-3* function. To reiterate, the proposed *hlh-3* dependent pathway specifies VC class fate and an *hlh-3* independent pathway promotes VC subclass diversity.

2.5.3 The LIM homeodomain transcription factor LIN-11 in VCs is downstream of and positively regulated by *hlh-3*

As shown by others, the gene encoding LIN-11 is expressed from L2 through adulthood (Hobert et al., 1998). We have observed this as well with the translational reporter *wgIs62 (lin-11::TY1::EGFP::3xFLAG + unc-119(+))* (data not shown). Since our analysis indicates that *hlh-3* is expressed before *lin-11*, we characterized the expression of a *lin-11* transcriptional reporter *(plin-11::mCherry)* in the absence of *hlh-3* function. We showed that *hlh-3(lf)* mutants exhibit reduced *lin-11* transcriptional activity in VCs (Figure 2.9). It is likely that *hlh-3* directly targets *lin-11*, but further work will determine whether this effect is indirect or indirect. Interestingly, the ortholog ASCL1 has been shown to directly target the

lin-11 ortholog, Lhx1, in a ChIP-seq analysis of the ventral telencephalon (Raposo et al., 2015; Kim et al., 2018).

Our analysis of *lin-11* expression in *hlh-3(lf)* also revealed that the proximal VCs are less affected than the distal VCs by the absence of *hlh-3* function (Figure 2.9A). The proximal VCs express *plin-11*::*mCherry* at higher proportions than the distal VCs. This prompted us to ask whether the presence of *lin-11* transcriptional activity is dependent on a secondary pathway other than one that is mediated by *hlh-3*. Given that others have shown *lin-11* acts downstream of EGF, *lin-11* may be targeted by both a *hlh-3* dependent pathway and this secondary EGF-dependent pathway (Figure 2.12B; Zheng et al., 2013).

We propose that the reason *lin-11* transcriptional activity is observed in the proximal VCs of *hlh-3(lf)* individuals is that EGF-dependent signaling is acting in parallel to *hlh-3*. It is known that the proximal VCs acquire this subclass-specific identity feature (*unc-4*) in a time-dependent manner, occurring after EGF signaling, after mid-L4 development (Zheng et al., 2013). Our analysis suggests the EGF signaling pathway promotes *lin-11* transcription too. This would explain why, in the absence of *hlh-3*, there is still expression of *lin-11* (Figure 2.9). Lastly, our findings that *hlh-3(lf)* mutants also exhibit reduced *unc-4* transcriptional activity in the proximal VCs is a logical consequence of lower *lin-11* expression in the proximal VCs (Figure 2.5D, 2.12A). Our model shows that two pathways affect the expression of *lin-11* and other VC identity genes (Figure 2.12).

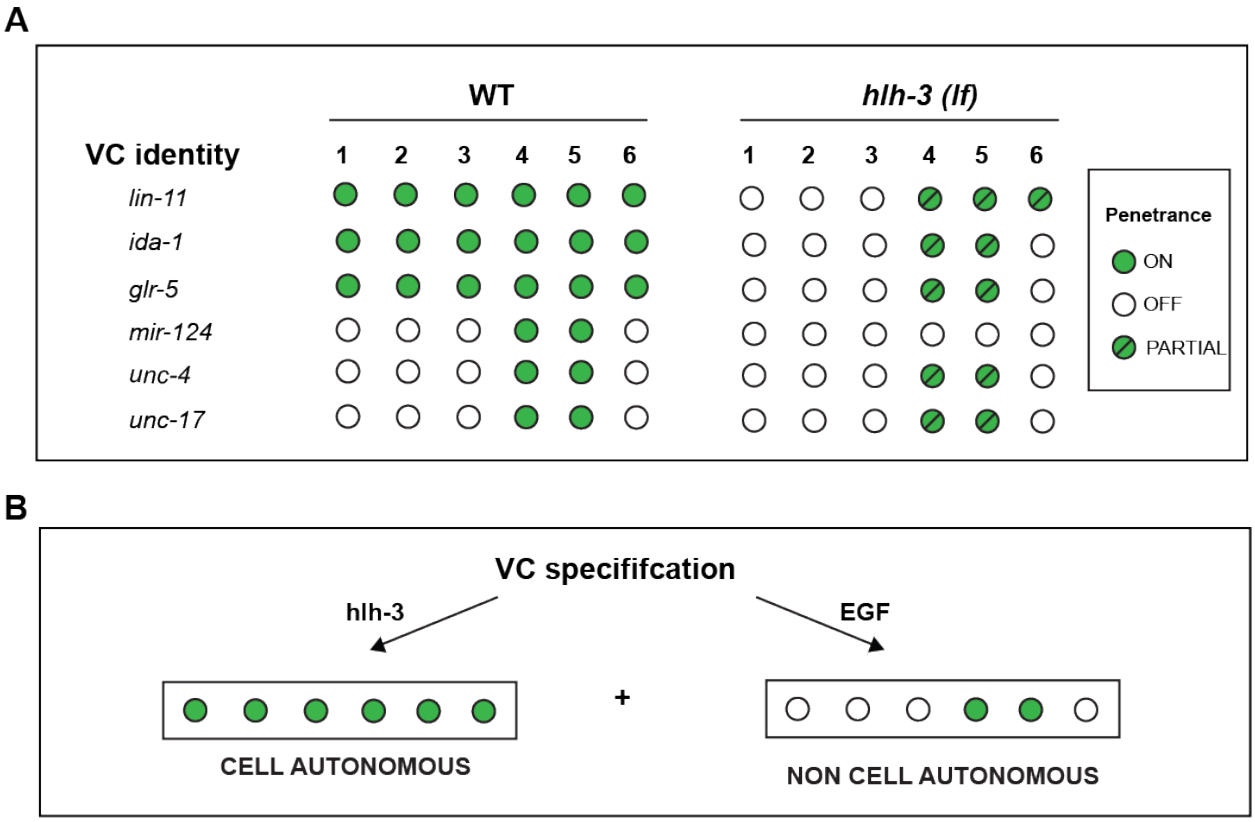


Figure 2.12. Two pathways promote the acquisition and maintenance of VC-class and VC-subclass genes

A) Expression of the VC identity features (*lin-11, ida-1, glr-5, mir-124*) require *hlh-3* function. B) The regulation of the VC identity features occurs in a cell-autonomous way prior and independently of EGF signaling during mid-L4 development. The alternative pathway, dependent on EGF, regulates the expression of *unc-4* (Zheng et al., 2013). We propose that the function of EGF signaling adds a secondary input to regulate *lin-11* levels in the proximal VCs, and affect *unc-4* and *unc-17,* as well as other VC identity features.

2.5.4 *hlh-3* may be a terminal selector of VC fate

Terminal selectors are factors that initiate and maintain the expression of effector genes, required in the final determination of neuronal subtype specification (Allan & Thor, 2015). *hlh-3* meets several criteria to be classified as a gene encoding a terminal selector, in the VCs First, it is expressed from the birth to the maturation of all VC features. Second, in its absence, all known VC class terminal identity features fail to be acquired. Lastly, it functions cell-autonomously. Since more than one terminal selector can function to regulate downstream effector genes, it is possible that another terminal selector may function with *hlh-3*. To confirm if *hlh-3* is a terminal selector, additional work will need to test for the direct regulation of VC identity genes by *hlh-3*.

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2.7 References

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3 Chapter III: hlh-10 has a role in repressing VC identity genes

CHAPTER III:
hlh-10 has a role in repressing VC identity genes

3.1 Introduction

3.1.1 HLH-10 is a predicted ortholog of the tumor suppressor protein TCF21

The Class II bHLH protein HLH-10 is encoded by four exons (Figure 3.1A) and has been shown to dimerize with the Class I bHLH protein HLH-2 (Grove et al., 2009). The HLH-10/HLH-2 heterodimer binds promiscuously to E-box motifs (CANNTG); it can bind "CACCTG," "CACCTG," "CAGCTG," "CATCTG," and "CATATG" (Grove et al., 2009). Among these the highest binding specificity is biased for the "CAT" half-site (De Masi et al., 2011).

As discussed in Chapter I, some bHLH proteins ie., HLH-2/Da and HLH-3/MASH-1/ASCL1, show evolutionary conservation in their sequence and function. The human ortholog of HLH-10 is TCF21 (Grove et al., 2009; Kim et al., 2018). An amino acid sequence alignment identifies 52% overall sequence identity, and 56% sequence identity (29/52) within the bHLH domain (position 4 to position 55) (Figure 3.1B). Initial reports of the ortholog TCF21 showed expression in the epithelium and mesenchyme of several organs including kidney (Quaggin et al., 1998), heart, and alveoli (Smith et al., 2006). TCF21 function has been implicated in the specification of cardiac fibroblasts and suggested to act as a transcriptional repressor (Acharya et al., 2012; Tandon et al., 2012).

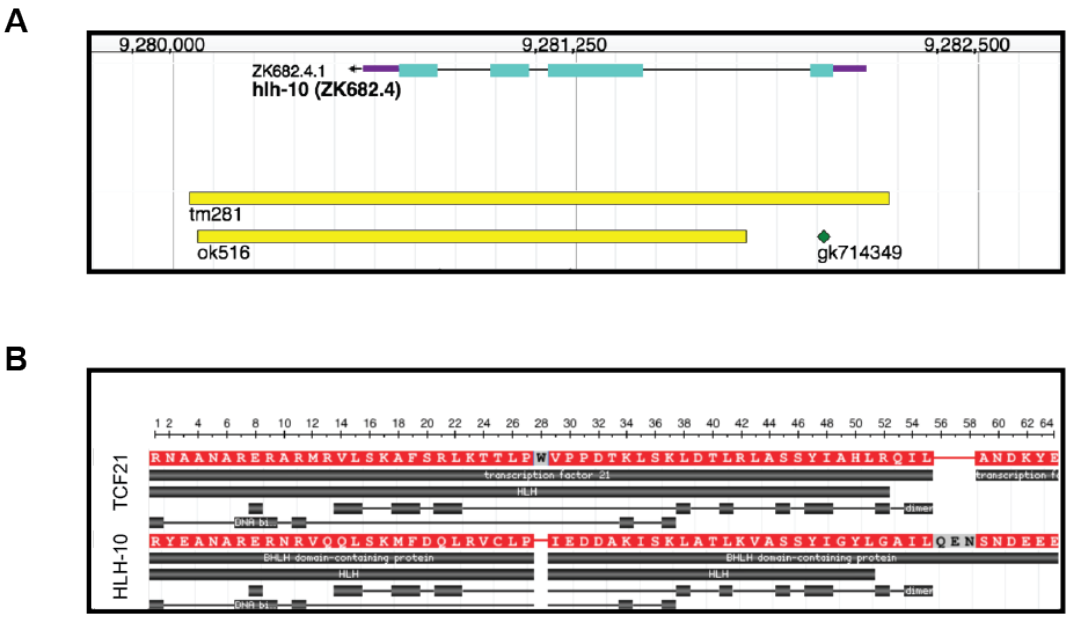


Figure 3.1. *hlh-10(ok516)* spans the bHLH domain.

A) Diagram of *hlh-10* locus (V: 9280590..9282154). Teal rectangles represent coding exons. Yellow boxes indicate the extent of deleted DNA in two different alleles, the green diamond represents a point mutation in exon 1. The allele *ok516* represents a 1707 bp deletion, the allele *tm281* represents a 2174 bp deletion (diagram rendered in JBrowse by Wormbase). In this work we use allele *hlh-10(ok516)* B) Alignment of amino acid sequence of the bHLH domain of *H. sapiens* TCF21 and *C. elegans* HLH-10 obtained from NP_003197.2 and NP_505401.2, respectively (diagram rendered with NCBI by BLASTP using standardized scoring parameters with BLOSUM62 Matrix: Multiple Sequence Alignment Viewer). There is 52% sequence identity (33/64), 6% gaps (4/64), and 88% positive matches (44/64).

3.1.2 In *C. elegans* the expression of *hlh-10* is restricted to post-embryonic developmental stages

In *C. elegans*, the expression of *hlh-10* is primarily observed in the head and tail regions of the hermaphrodite. Two laboratories have cataloged the *hlh-10* spatial expression using the same transcriptional reporter (*hlh-10::yfp*). They observed expression in the shared pairs of chemosensory neurons ASE and AFD (Etchberger et al., 2007) and the male-specific tail ray neurons (A and B type) 2-5 (*hlh-10::yfp*) (Portman & Emmons, 2004). Additionally, a separate study showed *hlh-10* is also expressed in the ADL head neurons of larvae and adults, because *phlh-10::gfp* is co-expressed with *psri-51::mCherry* an ADL marker (Gruner et al., 2016). Lastly, *hlh-10* transcriptional activity was monitored over development by Grove et al., 2009. They used an extrachromosomal transcriptional reporter *leEx1432 [hlh-10::GFP + unc-119(+)]*, this analysis was the first to reveal post-embryonic expression in the VCs as well as other areas of the nervous system including in head and tail neurons.

3.1.3 A function of *hlh-10* has yet to be determined in *C. elegans* development

The function of *hlh-10* has not been established as the homozygous mutant strains harboring any of the two existing deletions (Figure 3.1A) are viable and without an overt behavioral phenotype. Moreover, there is limited information on the mutant phenotype of the total loss of function alleles in the context of the nervous system. Knockdown of *hlh-10* reduces but does not abolish expression of the transcriptional reporter *psrh-24::gfp* in the ADL neurons (Gruner et al., 2016). This observation led to the suggestion that HLH-10 functions redundantly with other bHLHs. Given that HLH-10 is a Class II bHLH capable of promiscuous binding (Grove et al, 2009), it is not unreasonable to suggest it could bind to (creates an opportunity to bind) and potentially regulate many shared targets with other Class

II bHLH factors. In summary, there is no published data to inform if *hlh-10* has a role in the development of neuronal differentiation.

To address whether *hlh-10* has a role in the nervous system I examined two easy to score behaviors, locomotion and the ability to lay eggs, in hermaphrodites harboring *hlh-10(ok516) V* an allele generated by the *C. elegans* Gene Knockout Project harboring a 1707 bp deletion which removes most of the coding region including the bHLH domain (Figure 3.1 A). At first glance, homozygous mutant hermaphrodites do not show an overt behavioral phenotype, they are not uncoordinated or egg-laying defective. In contrast, *hlh-3(tm1688)* hermaphrodites retain eggs in utero and sometimes become bags of worms (Doonan et al., 2006) (Doonan et al., 2008).

However, since *hlh-10* is expressed in VCs and my analysis (Chapter II) suggests that VC differentiation is dependent on other regulators that work redundantly or in parallel with *hlh-3*, I sought to test whether *hlh-10* function is required for the differentiation of the VCs. The reasons *hlh-10* was a reasonable candidate to test were: (1) it encodes a Class II bHLH-containing protein capable of binding to E-boxes in effector genes that might be regulated by *hlh-3* (2) it is expressed post-embryonically and the only other Class II bHLH factor encoding gene expressed in the VC lineages (Grove et al., 2009). To determine whether *hlh-10* has a role in VC differentiation, I examined the expression pattern of VC markers in *hlh-10(ok516),* further referred to as a loss of function allele *"hlh-10(lf)*." Both VC class and VC subclass specific identity markers were analyzed.

3.2 Methods

3.2.1 Strains and maintenance

All strains were maintained at 22°C on nutrient growth media using standard conditions (Brenner, 1974). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *hlh-3(tm1688)* was isolated by the National Bioresource Project of Japan. The alleles reported herein include: LGII: *hlh-3(tm1688)*, LGV: *dpy-11(e224)* and *hlh-10(ok516)*. The integrated transgenes reported herein include: *IeEx1432* [*hlh-10::GFP + unc-119(+)], inIs179 [pida-1::gfp], otIs456 [pmyo-2::gfp, plin-11::mCherry]*, *uIs45 [punc-4::MDM2::gfp + rol-4(+)]*, *vsIs48 [punc-17::gfp]* and *punc-47::gfp* (kindly shared by Dr. Ilya Ruvinsky).

3.2.2 Microscopy

Animals were mounted on 3% agarose pads containing droplets of 10mM levamisole. Fluorescent images were acquired with AxioVision on Zeiss Axioskop 2 microscope. Following the collection of images, some conversions were made with FIJI version 2.0.0 (grayscale images were converted with Lookup tables: Red or Green) and processed into Adobe Illustrator for formatting. Fluorescent reporters were observed under confocal microscopy for the presence or absence of a fluorescent protein signal in transgenic lines. This study does not report quantification of intensity for any fluorescent reporter observed.

3.3 Results

3.3.1 Expression of *hlh-10::gfp* in the ventral nerve cord is dynamic

First, we sought to characterize the expression of *IeEx1432* [p*hlh-10::GFP + unc-119(+)]* in the ventral cord of hermaphrodites during larval development. Expression of the extrachromosomal array *IeEx1432* is absent in the ventral cord before L4 (Figure 3.2A) and turns on at low levels during L4 development in many, unidentified, motor neurons (Figure 3.2B). As hermaphrodites mature and transition into adulthood, expression diminishes until extinguished from the unidentified neurons but remains detectable in six ventral cord neurons (Figure 3.2C). We presumed these *IeEx1432* expressing neurons are VCs based on position and in agreement with previously reported observations (Grove et al., 2009). Notably, the neurons that flank the vulva appear to have lower levels of *IeEx1432* expression than the neurons that are distal to the vulva. It is not clear whether this is a reflection of transcriptional activity differences within the VCs, or if this is an artifact of mosaic expression of an extrachromosomal array. To confirm that *IeEx1432* is expressed in VCs, I created a strain that also carries a known VC marker *(plin-11::mCherry)* to co-label these neurons. Unexpectedly, few animals show overlapping expression of *phlh-10::gfp* and *plin-11::mCherry* in VCs during adulthood (Figure 3.2D) suggesting they may be regulated by the same factor (i.e, a titration effect).

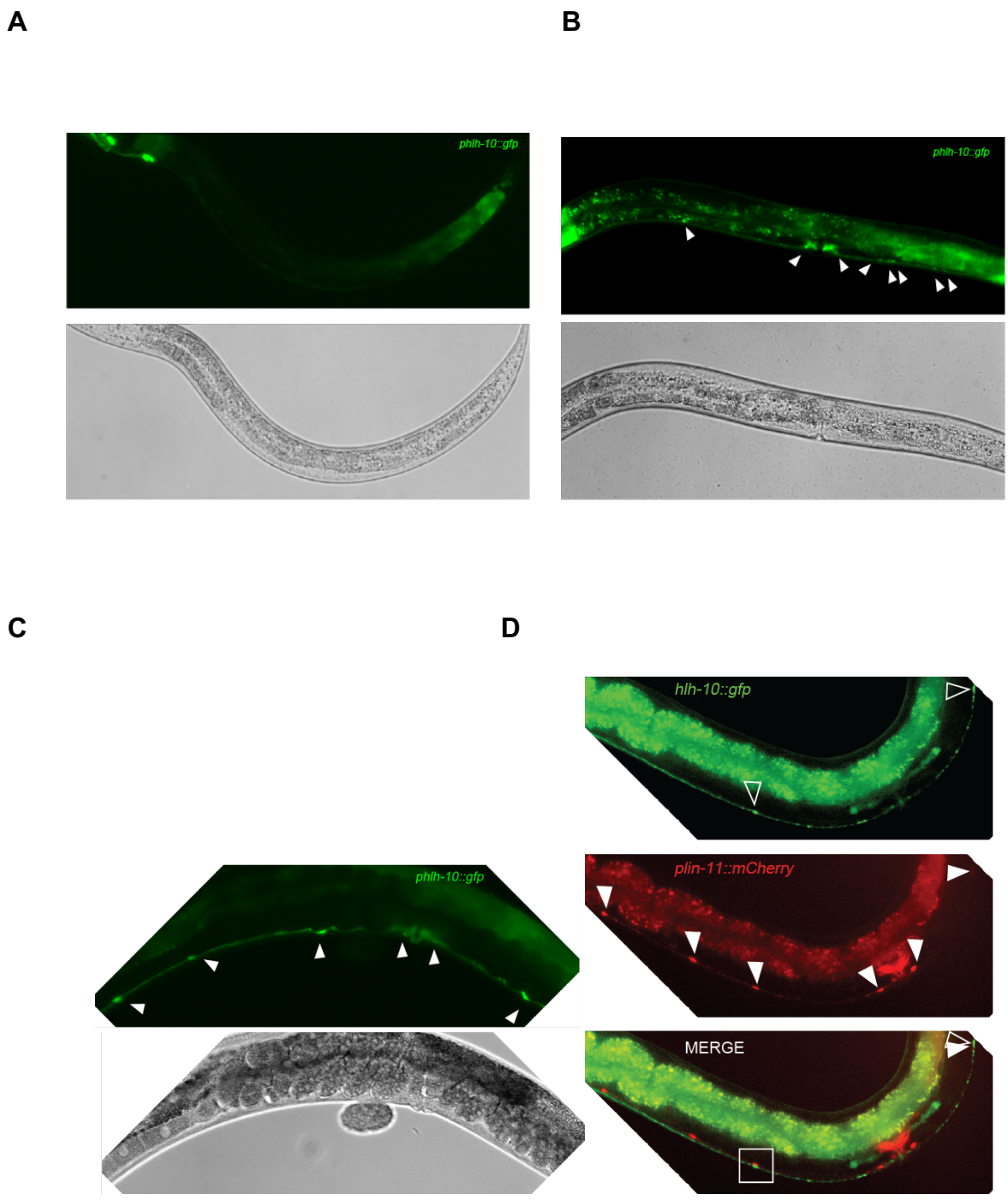


Figure 3.2. *phlh-10::gfp* expression is restricted to the VC nuclei in adulthood.

A) Representative image of an L2 individual (bottom panel) harboring *IeEx1432* [*phlh-10::GFP + unc-119(+)]* but not reporting expression in the ventral nerve cord (top panel) (n>5).

B) Representative image of L4 individual (bottom panel) reporting expression of *IeEx1432* along the ventral nerve cord (white arrowheads top panel) (n > 10). C) Representative image of adult (bottom panel) reporting expression of *IeEx1432* [*hlh-10::GFP + unc-119(+)]* in all VCs cells. This expression appears most intensely in distal VCs (top panel). D) Representative image of adult hermaphrodite harboring *IeEx1432* [*hlh-10::GFP + unc-119(+)]* (top panel) and *plin11::mCherry* (middle panel). VC3 expresses both reporters (bottom panel).

3.3.2 *hlh-10* does not regulate expression of *hlh-3* in VCs

Since *hlh-10* transcriptional activity was detected in the ventral nerve cord from L4 through adulthood, a time when VCs are maturing, and *hlh-3* function is present, I sought to address the possibility that *hlh-10* has a function in regulating *hlh*-3 in the ventral nerve cord. Given that *hlh-3* expression precedes *hlh-10* expression it is unlikely that HLH-10 regulates *hlh-3* (Chapter II Figure 2 and Figure 3.2*)*. However, I proceeded to test this assumption. I examined the expression of *ic271* [*hlh-3::gfp]* in *hlh-10(lf)*. As shown in Figure 3.3, expression of *ic272 [hlh-3::gfp]* is still detectable in the VCs of *hlh-10(lf)* individuals at the L4 developmental stage. Since the expression of *ic272* is still observed in the absence of *hlh-10* function, HLH-10 does not regulate *hlh-3* expression in VCs.

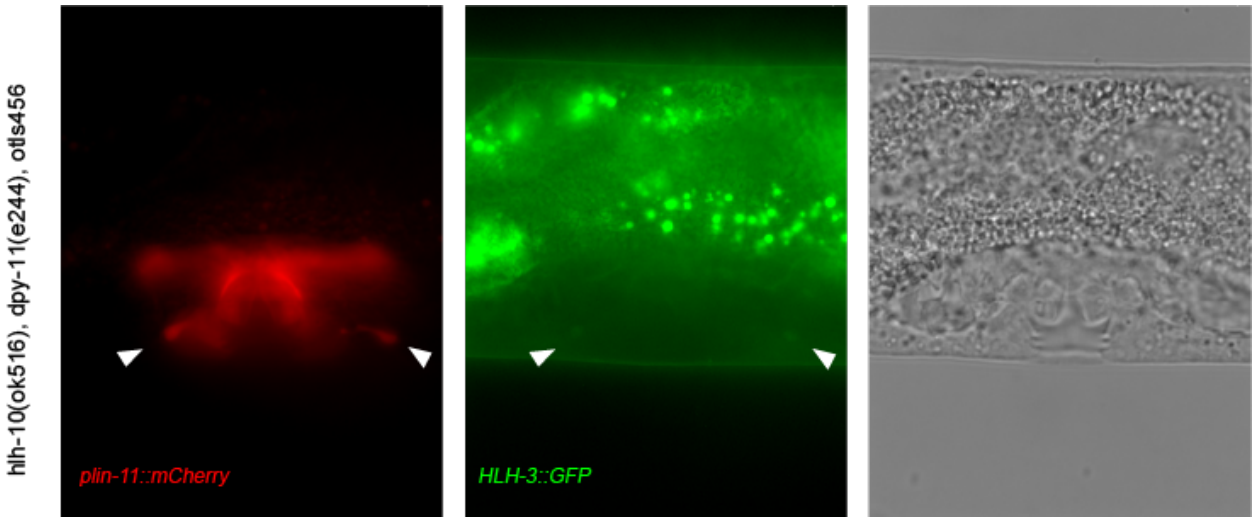


Figure 3.3. Expression of HLH-3::GFP is not affected by *hlh-10(ok516)*.

Representative images of the proximal VCs (white arrows) of an *hlh-10(ok516), dpy-11(e244)* individual in mid L4 (right panel, DIC) harboring a *plin-11::mCherry* reporter (left panel) and the *hlh-3(ic271)* allele (middle). 3/3 L3s, 5/5 L4s, and 2/2 adults express HLH-3::GFP in VCs. The transgene *otIs456* carries the selectable marker *myo-2::GFP* and *plin-11::mCherry*. Vulval tissue as well as the VCs express the *plin-11::mCherry* reporter (left panel). Gut granules autofluorescence are detected in the GFP channel (middle panel).

3.3.3 The class-specific feature *ida-1* is expressed in VCs and non-VCs in *hlh-10(ok516)*

To test whether *hlh-10* could have a role in the acquisition of VC fate I characterized the expression of a VC identity gene, *ida-1* in animals lacking *hlh-10* function. I find that expression of *InIs179 [ida-1::gfp]* is detectable in all VCs of *hlh-10(lf)* adults to the same extent as in WT individuals (Figures 3.4A and Figure 3.4B). Therefore, *hlh-10* function is not required for the expression of this one VC identity gene within VCs.

Notably, *hlh-10(lf)* animals displayed ectopic expression of *InIs179* along the ventral nerve cord in a reproducible pattern, immediately adjacent to a distal VC. Figure 3.4C highlights abnormal expression of *InIs179* near VC6. The penetrance for this ectopic expression was highest near VC6 (WT = 1/39 and *hlh-10(lf)* = 6/39) and occurred less frequently near VC3 (Figure 3.4D).

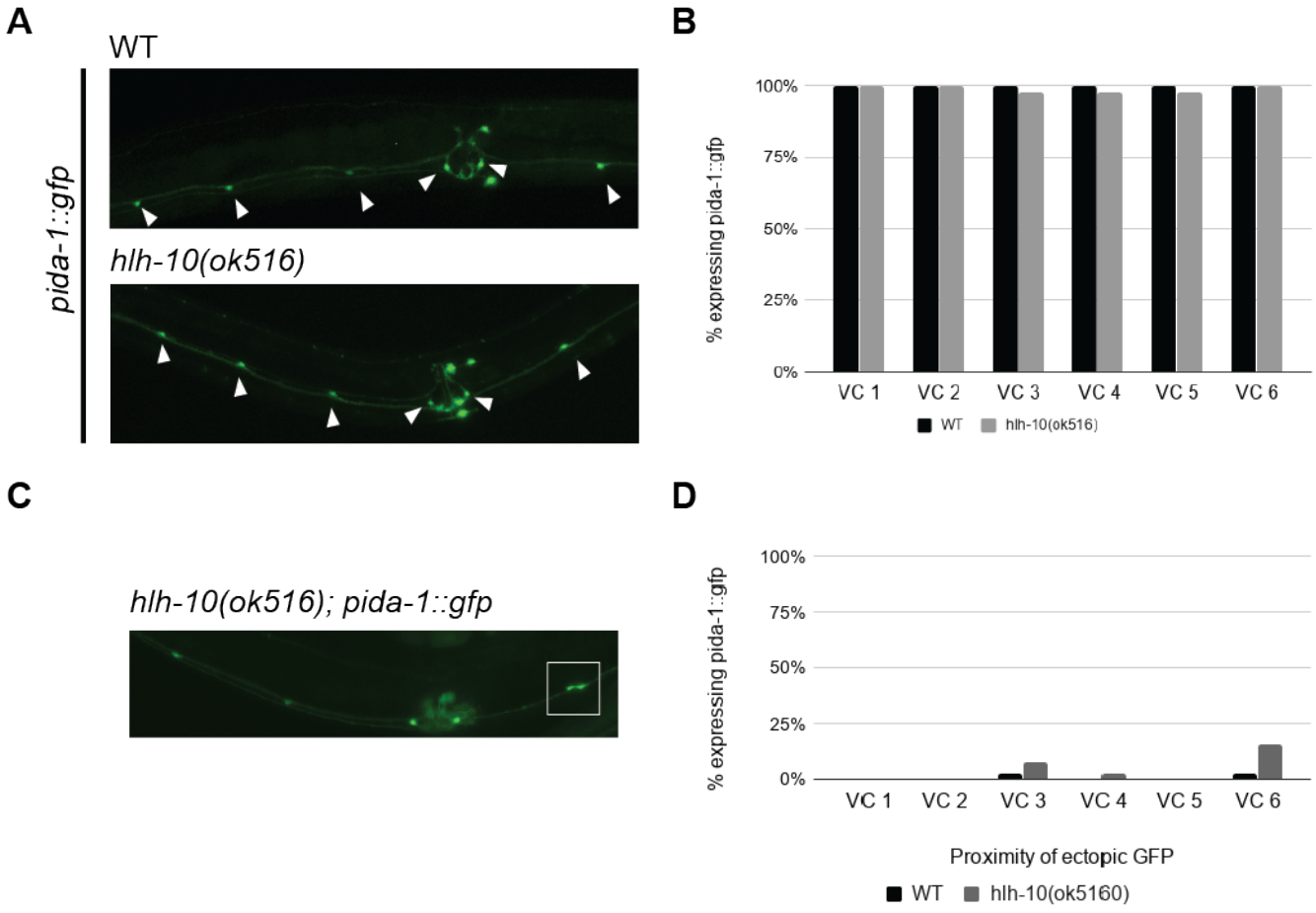


Figure 3.4. All VCs express the VC identity gene *ida-1* in *hlh-10(ok516)* mutants.

A) Representative images of adults from the indicated genotypes harboring *inIs179* [*pida-1::gfp*]. VCs are marked by white arrowheads, in WT (top), and in *hlh-10(ok516*) adults (bottom). B) Quantification of the percent of animals expressing *pida-1::gfp* each VC of WT [*him-8(e1489); inIs179*] and *hlh-10(ok516); inIs179* one day old adults. C) Representative image of a *hlh-10(lf)* adult reporting ectopic expression of *pida-1::gfp* in a cell proximal to VC6, outlined by white box. D) Quantification of the percent of animals expressing ectopic *pdida-1::gfp* near any VC in WT (n = 39) and *hlh-10(lf)* (n = 39).

3.3.4 The GABAergic and cholinergic fate of ventral cord motor neurons is not affected by *hlh-10(ok516)*

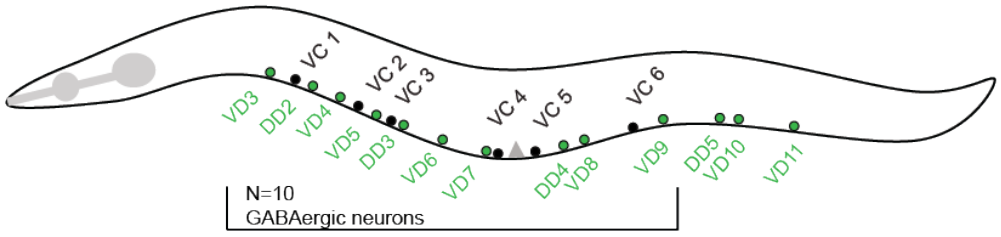
Given that the expression of *hlh-10* was detected in non-VC ventral cord motor neurons during the fourth larval stage (L4) (Figure 3.2), I sought to test whether *hlh-10* might have a function in the differentiation of these unidentified non-VC neurons. Do the sex shared motor neurons of the ventral cord differentiate properly in the absence of *hlh-10* function? One way to assess whether there is a role for *hlh-10* in the ventral cord is to observe differentiation markers for the non-VC motor neurons. Therefore, I analyzed the expression of known GABAergic and cholinergic fate markers to quantify the number of GABAergic or cholinergic neurons in the ventral cord of *hlh-10(lf)* mutants.

GABAergic neuron classes VD and DD make up the inhibitory neurons in the *C. elegans* ventral cord (Gendrel et al., 2016; Mclntire et al., 1993). The DD class is embryonically generated whereas the VD class is post-embryonically derived from the ectodermal like P blast cells making up the GABAergic neurons of the ventral cord (6 DDs and 13 VDs). To identify the GABAergic neurons, I looked at the expression of a transcriptional GFP reporter where the promoter of *unc-47* drives expression of GFP (kindly shared by Ilya Ruvinsky [Strain YR333]). *unc-47* encodes UNC-47, a synaptic vesicle membrane GABA transporter (Hobert, 2013; Mclntire et al., 1993). I focused on the 10 GABAergic neurons between VD3 and VD9 (Figure 3.5 A). I find that regardless of genotype, the reporter is detected in ten GABAergic ventral cord motor neurons (Figure 3.5 B).

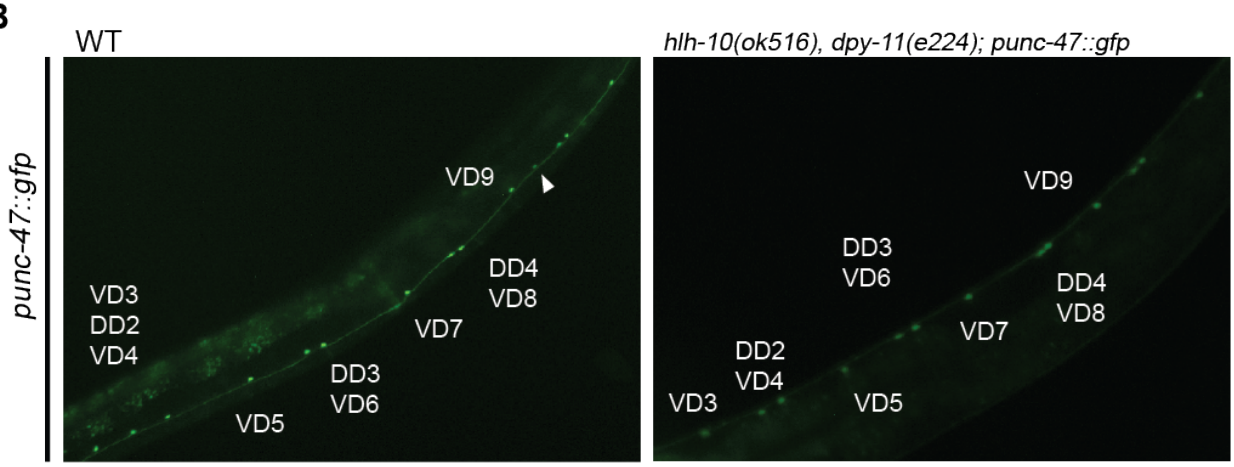
Next, I characterized the average number of sex-shared cholinergic ventral cord motor neurons (VA, VB, AS, DA, and DB) (Figure 3.6A) within the anterior half of the midbody and in the absence of *hlh-10* function. I specifically scored how many nuclei were detectable in the

region from VC1 through VC4 (excluding quantification of VCs). To visualize these cholinergic neurons, I characterized the expression of *vsIs48 [punc-17::gfp]*, a transcriptional reporter where the promoter of *unc-17* drives expression of GFP. The gene *unc-17* encodes a vesicular acetylcholine transporter required in all neurons that secrete the neurotransmitter acetylcholine (ACh) (Alfonso et al., 1993). I quantified the average number of *vsIs48* positive sex-shared motor neurons, ie., non-VCs, in *hlh-10(lf)* mutants (Figure 3.6B). I find the average number of neurons expressing *vsIs48* in *hlh-10(lf)* was equivalent to WT animals (Figure 3.6C).

A



B



C

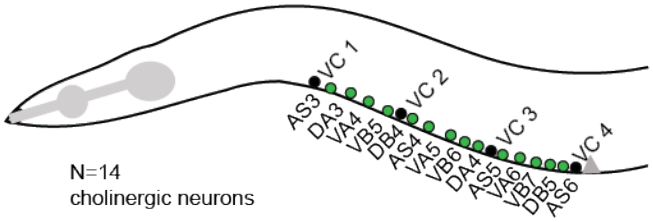
Genotype	Average # GABAergic VNC neurons
WT	10
hlh-10(ok516), dpy-11(e224)	10

Figure 3.5. Differentiation of VNC GABAergic neurons in the midbody is not affected by the absence of *hlh-10* function.

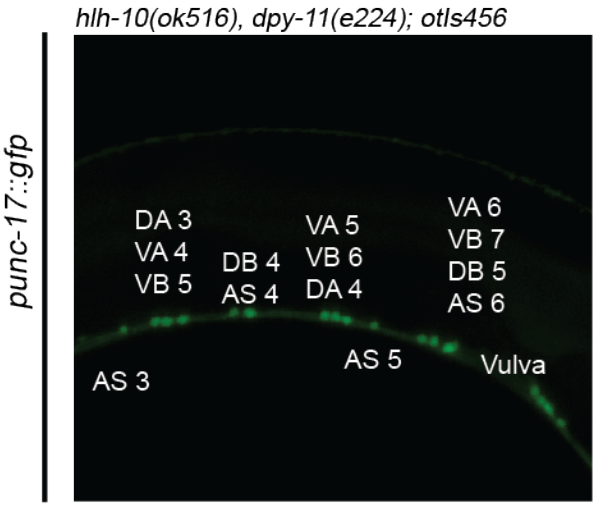
A) Diagram of the position of sex-shared GABAergic and sex-specific VC class neurons (illustrated by green and black circles, respectively). The black bracket highlights the scoring zone used for quantification of an integrated array *punc-47::gfp* from VD 3 through VD 9, as this is the region wherein VC1- VC6 are positioned. B) Representative images of adult

hermaphrodites (20-24 hours post-mid-L4) from the indicated genotypes harboring *punc-47::gfp*. The white triangle identifies an unexpected, likely ectopic cell expressing GFP in a WT adult animal. C) Quantification of WT (n = 17) and *hlh-10(ok516), dpy-11(e244)* (n = 19) adults show equivalent averages of GABAergic VNC neurons in the scoring region. The strain harboring *punc-47::gfp* was kindly shared by Dr. Ilya Ruvinsky.

A



B



C

Genotype	Average # cholinergic VNC neurons
WT *[otIs456; vsIs48]*	13.7
hlh-10(ok516), dpy-11(e224); otIs456; vsIs48	13.7

Figure 3.6. Anterior VNC cholinergic neurons in the midbody are not affected by the absence of *hlh-10* function.

A) Diagram of the position of sex-shared cholinergic and sex-specific VC class neurons, illustrated by green and black circles, respectively. The schematic highlights the scoring zone for quantification of *vsIs48 [punc-17::gfp]* from AS3 through VB7, as this is the region wherein

VC1- VC6 are positioned. B) Representative image of a one-day-old adult hermaphrodite harboring the cholinergic marker. AS6 is the most posterior neuron that is adjacent to the vulva and its detection is often occluded by DB5, although it is always observed with adjustments to the focal plane. C) Quantification of WT *(vsIs48)* (n = 10) and *hlh-10(ok516), dpy-11(e244); otIs456; vsIs48* (n = 22) adults show equivalent averages of cholinergic VNC neurons in the scoring region. WT data as shown previously in Chapter II Supplementary Figure 1.

3.3.5 A negative role for *hlh-10* in the regulation of VC class and VC subclass genes

The *hlh-10(lf)* single mutants do not appear to have differentiation defects in the VCs or the sex- shared ventral cord motor neurons, as evidenced by expression analysis of the *ida-1* VC class identity gene (as previously shown sections). However, I wondered whether the VC differentiation defects seen in *hlh-3(lf)* individuals would be exacerbated if *hlh-10* function was missing too. To address this question, I created a *hlh-3(lf); hlh-10(lf)* double mutant to assess expression for two additional VC identity genes: the VC class gene *lin-11* and the VC subclass gene *unc-4*.

First, I characterized the expression of these markers in *hlh-10(lf)* alone. The presence of the *hlh-10* mutation was tracked with a Dumpy mutation because *hlh-10(lf)* mutants do not show a visible phenotype. We used *dpy-11(e224)* on chromosome V as a visible, linked marker. To ensure *dpy-11(e224)* does not affect the expression of these VC markers, I first characterized their expression in the single mutant. I find that *dpy-11(e224)* adult hermaphrodites showed an equivalent expression pattern of the *lin-11* reporter when compared to WT, non-Dpy animals (Figure 3.7A). Next, I characterized the expression of *lin-11* in the *hlh-10(lf); dpy-11(e224)* strain. Consistent with our previous finding (Figure 3.4) where expression of the VC class gene *ida-1* was not altered in a *hlh-10(lf)* hermaphrodite, adult *hlh-10(ok516); dpy-11(e224)* hermaphrodites also showed no alteration in the expression of *lin-11* in the VCs (Figure 3.7A and Figure 3.7B). Interestingly, we also observed a few cases, where ectopic expression is seen in VC-like cells along the ventral nerve cord. This ectopic expression had already been documented when characterizing the expression of *ida-1::gfp* in *hlh-10(lf)* individuals (Figure 3.4D). In summary, loss of *hlh-10* function did not affect the pattern of expression of *plin-11::mCherry* in VCs.

Next, I sought to examine the effect of the absence of both *hlh-3* and *hlh-10* function. To our surprise, one day old adult *hlh-3(lf); hlh-10(lf); dpy-11(e224)* hermaphrodites showed expression of *plin-11::mCherry* in anterior VCs, wherein they are rarely detected in *hlh-3(lf)* alone. The recovery of expression in the distal VCs- when compared to the expression levels in *hlh-3(lf)* individuals- was the most robust in the anterior VC1-3 (Figure 3.7A and Figure 3.7C). Additionally, expression of *plin-11::mCherry* is more robust in the distal VC6 of *hlh-3(lf); hlh-10(lf); dpy-11(e224)* mutants compared to *hlh-3(lf)* single mutants. Thus, removal of *hlh-10* function in the absence of *hlh-3* function relieves an inhibitory process and these cells (VC-like) now can express *lin-11*. I did not detect notable differences in the proximal VCs.

Next, I characterized the expression of the VC subclass (proximal) identity gene *unc-4* in double mutants *hlh-3(lf); hlh-10(lf)* and single mutants *hlh-10(lf)*. I find that the percent of animals expressing *uIs45 [punc-4::MDM2::gfp]* in proximal VCs is higher in the double mutants than in single mutants *hlh-3(lf),* and highest in *hlh-10(lf)* when compared to WT individuals (Figure 3.8A-C).

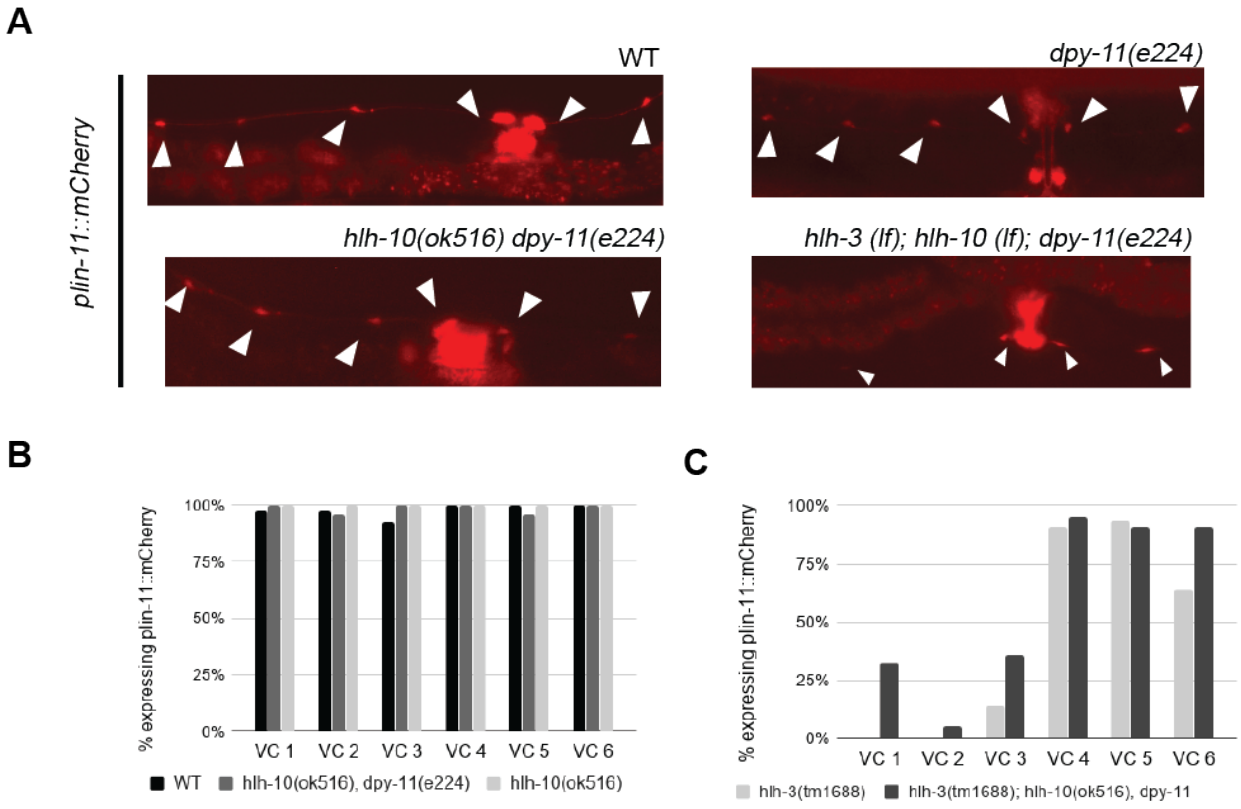
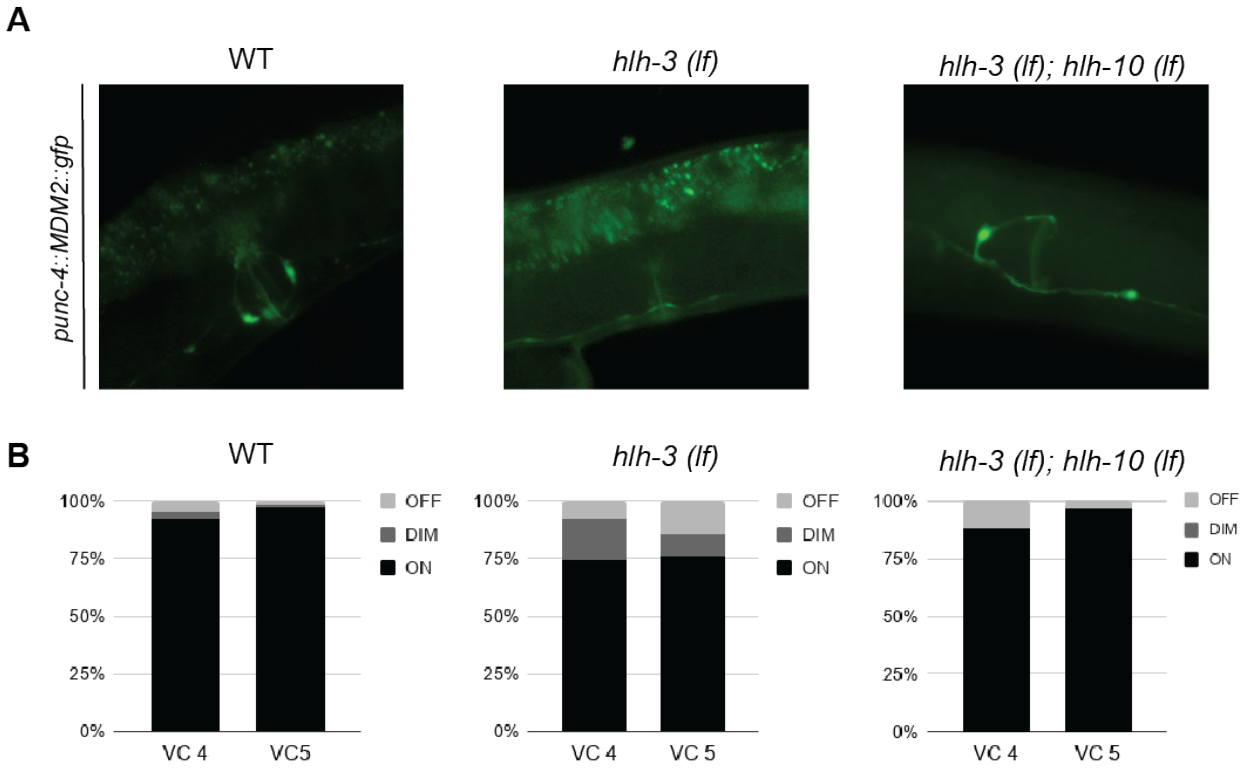


Figure 3.7. The absence of both *hlh-3* and *hlh-10* restores *lin-11* expression in distal VCs.

A) Representative images of adult hermaphrodites of the indicated genotypes harboring a *plin-11:mCherry* reporter. This reporter is expressed in VCs, marked by white arrowheads. B) Quantification of the percentage of one day adults expressing *plin-11::mCherry* in WT [*otIs456*] (n = 40), *hlh-10(ok516), dpy-11(e224), otIs456* (n = 28), *and hlh-10(ok516); otIs456* (n = 15) individuals. C) Quantification of the percentage of adults expressing *plin-11::mCherry* in *hlh-3 (tm1688); hlh-10(ok516), dpy-11(e224), otIs456* (n = 22) compared to *hlh-3(tm1688)* (n = 44) individuals.



C

Genotype	% Abnormal Axon Branching in Proximal VCs
WT	0 %
hlh-3 (lf)	42 %
hlh-3 (lf); hlh-10 (lf)	27 %

Figure 3.8. The absence of both *hlh-3* and *hlh-10* function restores expression of proximal VC differentiation markers and morphology.

A) Representative images of expression pattern of *uIs45* [*punc-4::MDM2::gfp; rol-6(su1006)*] in proximal VCs of indicated genotypes. This reporter also allows for quantification of proximal VC axonal morphology. B) Quantification of *uIs45* detection in WT (n = 66), *hlh-3(lf)* (n = 62), and *hlh-3(lf); hlh-10(lf)* (n = 33) adult hermaphrodites. Animals expressing *uIs45* are scored as

"ON" (black), OFF (dark grey), or DIM (light grey). C) Quantification of the percent of individuals from the indicated genotypes with abnormal axon branching as detected with *uIs45* (normal axon branching was defined as a complete ring-like structure around the vulva) Number of adult individuals analyzed are in WT (n = 15), *hlh-3(lf) (n = 24),* and *hlh-3(lf); hlh-10(lf)* (n = 15). The WT and *hlh-3(lf)* data was reported in Chapter II Figure 5 and included here for comparison.

3.4 Discussion and Future Directions

3.4.1 *hlh-10* may be a direct transcriptional repressor of VC identity genes

The role of the HLH-10 protein in the development of function of the *C. elegans* nervous system is not well understood. With the work described in this chapter, I confirm that *hlh-10* is expressed in the sex-specific ventral cord motor neurons, the VCs, and provide new information about the consequences of lacking *hlh-10* function in the differentiation of the VCs and other sex-shared neurons in the ventral nerve cord. Since VC differentiation markers are re-expressed in *hlh-3(lf); hlh-10(lf)* mutants when compared to *hlh-3(lf)* single mutants, the inhibitory function of *hlh-10* is only revealed in the absence of *hlh-3*: expression of VC terminal identity genes are improved and axonal branching morphology is less affected (Figures 3.7 and 3.8). This indicates that removal of *hlh-10* function has a positive effect when *hlh-3* function is absent.

Our findings suggest that the VC class identity gene, *lin-11*, and the VC subclass identity gene, *unc-4,* are negatively regulated by *hlh-10*. Although there is no evidence that *lin-11* is a direct target of the HLH-2/HLH-10 heterodimer (Grove et al., 2009) a *lin-11* VC enhancer, required to drive expression in VCs contains an E-box (identified in this work and described in Chapter IV). It is intriguing that this E-box motif, CATCTG, contains "CAT" the preferred half-site of HLH-10 (Grove et al., 2009; De Masi et al., 2011). Chapter IV will describe the work performed to dissect the role of this E-box motif on the regulation of *lin-11* expression in VCs.

With respect to *unc-4*, *hlh-10* may indirectly affect expression through the regulation of *lin-11*. Others have reported that *unc-4* expression in VCs requires LIN-11 (Zheng et al., 2013). Therefore, we propose that improved *unc-4* expression in an *hlh-3(lf); hlh-10(lf)* individual, compared to *hlh-3(lf)* individual, could be explained by increased levels of LIN-11. Future work

could quantitatively examine intensity differences of a *lin-11* reporter in the VCs in these genotypes.

The analysis of GABAergic and cholinergic motor neuron differentiation markers in *hlh-10(lf)* indicates that *hlh-10* does not have a role (negative or positive) in the expression of the transcriptional reporter genes *unc-47::gfp* and *unc-17::gfp* in these motor neurons (Figures 3.5 and 3.6). With respect to the VCs, *hlh-10* function is not that of a positive regulator. Currently all we can say is that it is a suppressor of *lin-11* activity and as a result an inhibitor of VC-like features.

Furthermore, with respect to the role of *hlh-3* in the VC neurons, this work suggests that *hlh-10* function is more robust in the distal subclass neurons, since the expression of VC identity genes is improved in the distal VCs, primarily. This could fit into a model whereby *hlh-10* dampens overall levels of VC class identity genes in the distal VCs following EGF signaling, so as to restrict downstream signaling pathways that would instead upregulate these genes in the proximal VCs. One could quantify the levels of HLH-10 between distal and proximal VCs to interpret if it is required in one subclass more than the other.

3.3.2 The inhibitory function of *hlh-10* and *TCF21* may be conserved over evolution

This work shows that the inhibitory role of *hlh-10* is revealed in the absence of *hlh-3*. Its human ortholog, TCF21, has been shown to repress transcriptional activity of the SF-1, AR, and p21 promoters (Cheol et al., 2005; Funato et al., 2003; Käser-Pébernard et al., 2014). In adrenocortical cancer, the overexpression of TCF21 inhibits the function of SF-1 E-box elements (Malheiros França et al., 2013). Second, it has been suggested that TCF21 (formerly POD-1) negatively regulates the androgen receptor (AR) in prostate cancer by obstructing the function of

an E-box in the promoter, known to be an activator binding site (Cheol et al., 2005). Lastly, TCF21 has been shown to recruit a histone deacetylase, HDAC1, and interact with AR in-vivo, forming a complex at the AR promoter to repress AR (Cheol et al., 2005).

The role of TCF21 in cancer biology is intriguing when considering the mechanisms regulating neuronal diversity in *C. elegans*. The expression of the VC (proximal subclass) identity gene *unc-4* is in a constant state of repression until the larval L4 developmental EGF cue induces de-repression in the proximal VCs requiring the function of histone-modifying factors (Zheng et al., 2013). A histone modifier, the conserved histone demethylase SPR-5/LSD-1 is linked with de-repression of *unc-4*: the *spr-5* mutants have less expression of *unc-4* in proximal VCs. Intriguingly, SPR-5 and the homolog HDAC-1 in *C. elegans*, HDA-1, have been shown to physically interact, albeit in embryonic development (Käser-Pébernard et al., 2014). SPR-5 encodes a histone demethylase and HDAC-1 encodes a histone deacetylase, both of which are epigenetic factors responsible for removing histone methyl and acetyl groups, respectively, resulting in open and accessible chromatin or condensed chromatin. Nevertheless, this evidence prompts us to propose that a SPR-5/HDA-1 complex could be formed at other stages of development such as L4 development, and potentially associate with each other to promote the expression of genes like *unc-4*.

4 Chapter IV: Analysis of the lin-11 VC enhancer element reveals novel regulators

CHAPTER IV:
Analysis of the *lin-11* VC enhancer element reveals novel regulators

4.1 Introduction

4.1.1 *lin-11* is a conserved LIM homeodomain factor

In *C. elegans*, *lin-11* is one of several well-known LIM-homeobox domain protein-encoding genes. They are so classified because the structure of the LIM-homeodomain is well conserved between the founding members, the proteins encoded by the genes *lin-11*, *isl-1*, and *mec-3*. This structure comprises one homeodomain (the DNA binding domain) and two cysteine-rich motifs known as LIM1 and LIM2 (the protein-protein interaction domains) (Freyd et al., 1990; Hobert & Westphal, 2000). Interestingly, while the homeodomains are highly similar (75%, 45/60) among the *C. elegans* proteins LIN-11 and MEC-3, the LIM motifs are not as similar (32%, 35/111). In contrast, the two LIM motifs of LIN-11 and the vertebrate protein ISL-1 are slightly more conserved (43%, 48/111) (Freyd et al., 1990). Additionally, homeodomain sequence identity analysis indicates that LIN-11 is more closely related to the subclass LHX1/LHX5 (Hobert et al., 1998). Similar to the ortholog LHX1, the LIN-11 DNA binding homeodomain is split between two exons (Bozzi et al., 1996; Freyd et al., 1990).

In *C. elegans*, the LIM-homeodomain protein encoding genes include *mec-3*, as mentioned, as well as *ttx-3,* and *ceh-14* (Hobert & Westphal, 2000). *mec-3* function is associated with mechanosensory behavior, it is expressed in and required for the terminal differentiation of the touch receptor neurons; all features specific to these cells are lost in *mec-3(lf)* mutants (Way & Chalfie, 1989; Way & Chalfie, 1988). *ttx-3* was first associated with a role in the functional specification of the AIY neurons, interneurons implicated in the integration of thermal cues (Hobert et al., 1997). Subsequent work lead to its classification as a terminal selector for AIY, as

well as the AIA, and NSM neurons, as it is required for the expression of genes that are unique to each of these neuronal classes (Altun-Gultekin et al., 2001; Wenick & Hobert, 2004; Zhang et al., 2014). Finally, *ceh-14* is unlike *mec-3* and *ttx-3*, in that its activity is only required for one of the functions of the AFD neurons, thermotaxis (Cassata et al., 2000). In summary, the reported roles of *mec-3, ttx-3 and ceh-14* demonstrate that LIM homeodomain proteins, in the context of *C. elegans*, are required for acquisition of terminal neuronal features and function but not generation.

4.1.2 *lin-11* has function in the specification and differentiation of neuronal and nonneuronal cells

Specification of both neural and non-neural cells is regulated by *lin-11*. Absence of *lin-11* function primarily disrupts cell lineage specification and morphogenesis of the vulval and uterine tissues in the hermaphrodite resulting in a "lineage defective" phenotype (i.e., *lin-11*) (Ferguson et al., 1987; Gupta & Sternberg, 2002; Newman et al., 1999). In addition to its role in these non-neural tissues, *lin-11* has function in the specification of neurons and in the development of axon morphology discussed below.

The expression of a *lin-11* transcriptional reporter is detected in several pairs of sensory neurons, including head neurons AWA, ASG, AIY, ADL, AVG, AIZ, and the tail phasmid pair of PHA (Hobert et al., 1998; Sarafi-Reinach, 2001). Absence of *lin-11* function does not interfere with the generation of neurons in which *lin-11* expression has been detected, implying its role is dispensable for neurogenesis (Hobert et al., 1998). Consistent with its expression in the head neurons AWA and the ASG, *lin-11* has a role in promoting activation of cell-specific markers in both neurons, but is only sufficient for the specification of the ASG neuron (as ectopic expression of *lin-11* in other cells drives ASG specific gene expression) (Sarafi-Reinach, 2001).

Whereas in the AWA cells, it is required for correct gene expression of *odr-7*; ODR-7 is a transcription activator of AWA function, and a nuclear receptor family member essential for AWA differentiation (Colosimo et al., 2003; Sarafi-Reinach, 2001; Sengupta et al., 1994). However, ectopic expression of *lin-11* does not drive AWA specific gene expression. Further analysis has indicated other neurons, beyond head sensory neurons, express and require *lin-11* function, including AVG, PVK and VC cells discussed below.

In the ventral nerve cord, *lin-11* is expressed and has a function in AVG neurons along the ventral cord including the AVG pioneer neuron, the PVPR pioneer interneuron, and the sex specific ventral cord motor neurons, VCs (Durbin, 1987; Hobert et al., 1998). In AVG it is required for the outgrowth of its right tract neuronal process in the ventral cord and the absence of *lin-11* function impairs AVG and PVP's differentiation (Hutter, 2003). *lin-11* function is also implicated in the outgrowth of the PVQL processes; a defect in this process, in the absence of *lin-11* function, results in a defect in the fasciculation of other neurons, including the HSN left axon (Garriga et al., 1993). Lastly, *lin-11* function is required for axon fasciculation of the VCs, but it is not necessary for their generation (Hobert, 1998).

Overall, these later roles of LIN-11 in the *C. elegans* ventral nerve cord hint at conserved roles in motor neurons. For example, Lxh1 mediates migration of spinal cord motor neurons (Palmesino et al., 2010), and is required for maintenance of neurotransmitter identity in spinal cord GABAergic interneurons (Pillai et al., 2007); and ISL-1 promotes cholinergic motor neuron identity in the spinal cord (Cho et al., 2014). These observations, that function of Lhx1 and Isl-1 are required for motor neuron function, provided an opportunity to address whether the regulation of the Lhx1 ortholog in worms, *lin-11,* is also conserved. In the mouse ventral telencephalon tissue, ChIP-Sequencing analysis revealed that ASCL1 directly binds to the Lhx1

locus (Raposo et al., 2015), suggesting it acts as a transcriptional regulator of Lhx1. Herein I seek to determine whether HLH-3, an ortholog of ASCL1, regulates *lin-11* and test one mechanism for this proposed regulation (discussed below).

4.1.3 Identifying regulators of *lin-11* in VCs

The regulators that control expression of *lin-11* in VCs are unknown. Here I set out to test whether HLH-3 and HLH-10, bHLH proteins detected in the VCs, regulate *lin-11* expression in VCs. I show they do by (1) confirming the spatiotemporal expression pattern of a *lin-11* translational reporter (2) testing whether a fragment (510 bp) in the *lin-11* VC enhancer drives expression in the VCs, and recapitulates the expression pattern of the full length regulatory fosmid reporter (3) determining whether expression of the *lin-11* fragment is affected by loss of function mutations in the genes encoding the predicted regulators HLH-3 and HLH-10 (4) examining the role, if any, of an E-box in the *lin-11* fragment by analyzing expression in its absence and within several distinct genotypes. Lastly, I tested whether signaling via the LIN-3/EGF pathway can regulate expression of *lin-11* driven by the 510 bp fragment. I characterized the expression in the reduction of function *lin-3(n1417)* mutant background referred to as *lin-3(rf)*.

Overall, the work in this chapter identifies regulators of *lin-11* expression in VCs. I provide additional evidence that HLH-3 promotes expression of *lin-11* in VCs, and HLH-10 represses expression of *lin-11* in non-VC cells. I also show that an E-box in the 510bp VC enhancer fragment of *lin-11* is a repressor binding site. The E-box found in this fragment, CATCTG, is a preferred motif of the Class II bHLHs (Grove et al., 2009). My work aims to address whether this site is critical to promote expression of *lin-11* in VCs (in a WT context).

4.2 Materials and Methods

4.2.1 Strains and maintenance

All strains were maintained at 22°C on nutrient growth media using standard conditions (Brenner, 1974). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *hlh-3(tm1688)* was isolated by the National Bioresource Project of Japan. The alleles reported herein include: LGII: *hlh-3(tm1688)*, LGV: *dpy-11(e224)* and *hlh-10(ok516)*. The integrated transgenes reported herein include: *icIs272 [pmyo-2::mCherry, plin-11::gfp], icIs273 [pmyo-2::mCherry, plin-11ΔE-box::gfp], otIs456 [pmyo-2::gfp, plin-11::mCherry]*, *uIs45 [punc-4::MDM2::gfp + rol-4(+)]*, *vsIs48 [punc-17::gfp]*, and *wgIs62 [lin-11::TY1::EGFP::3xFLAG + unc-119(+)]*.

4.2.2 Construction of transcriptional reporters for E-box analysis

Transgene *icIs272 [plin-11::gfp; pmyo-2::mCherry]:* pLP03 was created with New England Biology Laboratory Gibson Assembly Master Mix using manufacturer's guidelines. A 510 bp fragment of *lin-11* was cloned into an Andy Fire vector pPD95.75 following blunt end restriction enzyme digestion with SmaI. The PCR product amplified from the *lin-11* VC enhancer was created with New England Biology Laboratory Phusion High Fidelity Master Mix and protocol with the following primers: UIC 399 [actctagaggatcccccatactcaatgccacgtc] and UIC 400 [tcctttggccaatccccacctcctccgaccgtattc]. This modified plasmid was submitted for sequencing to validate successful construction to the University of Illinois Research Resources Center. Plasmid pLP03 was injected at 20 ng/uL and the selectable marker PCFJ90 was injected at 2.5ng/uL into N2 hermaphrodites. This line was treated with UV-TMP for integration, creating *icIs272* [*pmyo-2::mCherry; plin-11::GFP*].

Transgene *icIs273 [plin-11Δe-box::gfp; pmyo-2::mCherry]*: pLP04 is a derivative of pLP03 lacking the E-box CATCTG. The E-box was removed by using New England Biology Laboratory Q5 Site-Directed Mutagenesis Kit following manufacturer's protocol with the primers UIC 401 [gtgtcggtttctcacatttcc] and UIC 402 [gaaataacccaatgatggaac]. Plasmid pLP04 was injected at 20 ng/uL and the selectable marker PCFJ90 was injected at 2.5ng/uL into N2 hermaphrodites. This line was treated with UV-TMP for integration (see integration procedure below), creating *icIs273* [*pmyo-2::mCherry; plin-11ΔE-box::GFP*].

4.2.3 Integration of extrachromosomal arrays

The transgenic strains harboring *icIs272* and *icIs273* were generated by exposing a plate of pre-washed (with M9) L4 hermaphrodites to UV-TMP (350microJoules x 100 on a Stratagene UV Stratalinker 1800; 0.03microgram/microliter TMP. Irradiated animals were placed onto seeded NGM plates and transferred the next day to freshly seeded NGM plates (3 Po/plate). These were followed to clone F1s (~150) and then three F2s were cloned per each F1.

4.2.4 Microscopy

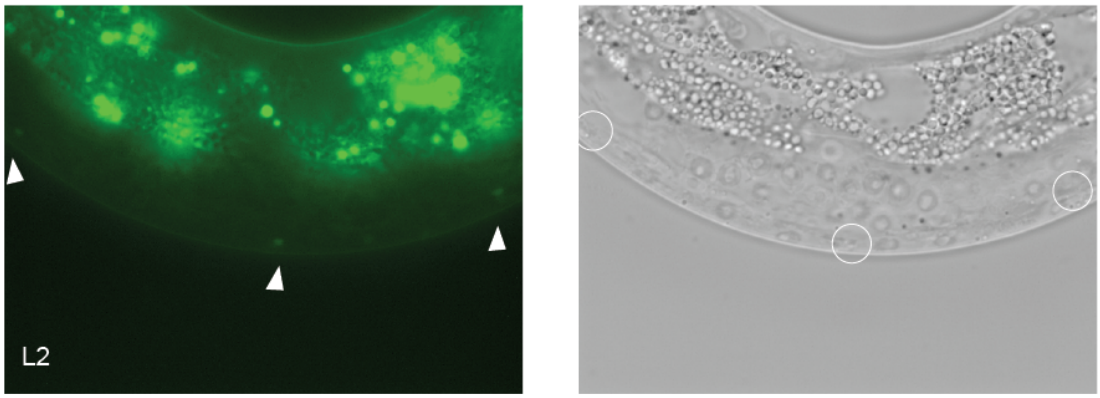
Animals were mounted on 3% agarose pads containing droplets of 10mM levamisole. Fluorescent images were acquired with AxioVision on Zeiss Axioskop 2 microscope. Following the collection of images, some conversions were made with FIJI version 2.0.0 (grayscale images were converted with Lookup tables: Red or Green) and processed into Adobe Illustrator for formatting. Fluorescent reporters were observed under confocal microscopy for the detection of a fluorescent protein signal (presence or absence) in transgenic lines. This study does not report quantification of intensity for any fluorescent reporter observed.

4.3 Results

4.3.1 Characterization of the *lin-11^{fosmid}::gfp* in VCs

To determine as close as possible the endogenous pattern of expression for *lin-11*, I characterized the expression of the fosmid (*wgIs62*) which includes 30-40 kbs of coding region and flanking sequences and likely to include all of the regulatory elements driving GFP *[lin-11::TY1::EGFP::3xFLAG + unc-119(+)]* (Niu et al., 2011). I find that individuals express *lin-11* in the VCs as soon as the second larval stage (L2) (Figure 4.1A). However, the LIN-11::GFP intensity is low and perhaps indicative of low protein levels; this could explain why I am only able to detect a signal in ⅖ individuals at the L2 stage. However, as the animal matures into adulthood, LIN-11::GFP detection increases and is confined to the VCs in the ventral nerve cord (Figure 4.1 A and B). Moreover, in adulthood the expression of LIN-11 appears more intense than when it is first detected in L2 (Figure 4.1 A and B)

A



B

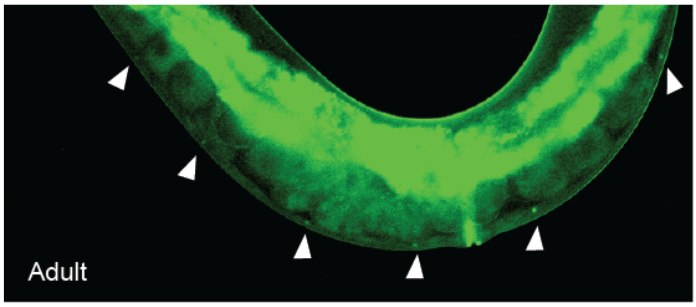


Figure 4.1. LIN-11::GFP is detectable from L2 through adulthood in VCs.

A) Representative image of L2 individual harboring *wgIs62*. LIN-11::GFP positive nuclei, are denoted by arrowheads (left panel) and their cell bodies outlined by white circles (right panel). B) Representative image of a two-day old adult hermaphrodite. Arrows point to LIN-11::GFP positive. Gut granules exhibit autofluorescence (upper half of the body).

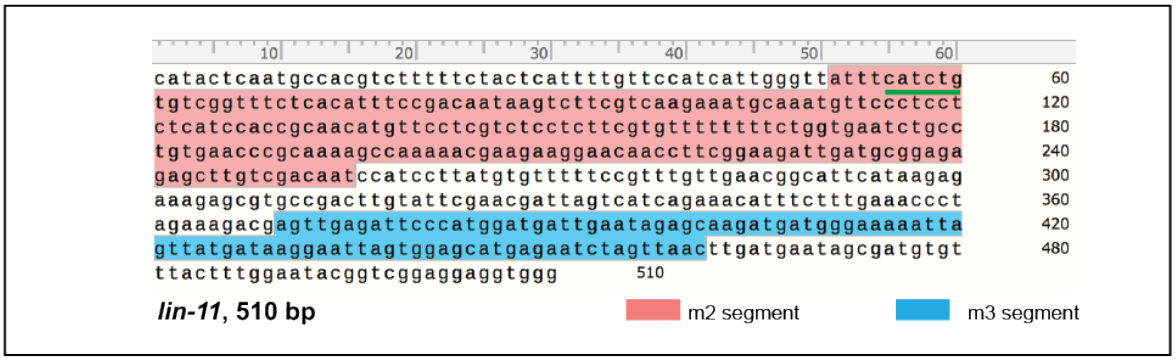
4.3.2 A 510 bp fragment of *lin-11* recapitulates *lin-11^{fosmid}::gfp*

To address the question of whether *lin-11* expression in the VCs is directly regulated by the HLH-3/HLH-2 heterodimer, I scanned the upstream region of the genomic *lin-11* sequence for motifs that HLH-3 preferably binds to: CACCTG, CAGCTG, and CATCTG (De Masi et al., 2011; Grove et al., 2009). I specifically looked for these motifs within a region shown to drive expression in VCs; this region harbors structurally conserved segments between three other *Caenorhabditis* species (Marri & Gupta, 2009). Within the segment that drives expression in VCs, referred to as m2, one can find an E-box of the type that Class II bHLH proteins, including HLH-3, prefer to bind (CATCTG) (Figure 4.2 A).

Since the sequence m2 drives reporter gene expression of *lin-11*, and it contains an E-box, this provided an opportunity to address whether it was a target site for HLH-3 binding. To characterize its role, I first created a transcriptional reporter (*icIs272*) to address whether the VC enhancer element recapitulates the expression pattern of the *lin-11* fosmid in VCs. I cloned a 510 bp fragment containing m2 as well as m3, into an Andy Fire vector, pD97.75, upstream of the GFP coding sequence (Figure 4.2 B). The m3 segment, which drives expression in the pi cell lineage, was included to ensure that the transcriptional reporter can be tracked in non-VC cells. This is because expression in the *hlh-3(lf)* background might fail to report any expression of this transcriptional reporter in any of the VCs as previous data has shown (Chapter 3) (Marri & Gupta, 2009). Next this construct was co-injected with a marker *(pmyo-2::mCherry),* then integrated, and outcrossed two times resulting in animals carrying the array *icIs272* [*pmyo-2::mCherry; plin-11::GFP*] (See Methods). Herein this construct is referred to as *plin-11::gfp*. Importantly, the sequence used to generate *plin-11::gfp* is distinct from the sequence used to generate *plin-11::mCherry*; they may have overlapping regions, but are not identical.

First, I assessed the expression of this transgene in WT animals before assessing its expression in any genetic mutant background. Synchronized populations of individuals from the first larval stage through adulthood were analyzed. Expression of *plin-11::gfp* is first observed in L2 development and maintained throughout development in VCs (Figure 4.2). This expression pattern recapitulates the pattern of the *lin-11*^{fosmid}*::gfp* expression in time and space. However, expression of *plin-11::gfp* is more robust in larval development than that of *lin-11*^{fosmid}*::gfp* (dim).

A


10
20
30
40
50
60
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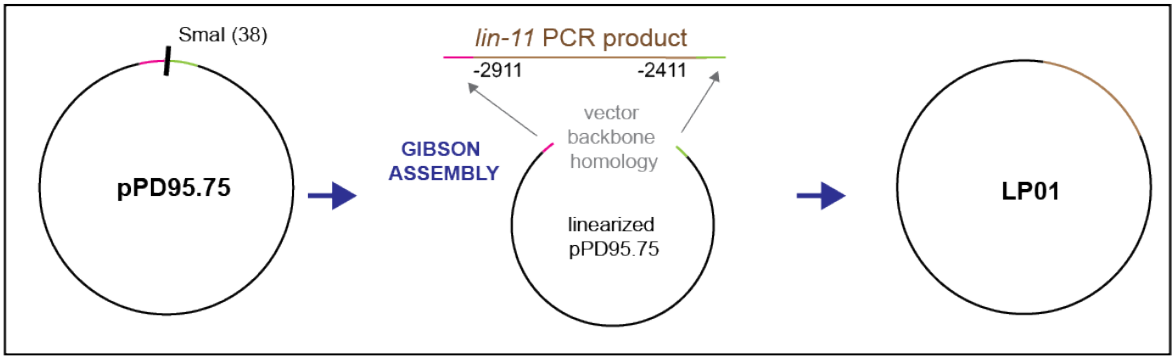
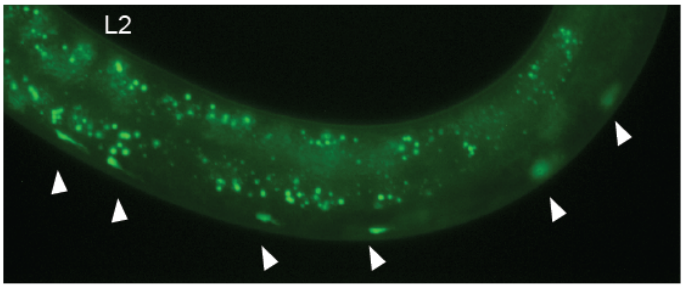
ctcatccaccgcaacatgttcctcgtctcctcttcgtgtttttttctggtgaatctgcc 180
tgtgaacccgcaaaagccaaaaacgaagaaggaacaaccttcggaagattgatgcggaga 240
gagcttgtcgacaatccatccttatgtgtttttccgtttgttgaacggcattcataagag 300
aaagagcgtgccgacttgtattcgaacgattagtcatcagaaacatttctttgaaaccct 360
agaaagacgagttgagattcccatggatgattgaatagagcaagatgatgggaaaaatta 420
gttatgataaggaattagtggagcatgagaatctagttaacttgatgaatagcgatgtgt 480
ttactttggaatacggtcggaggaggtggg
510
lin-11, 510 bp
m2 segment
m3 segment

B


SmaI (38)
pPD95.75
lin-11 PCR product
-2911
-2411
GIBSON ASSEMBLY
vector backbone homology
linearized pPD95.75
LP01

C


L2

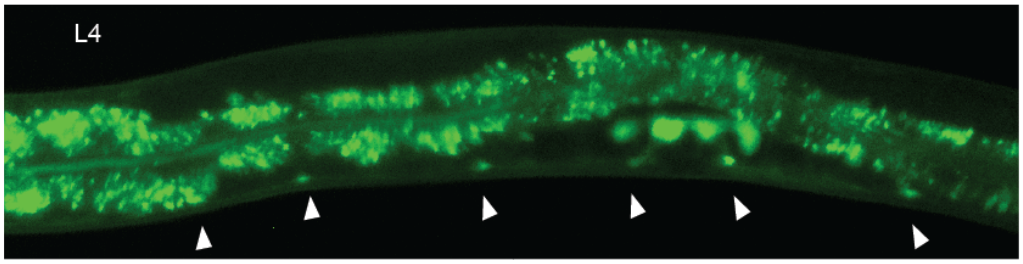

L4

Figure 4.2. *plin-11::gfp* drives expression in VCs during development.

A) Annotated sequence of the 510 bp fragment containing regulatory sites that drive expression in both VCs (m2) and vulval tissue, the pi cells (m3). An E-box, CATCTG, is present in the m2 segment, underlined in green. Sequence annotated in SnapGene Viewer Version 4.3.11 (Adapted from Marri & Gupta 2009). B) Strategy to clone the 510bp fragment into an Andy Fire vector pPD95.75. The plasmid was digested with restriction enzyme SmaI to generate a linearized fragment. The 510bp fragment was generated by PCR. Both molecules were ligated using Gibson Assembly (See Materials and Methods). C) Representative images of individuals harboring *plin-11::gfp;* L2 (top panel) and L4 (bottom panel). Expression is observed in all six VCs, denoted by white arrowheads, in both developmental stages. Additionally, GFP is observed in vulval cells of L4 individuals (mid body).

4.3.3 *hlh-3* positively regulates *plin-11::gfp* within the VC enhancer element

In Chapter III, I showed that *hlh-3(lf)* hermaphrodites fail to properly express a *lin-11* transcriptional reporter, *plin-11::mCherry (otIs456)*, in L4 and through adulthood. This reporter, kindly provided by Dr. Oliver Hobert's laboratory was not accessible for modification and mutagenesis, thus why I created the *plin-11::gfp* reporter (*icIs272*).

To determine whether the 510 bp fragment could also be regulated by HLH-3, I characterized the expression of *plin-11::gfp* in *hlh-3(lf)* adult hermaphrodites. Here I found that the percent of animals expressing *plin-11::gfp* in any of the VCs in *hlh-3(lf)* is lower than in WT adults (Figure 4.3A-B, top and middle panels). This is in agreement with previous findings reported in Chapter III in which the *plin-11::mCherry* reporter was used.

Because of further work discussed later, I also scored *hlh-10(lf)* adults for reporter expression in VCs. I find that all animals expressed *plin-11::gfp* in each VC (Figure 4.3, bottom panels). Few animals reported ectopic expression (reported and discussed in the next section). Therefore, *hlh-10* function is not required for expression of this reporter in VCs.

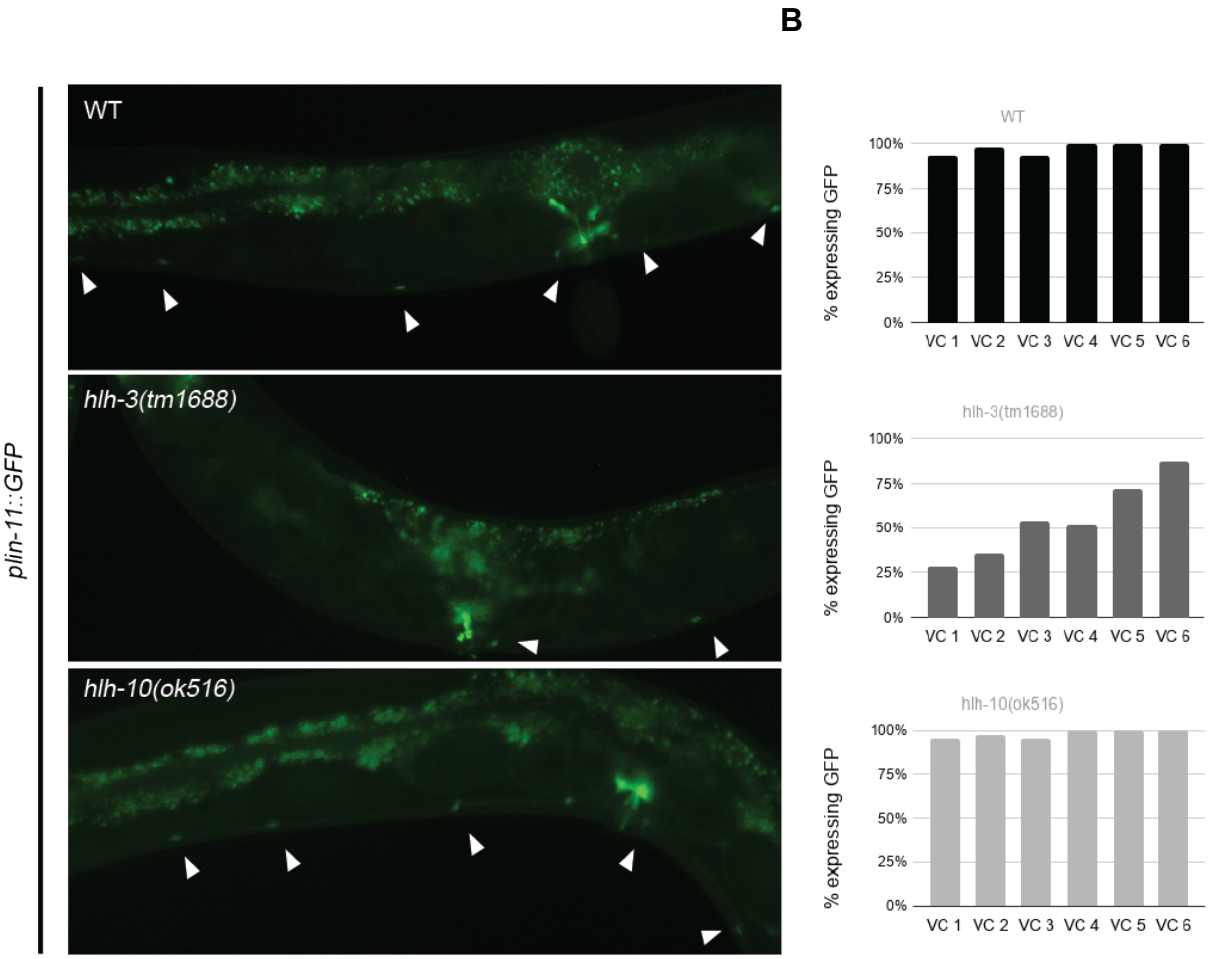


Figure 4.3. *plin-11::gfp* expression is reduced in the absence of *hlh-3* function but maintained in the absence *of hlh-10*.

A) Representative images of one day adults expressing *plin-11::gfp* in the VCs (indicated by white arrowheads) of the indicated genotypes B) Quantification of percent of one day adults expressing the reporter in each of the VCs of the indicated genotypes WT (n = 46), *hlh-3(lf) (*n = 39*), and hlh-10(lf)* (n = 41).

4.3.4 An E-box in the VC enhancer element of *lin-11* negatively regulates expression in the ventral nerve cord

To address whether the "CATCTG" E-box found in the VC enhancer region of *lin-11* is required to drive expression in VCs, I systematically analyzed the effect of the E-box containing (*icIs272*) and E-box deleted (*icIs273*) constructs in a control wild type background, as well as in the *hlh-3(lf)*, and *hlh-10(lf)* genotypes. To create animals lacking E-box in the 510 bp fragment of *plin-11:gfp*, I mutagenized the E box from the construct pLP03 used to create *icIs272*, containing *plin-11::gfp*. Using site-directed mutagenesis, the E-box sequence "CATCTG" was removed (Figure 4.4 A), producing a new construct pLP04. pLP04 was co-injected with plasmid pCFJ90 carrying the marker (*pmyo-2::mCherry*) into WT animals, then integrated, and outcrossed to a WT background to generate the strain harboring *icIs273* [*pmyo-2::mCherry; plin-11ΔE-box(-)::GFP*] (for more details see Materials and Methods) (Figure 4.4A). For ease of distinguishing between the two *lin-11* transcriptional reporters, *icIs272 [plin-11::gfp; pmyo-2::mCherry]* is referred to as "*E-box(+)::gfp*" and *icIs273* [*plin-11ΔE-box::gfp; pmyo-2::mCherry*] is referred to as "*E-box (-)::gfp*" throughout the rest of this chapter.

First, WT adults were examined and the total number of one day old adults expressing *E-box(+)::gfp* and *E-box (-)::gfp* in the ventral nerve cord was quantified. About 90% of WT animals harboring *E-box (+)::gfp* showed expression in all six VCs (Figure 4.3A, B top panels) the remaining had expression in only four to five VCs (Figure 4.4A, top panel). WT animals harboring *E-box (-)::gfp* showed an expansion of reporter expression in the ventral nerve cord; the majority of individuals had six or seven GFP-positive cells, but some had as many as ten. This suggests that the E-box is required for repression in other cells in the ventral nerve cord (Figure 4.4A, top panel). Additionally, animals that lack this E-box in the VC enhancer fragment

also appeared to have higher GFP intensity (not quantified). Finally, although the ectopic expression is restricted to the midbody of the ventral nerve cord, it is randomly dispersed, lacking any obvious pattern.

Next, *hlh-3(lf)* adults were examined and the total number of individuals expressing *E-box(+)::gfp* and *E-box (-)::gfp* in the ventral nerve cord was quantified. As expected, *hlh-3(lf)* adults harboring the *E-box(+)::gfp* construct contain less than six GFP-positive cells; the total number ranges from zero to six cells (Figure 4.4 B and C, middle panels). In contrast, *hlh-3(lf)* adults harboring *E-box(-)::gfp* showed more than 6 GFP-positive cells, consistent with this site serving as a repressor binding site. The ectopic expression of *E-box (-)::gfp* in the ventral nerve cord of *hlh-3(lf)* also indicates repression is independent of *hlh-3* function.

Lastly, *hlh-10(lf)* adults were examined and the total number of individuals expressing *E-box(+)::gfp* and *E-box(-)::gfp* were quantified. We find that *hlh-10(lf)* adults harboring *E-box(+)::gfp* mostly showed expression in six cells (Figure 4.4B,C, bottom panels). In contrast, *E-box(-)::gfp* harboring individuals had a range of GFP-positive nuclei. Most had six or seven GFP-positive cells, and few had as many as nine. With respect to WT individuals harboring *E-box(-)::gfp,* the *hlh-10(lf)* individuals harboring *E-box(-)::gfp* show less total VNC expression, demonstrating that the absence of *hlh-10* slightly decreases ectopic expression in the VNC; this is consistent with *hlh-10* having a repressive role on the expression of *lin-11*.

In summary, regardless of the genetic background [WT, *hlh-3(lf),* or *hlh-10(lf)*)], about 25% of animals harboring the *E-box (-)::gfp* construct demonstrated ectopic expression (total cells > 6). This analysis suggested that the E-box site mediates repression of *lin-11* in non- VCs. Since in *E-box (-)::gfp* animals, it is not straightforward to distinguish the VCs themselves from other cell(s) that are GFP-positive, I next tried to distinguish the identity of VCs from non VCs.

To confirm that *E-Box(-)::gfp* reporter is expressed in VCs, and to try to distinguish between VC cells and VC-like cells in individuals expressing *E-box(-)::gfp*, I asked whether the VC marker *plin-11::mCherry* was co-expressed in the GFP-positive cells. To address this question I characterized a strain harboring both *hlh-10(lf)* and *otIs456* (*pmyo-2::gfp; plin-11::mCherry)*. I find that some of the GFP-positive cells are not VCs, as expected, that is they do not express *plin-11::mCherry*. As shown in Figure 4.5 A (top panel), this individual expresses *E-box(-)::gfp* in four cells along the ventral cord. But only three of those cells also express the VC marker *plin-11::mCherry*, (Figure 4.5 A, top right). In this individual, VC2, VC3 and VC4 co-express *E-box(-)::gfp* and *plin-11::mCherry*, however there is a cell anterior to VC3, inappropriately expressing the *E-box(-)::gfp* reporter (Figure 4.5 A, bottom left). I do not yet know the identity of this cell, the cells that are closest to VC3 on the anterior side are VA5, VB6, DA4, and DD3. To assign an identity to this cell, one would need to find other molecular markers unique to the neighboring cells to VC3. However, based on position, and as an example, an animal harboring both *plin-11::mCherry* and the cholinergic marker *punc-17::gfp* suggest that the cell expressing *E-box(-)::gfp* could be VA5, VB6, or DA4 (Figure 4.5B).

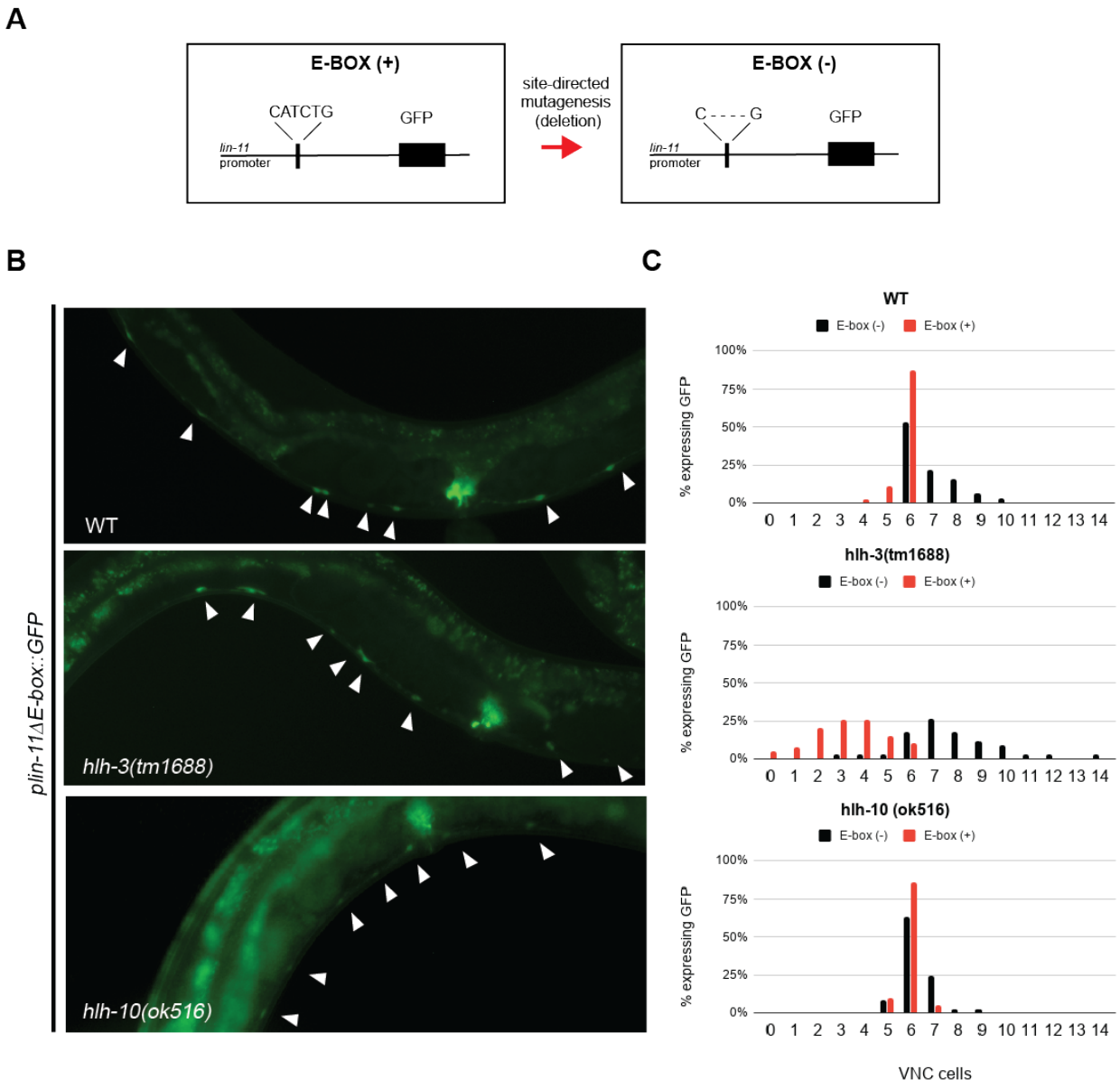
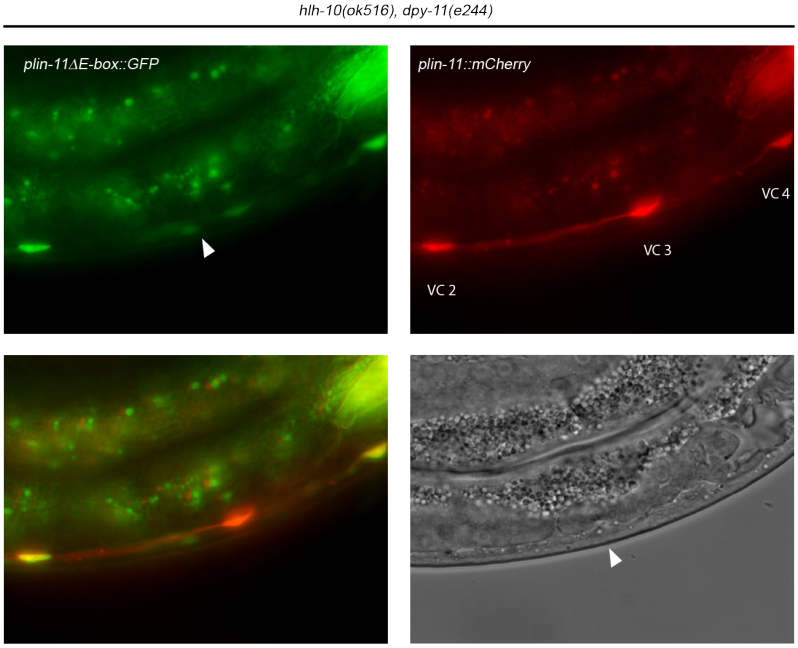


Figure 4.4. Deletion of an E-box in the *plin-11::gfp* VC enhancer element expands GFP expression in the ventral nerve cord.

A) Illustration of the site directed mutagenesis modification performed to delete the E-box containing nucleotides in the *lin-11* VC enhancer fragment. B) Representative images of one-day- old adults expressing *icIs274* [*plin-11ΔE-box::GFP; pmyo-2::mCherry]* in the indicated genotypes C) Quantification of the percent of animals that express GFP in any number of cells

along the ventral nerve cord (VNC). Expression of *plin-11::gfp and plin-11ΔE-box::gfp* was characterized for total in each one day old adult animal of *E-box (+)* (n = 46), *E-box (-)* (n = 33), *hlh-3(lf); E-box (+)* (n = 39), *hlh-3(lf); E-box (-)* (n = 34), *hlh-10(lf); E-box (+)* (n = 41), *hlh-10(lf); E-box (-)* (n = 49). Arrowheads point to all cells expressing *plin-11ΔE-box(-)::gfp* along the ventral nerve cord.

A



B

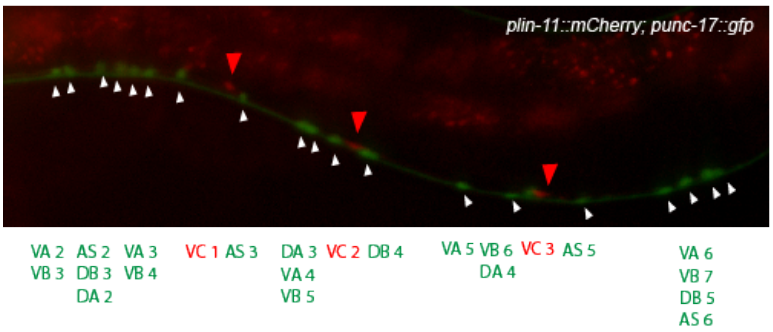


Figure 4.5. The E-box represses expression of *lin-11* in non-VCs.

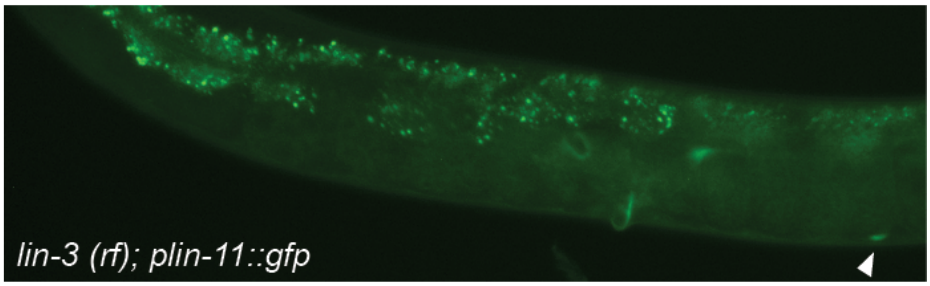
A) Ectopic expression of *icIs273* [*plin-11*Δ*e-box::GFP*] is adjacent to VC3 (white arrowhead). All VCs are labeled by the VC class identity marker *lin-11* carried by *otIs456 [pmyo-2::GFP; plin-11::mCherry]*. B) Annotated image of ventral cord motor neuron positions with respect to VCs. Ventral cord cholinergic motor neurons are marked by the transcriptional reporter *vsIs48 [punc-17::gfp*], shown by white arrowheads. VCs are marked by red arrowheads.

4.3.5 EGF/LIN- 3 function is required for the expression of *plin-11:gfp* in VCs

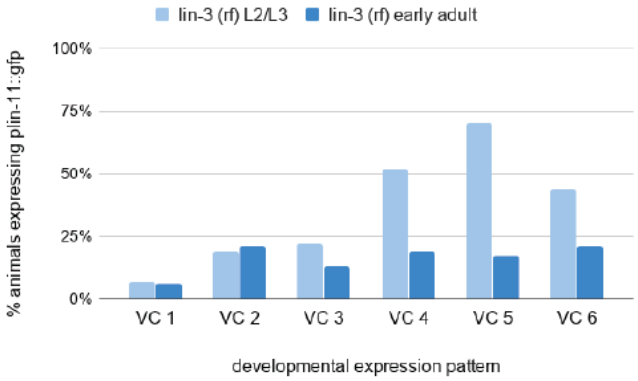
To date there is no published report indicating that expression of *lin-11* in the VCs can be regulated via a non-cell autonomous mechanism. So far, this work has investigated the effects of loss of function mutations and site-specific *lin-11* VC enhancer modification on the expression of *lin-11* in VCs. However, what remains to be tested is whether signaling via an extracellular cue like EGF can regulate *lin-11* expression in VCs. EGF, encoded by *lin-3*, is secreted by vulF cells, and is required for expression of the VC gene *unc-4*, a gene downstream of *lin-11* (Zheng et al., 2013). However, Zheng et al.,'s work did not address whether EGF regulates expression of *lin-11*. Here, I set out to test whether LIN-3/EGF signaling has a role in the regulation of *lin-11* in VCs and when this might occur.

To characterize the effects of loss of the extracellular cue EGF, I examined expression of *plin-11::gfp* (*E-box(+)::gfp*) in a *lin-3(n1417)* reduction of function mutant animal referred to as "*lin-3(rf)*." I find that expression of *plin-11::gfp* is nearly abolished in all VCs of *lin-3(rf)* mutants as they mature through early adulthood, indicating that *lin-3* function is required for expression of *lin-11* (Figure 4.6 A-C). The expression of *lin-11* is seen at higher proportions (50%), particularly in the more posterior VCs 4-6 in early larval stages compared to adults (Figure 4.6 A). As an early adult, expression of *plin-11::GFP* is only present in VC6 (Figure 4.6 A). This reduction in expression decreases to 25% occurring during L4 development, where *lin-3(rf)* mutants fail to report nearly WT levels of expression (Figure 4.6 B). In the earlier larval stages (L2-L3) expression of this construct is detectable in higher proportion in VCs that are closest to the vulva. This could be a result of a weak function (reduction of function) of *lin-3*. In summary, the reduction of expression throughout all VCs over development indicates that the 510bp fragment of the *lin-11* VC enhancer is responsive to signaling by EGF.

A



B



C

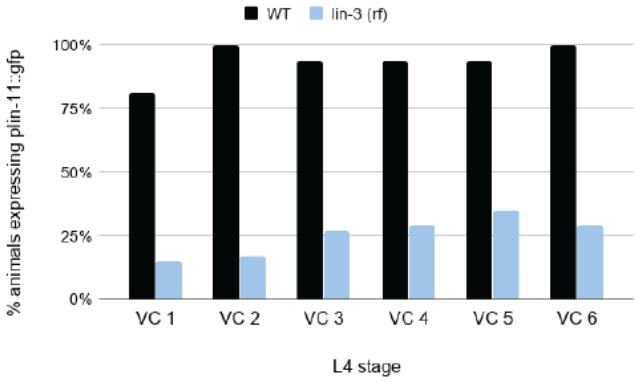


Figure 4.6. LIN-3/EGF mediated signaling positively regulates *plin-11::gfp* expression throughout development.

A) Representative image of early adult expressing *plin-11::gfp* in VC6 identified by white arrowhead (10 hours post mid L4 grown at 22°C) B) Quantification of the number of individuals

expressing the reporter in each of the VCs of L2/L3 and adult animals. L2/L3 animals are shown in light blue (n = 27); early adults (10 hours post L4) are shown in dark blue (n = 54). C) Quantification of the number of individuals expressing the reporter in each of the VCs of L4 individuals in WT (black bars, n = 48) and *lin-3(rf)* (light blue bars, n = 16) backgrounds.

4.4 Discussion and Future Directions

4.4.1 Analysis of *plin-11::gfp* reveals EGF as a novel regulator of *lin-11* expression

This chapter presents data identifying previously unknown regulators of *lin-11* expression in the VCs. Here I have provided further evidence that *lin-11* expression is dependent on *hlh-3* function (previously reported in Chapter II). Additionally, I have shown that *lin-3* function is also important.

This work supports the hypothesis that a parallel pathway, independent of *hlh-3* function, promotes expression of *lin-11* in VCs. This chapter summarizes the data that supports one such pathway, LIN-3/EGF dependent, which positively regulates *lin-11* reporter expression (Figure 4.6). As previously reported, LIN-3/EGF is secreted by vulF cells during L4 development and promotes *unc-4* expression in VCs (Zheng et al., 2013).

4.4.2 *lin-11* is repressed in other ventral cord motor neurons via an E-box

In addition to identifying positive regulators of *lin-11* expression in VCs, this work also reveals an E-box in the *lin-11* enhancer which mediates repression in non-VCs throughout the ventral cord. This work confirms that E-boxes can be binding sites for positive regulators as well as repressors. This work also supports the hypothesis that *hlh-10* has a repressive role in the expression of *lin-11*. In humans, TCF21 (the HLH-10 ortholog) has been shown to negatively repress transcription (Cheol et al., 2005). It is possible that HLH-10 could act on other E-boxes in the VC enhancer sequence or compete with other regulators at these sites. Therefore, future work can examine a mechanism of action for the repression of *lin-11* by HLH-10.

4.4.3 Identifying direct regulators that promote *lin-11* expression in VCs

This work identified positive regulators of *lin-11* in VCs. However, the primary limitation of these findings is whether this is a result of direct or indirect regulation. Therefore, future work should be conducted to identify direct regulators of *lin-11*. One candidate is LIN-39. In vulval cells, *lin-39* is a downstream target of the LET-23/EGFR/MAPK signaling pathway (Eisenmann et al., 1998; Maloof & Kenyon, 1998; Pellegrino et al., 2011). As mentioned previously, EGF acts on VCs, so it is presumed EGFR signaling activates RAS in VCs, and since LIN-39 is a known downstream target of RAS, it is possibly targeted in the VCs too. But, it remains to be understood whether *lin-11* is targeted by this pathway, or whether another factor is activated (such as LIN-39) whereby such a factor then activates *lin-11*. Others have recently shown that LIN-39 promotes the expression of one VC identity gene *(srb-16)* therefore it is possible that it may regulate other VC identity genes, although it does not regulate the VC class gene *ida-1* (Feng et al., 2020; Kalis et al., 2014). Furthermore, LIN-39 is a midbody HOX TF that has a more pronounced role in anterior VCs (Kalis et al., 2014). Therefore, this could explain why I found that anterior VCs exhibit less *lin-11* reporter expression in *lin-3(rf)* in L2/L3 individuals (Figure 4.6).

4.4.4 Elucidating the role of *lin-11* in VCs by testing downstream targets

To date, the function of *lin-11* in VCs is associated with axon fasciculation and the expression of *unc-4* in the proximal VCs. As a future direction, one could determine if any of the VC identity genes are regulated by *lin-11*. A ChIP-Sequencing dataset of LIN-11 binding events during larval L3 is publicly available. Within this list, some binding events occur upstream of VC identity genes including *ida-1* and *mir-124* (Niu et al., 2011). As a future direction, one

could assay reporter expression of *ida-1* and *mir-124* in the absence of *lin-11* function to determine that these genes are affected.

5 Chapter V: Conclusions

CHAPTER V:

Conclusions

5.1 *hlh-3* is required in later neurodevelopment for VC specification

In this work I characterized the expression pattern of *hlh-3* using a CRISPR-Cas9 engineered line. This work confirms that *hlh-3* is expressed in the VC neural precursors and the VCs themselves, throughout all stages of development. By examination of molecular markers, I found that the VCs fail to differentiate in the absence of *hlh-3* function. However, this work also provides evidence that these neurons are generated, highlighting a later, non-proneural role for this bHLH factor. Since the generation of VCs are not affected, but the acquisition of terminal features is defective, the function of *hlh-3* is required for VC class specification and differentiation. Additionally, this work also provides evidence that *hlh-3* is not required for the differentiation of the other sex-shared ventral cord motor neurons.

5.2 *hlh-3* functions in all VCs but may primarily induce distal VC subclass identity

In the characterization of *hlh-3* function on VC differentiation, subclass differences among distal and proximal VCs were accentuated. The absence of *hlh-3* function nearly abolishes expression of all reporter genes in the distal VCs, whereas the proximal VCs show less pronounced effect on reporter gene expression. This indicates that the distal VCs require *hlh-3* function for the acquisition of terminal features, and overall identity, whereas the terminal differentiation of the proximal VCs is regulated by additional, parallel pathway(s).

As discussed in Chapter II, one such parallel pathway is mediated by EGF signaling. The expression of proximal VC gene *unc-4* is known to depend on EGF signaling, but it also requires *lin-11* expression. Given that *hlh-3* mutants show expression of *unc-4*, but at decreased levels, it

is logical to presume that expression persists due to this superimposed EGF pathway. Therefore, this work emphasizes that at least two pathways promote VC subclass differentiation, an *hlh-3* dependent pathway acting on all VCs and an EGF pathway that converges with *hlh-3* on proximal VCs. It remains to be tested whether the proximal VCs express all other VC molecular markers because of the EGF signaling pathway, despite absence of *hlh-3* function. Future work should examine this possibility.

5.3 Direct targets of *hlh-3* remain to be identified

Our work identified VC identity genes (terminal and not terminally expressed) downstream of *hlh-3* and affected by the absence of *hlh-3* function. However, these analyses do not implicate *hlh-3* as a direct transcriptional regulator. Further work needs to address the direct targets of *hlh-3*. Using the *hlh-3:gfp* CRISPR-Cas9 engineered line, an unbiased approach can be taken to identify chromatin bound regions of by HLH-3::GFP using ChIP-Seq.

5.4 The role of *hlh-3* in *C. elegans* neurodevelopment

This analysis in VC motor neuron differentiation adds evidence that *hlh-3* function is not necessarily limited to proneural gene function, and in fact, demonstrates that it is required throughout the entire development of the VC motor neurons in the hermaphrodite. In fact, the work described in this thesis implicates *hlh-3* in later stages of neuronal subtype specification. Other work has demonstrated that *hlh-3* function is proneural in the generation of the HSN motor neuron and has shown that *hlh-3* functions at later stages of terminal differentiation to induce a serotonergic fate during the last larval stage of development (L4). Overall, whether *hlh-3* acts in early stages of neurogenesis, or in later stages of neurodevelopment, appears to be specific to neuronal cell type.

5.5 *lin-11* expression is regulated by several mechanisms

This work set out to address whether *lin-11* was a downstream target of *hlh-3* by testing one mechanism. This work has demonstrated that *hlh-3* positively regulates *lin-11* expression in the VCs, although through an unknown mechanism. In examining a potential mechanism for the regulation of *lin-11* in the VCs, a repressor of *lin-11* was identified (*hlh-10)* and a repressive site in the *lin-11* VC enhancer was identified (CATCTG). The examination of one E-box site is a limitation to this work, as another E-box is also found in this regulatory region driving expression in VCs.

6 Cited Literature

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7 APPENDIX

7.1 Appendix A

Chapter II contains work submitted as a preprint, "The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in *C. elegans"* in BioRxiv.

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Beyond proneural: emerging functions and regulations of proneural proteins

Author: François Guillemot,Bassem A Hassan

Publication: Current Opinion in Neurobiology

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Date: February 2017

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Lillian Perez, Ph.D. Candidate Phone: (773)407-1601 Email: lillianperez.0@gmail.com

EDUCATION

Ph.D. Neurobiology	University of Illinois at Chicago (UIC) Chicago, Illinois	2013-Present
B.S. Biological Sciences	DePaul University (DPU) Chicago, Illinois	2009-2013
	Latin School of Chicago Chicago, Illinois	2005-2009

GRADUATE RESEARCH

- My graduate research focused on elucidating the mechanisms regulating neuronal specification and differentiation of sex-specific neurons in the developing nematode *C. elegans*.
- Characterized the function of a proneural gene *hlh-3* and characterized the spatiotemporal expression pattern of *hlh-3* using CRISPR-Cas9 reporter tagging
- First author manuscript (submitted): "*The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in C. elegans*"
- Advisor: Dr. Aixa Alfonso

PROFESSIONAL SOCIETY MEMBERSHIPS

Women in Bio	2020
Society for Neuroscience	2013-2019
Graduate Women in Science (Chicago Chapter)	2019

PRESENTATIONS

The generation of the mystery cells of the male depends on HLH-3 function in *C.elegans* *(poster)*

Society for Neuroscience, Chicago, IL 2019

VCs require HLH-3 function to assume their terminal differentiation state (*poster*)

C. elegans Neurobiology Meeting, Madison, WI 2018
Chicago Area Worm Meeting, Chicago, IL 2018

HLH-3 has a role in the differentiation of the sex-specific descendants of the ectodermal-like P cells (*oral*)

Data Blitz Laboratory of Integrative Neuroscience, UIC 2016

Ventral cord VC neurons are abnormal in their differentiation in HLH-3 loss of function hermaphrodites (*poster***)**

Chicago Society for Neuroscience Meeting, Chicago, IL 2016
Midwest *C. elegans* Meeting, Grand Rapids, MI 2016

Differential effects of varied rehabilitative approaches on Nogo-A in an animal model of traumatic brain injury *(poster)*

Society for Neuroscience, San Diego, California 2013

Nature and Nurture: transcriptomic brain analysis of *Apis mellifera* sub-species (*poster and oral)*

Illinois Summer Research Symposium, Urbana, Illinois 2012

Exported molecules that distinguish normal and Juvenile Neuronal Ceroid Lipofuscinosis fibroblasts as a biomarker for JNCL disease progression and therapeutic efficacy (*poster*)

SACNAS National Conference, San Jose, California 2011

AWARDS AND HONORS

Biological Sciences Travel Award, UIC 2019
Laboratory of Integrative Neuroscience Travel Award, UIC 2018
NSF Bridge to the Doctorate Fellowship, UIC 2014-2015
NSF New Biology Program Research Fellow, UIUC 2012-2013
Biological Sciences Departmental Senior Appreciation Award, DPU 2013
Leadership Development Certificate, Student Leadership Institute, DPU 2013
ILSAMP Research Scholarship, DPU 2012-2013
Ronald E. McNair Post-baccalaureate Achievement Program Scholar, DPU 2012-2013
Arnold L. Mitchem Fellowship, DPU 2011-2012
Margaret O'Malley Scholarship, DPU 2011-2012
Genius Research Scholar, DPU 2011
Leadership Scholarship, DPU 2009- 2013

TEACHING EXPERIENCE

Lecture Courses

Human Embryology, BIOS326, UIC 2020

Biology of the Brain, BIOS286, UIC 2018, 2019
Advanced Mammalian Physiology, BIOS 443, UIC 2019

Laboratory Courses

Cellular Biology BIOS223, UIC 2016-2017
Introduction to Biological Sciences BIOS100, UIC 2013, 2016
Biostatistics BIO206, DePaul University 2012

Professional Development and Research Mentorship

Workshop Instructor, Latinos Gaining Access to Networks for Advancement in Science, UIC 2018-Present
Workshop Instructor, Summer Research Opportunities Program, UIC 2018

Laboratory Mentorship

I have mentored and assisted in laboratory research training to the following students for research projects pertaining to the lab and related to my thesis.

Alex Obafemi, Graduate Non-degree 2020

Valerie Pyrn, B.S. Neuroscience 2019-2020

Basil Muhanna, B.S. Biological Sciences 2019-2020

Freddy Jacome, B.S. Biological Sciences 2018-2019

Luis Enriquez, Biological Sciences & Psychology 2017-2018
Poster, *The effect of hlh-3 (lof) on body wall muscle in C. elegans*
Chicago Area Worm Meeting, 2018
The 1st Annual Midwest Regional SACNAS Research Conference, University of Chicago, 2018

Nadeen Shaban, B.S. Biological Sciences, Honors College Capstone 2016-2017
Poster, *The effects of hlh-10 on Ventral Cord Neuronal Differentiation*
The UIC Student Research Forum, 2017

Janeen Radwan, B.S. Biological Sciences 2016-2017

Zena Ibrahim, B.S. Biochemistry, Honors College Capstone 2016-2017
Poster, *Role of hlh-3 in MCM anatomy and function in C. elegans*
The UIC Student Research Forum, 2017

Carolina Castro, Summer Research Opportunity Program Scholar (UIC) 2016

Poster, *Elucidating the role of hlh-3 in the differentiation of sexually dimorphic neurons in the nematode C. elegans*
UIC SROP Symposium 2016
Annual Biomedical Research Conference for Minority Students, 2016

Cynthia Velazquez '16, B.S. Biological Sciences, Honors College Capstone 2015-2016
Poster, *Assessing the role of hlh-3 in the ventral cord motor neuron differentiation: an analysis using lin-11 as a differentiation marker*
SACNAS Chicago Symposium, Northwestern University, 2016

ACTIVITIES

Panelist, Rosalind Franklin University of Medicine and Science: Influence Student Potential and Increase Representation in Education (INSPIRE) STEM Career Panel 2018
Panelist, Life after Latin: Science Panel, Latin School of Chicago 2018
Panelist, Educational Transitions**,** Spring Symposium in STEM IL-SAMP Conference 2017
Volunteer Judge, Spring Symposium in STEM IL-SAMP Conference 2017
Panelist, STEM Alumni Showcase, DePaul University 2017
Contributing Writer, "The Science Café" Newsletter 2016-2017
President, SACNAS at UIC Chapter, University of Illinois at Chicago 2016-2017
Keynote Speaker, Graduate School Forum, DePaul University 2016
Volunteer, "Young Scientists" Chicago Cares, McCormick Elementary School 2016
Volunteer Judge, "STEM Forum," DePaul University 2016
Volunteer Judge, Research presentations, BIOS 286, University of Illinois at Chicago 2016
Volunteer Poster Judge, BIOS 286, University of Illinois at Chicago 2016
Co-President, Neuroscience Club, DePaul University 2012
President, DePaul SACNAS Chapter, DePaul University 2011

UNDERGRADUATE RESEARCH

DEPAUL UNIVERSITY, Chicago, IL
Student Researcher August 2012 – May 2013

UNIVERSITY OF ILLINOIS AT URBANA, Champaign, IL
Research Fellow, New Biology Fellowship Summer 2012, Summer 2013

ROSALIND FRANKLIN UNIVERSITY MEDICAL SCHOOL, Chicago, IL
Research Assistant June 2011 – May 2012