A ROLE OF THE LIN-12/NOTCH SIGNALING PATHWAY IN DIVERSIFYING THE NON-STRIATED MUSCULATURE OF THE EGG-LAYING SYSTEM IN *C. ELEGANS*

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A ROLE OF THE LIN-12/NOTCH SIGNALING PATHWAY IN DIVERSIFYING THE NON-STRIATED MUSCULATURE OF THE EGG-LAYING SYSTEM IN *C. ELEGANS*

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In order for an organ to develop and function, different cell types and tissues must be specified and integrated in a tightly controlled manner. An example of this is the *C. elegans* hermaphrodite egg-laying system, which requires coordination between the vulva, uterus, neurons, and musculature. The specification of the first three components are well understood from a genetic standpoint, however, very little is known about the molecular mechanisms responsible for the specification of the egg-laying musculature. The egg-laying muscles are non-striated in nature and consist of sixteen cells, four each of type I and type II vulval muscles and uterine muscles. These sixteen non-striated muscles possess unique morphology, location, synaptic connectivity and function. Through the use of an RNAi screen targeting the putative transcription factors in the C. elegans genome, I identified a number of novel factors important for the diversification of these distinct types of egg-laying muscles. In particular, I discovered that RNAi knockdown of lag-1, which encodes the sole *C. elegans* ortholog of the transcription factor CSL (CBF1, Suppressor of Hairless, LAG-1), an effector of the LIN-12/Notch pathway, led to the production of extra type I vulval muscles. Similar phenotypes were also observed in animals with down-regulation of the Notch receptor LIN-12 and its DSL (Delta, Serrate, LAG-2) ligand LAG-2. The phenotype of extra type I vulval muscles in

animals with reduced LIN-12/Notch signaling was the result of a cell fate transformation of type II vulval muscles to type I vulval muscles. I demonstrated that LIN-12/Notch was activated in the undifferentiated type II vulval muscle cells by LAG-2/DSL that is likely produced by the anchor cell (AC). These findings provide intriguing evidence highlighting the roles of LIN-12/Notch signaling in coordinating the formation of multiple distinct components of the functional C. elegans egg-laying system. I also identified multiple new factors that play critical roles in the proper specification of the different types of egg-laying muscles. Together, my work has provided insight into the key mechanisms that underlie the organogenesis of the egg-laying system and the specification of non-striated muscle.

Biographical Sketch

Jared J. Hale was born in North Jackson, Ohio, a small rural town to Daniel and Cynthia Hale. He was raised near his extended family and was encouraged by all to become educated. He took the opportunity to explore science during primary education and was awarded a full scholarship to The Ohio State University where he studied Molecular Genetics. After graduating with Honors and Research Distinction he became a graduate student in the department of Molecular Biology and Genetics at Cornell University in 2009. There he has developed his teaching and research skills while pursuing his PHD.

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Chapter 1: Introduction

The development of multicellular organisms begins from a single fertilized egg that will eventually give rise to all of the distinct cell types of the adult organism. This is undoubtedly a complicated process, as the cell must be capable of detecting and responding to different temporal and spatial developmental cues. The cells must coordinate and regulate the intrinsic and extrinsic signaling that allows for the establishment of the cell's unique fate including gene expression, location and morphology. Consequently, one of the most fundamental and yet most fascinating questions in developmental biology is a how a single cell can develop into a multicellular organism with the amazingly complex array of different tissues and cell types that comprise the adult animal.

During development, early pluripotent or multipotent cells become more restricted as they are exposed to a variety of intrinsic and extrinsic signals. These signals can force previously equipotent cells into distinct cell fates. A surprisingly few number of highly-conserved signaling pathways are used throughout development, such as the Notch, TGFβ and Wnt signaling pathways. Through the variation of temporal, spatial and cellular contexts these few signaling pathways are able to account for many different developmental outcomes. The focus of this work highlights one pathway in particular: the Notch signaling pathway.

The Notch pathway

The Notch pathway is highly conserved throughout development across Metazoa (Artavanis-Tsakonas, 1999). The Notch pathway has been well studied in a variety of organisms and was originally named for its identification as a haploinsufficient “notched” wing phenotype in *D. melanogaster* nearly a century ago (Mohr, 1919). The homozygous null mutants caused lethality during embryogenesis due to a pro-neurogenic phenotype at the expense of ectoderm and yielded dead embryos that had numerous cell fate transformations in multiple cell types (Poulson, 1939).

The canonical Notch signaling pathway has been thoroughly studied and is known to begin when a Delta ligand binds a Notch receptor. This interaction allows for several subsequent cleavage events that ultimately results in the release of the intracellular domain of Notch. The intracellular domain is then free to shuttle to the nucleus where it can interact with transcription factors and other cofactors to affect transcription (Mumm and Kopan, 2000).

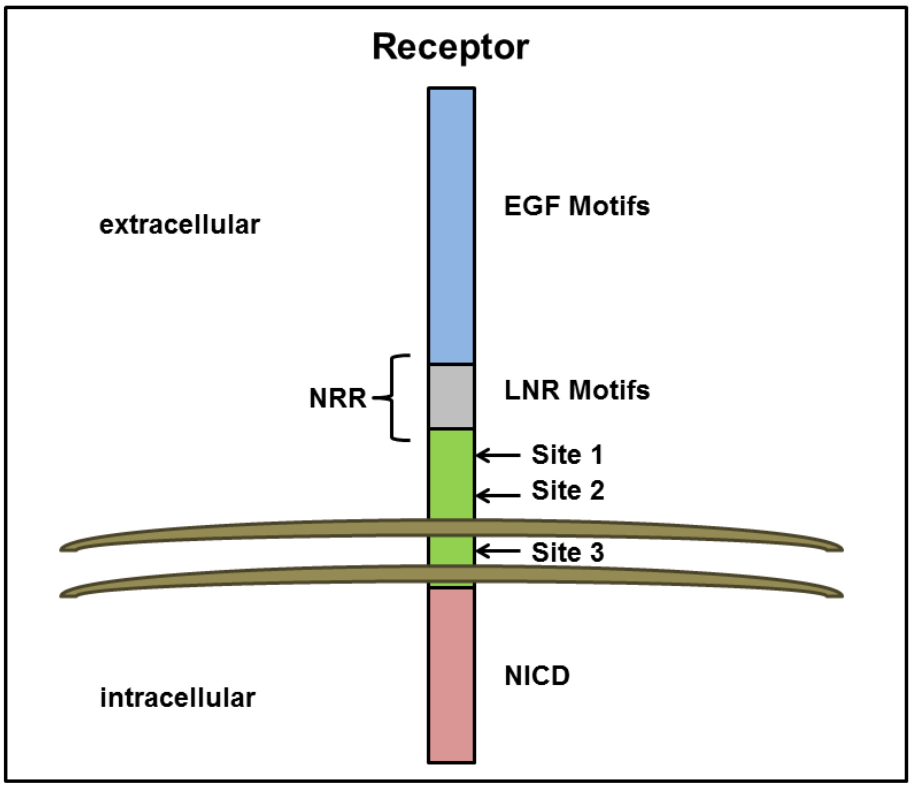
In *C. elegans* there are two canonical Notch receptors LIN-12 and GLP-1. In certain cell fate decisions the two receptors have been shown to be functionally redundant (Lambie and Kimble, 1991). However many other cell fate decisions, such as specification of the VPCs (vulval precursor cells) or the regulation of germline specification, rely upon only lin-12 or glp-1 respectively (Greenwald et al., 1983; Austin and Kimble, 1987). Sequence analysis has revealed that *lin-12* and *glp-1* likely arose through a gene duplication event, lending credence to the functional redundancy found in certain cell fate decisions (Kimble and Rudel, 2002). While many cell types utilize

only LIN-12 or GLP-1 exclusively, it has been shown that using the *lin-12* promoter to drive *glp-1* expression in *lin-12(0)* animals which lack endogenous *lin-12* activity was able to rescue the *lin-12(0)* phenotype. This work demonstrates that there is biochemical similarity and functional redundancy between the two receptors (Fitzgerald et al., 1993).

Signal transduction by Notch receptors such as LIN-12 or GLP-1 is facilitated through several different protein motifs. Both LIN-12 and GLP-1 are type 1 transmembrane proteins containing multiple EGF-like repeats and three LIN-12/Notch repeats (LNR) which define their classification as Notch receptors (Yochem et al., 1988; Yochem and Greenwald, 1989). In vertebrates, the spacer region that separates the LNR repeats and the transmembrane domain contains a pair of conserved cysteine residues and has been shown to be cleaved by a furin-like convertase during the protein maturation process at the site 1 (S1) cleavage site, resulting in a heterodimeric organization between the two Notch fragments at the surface of the cell. This cleavage event is critical for proper maturation and processing of Notch receptor in vertebrates because this cleaved biologically-relevant form of Notch is the only form found at the surface of the cell membrane in vivo (Logeat et al., 1998). However, it remains unclear whether or not this S1 furin-cleavage is conserved in *C. elegans*. Nonetheless, later cleavage events have been shown to be conserved in the signal transduction of Notch (Figure 1.1).

Figure 1.1: Diagram of *C. elegans* LIN-12/Notch Receptors

C. elegans has two LIN-12/Notch receptors, LIN-12 and GLP-1, both of which are type 1 transmembrane proteins. The conserved motifs include: Epidermal Growth Factor-like motifs (EGF), LIN-12/Notch Repeat motifs (LNR), cleavage Site 1 (S1) cleaved by furin in vertebrates, cleavage Site 2 (S2) cleaved by ADAM metalloproteases, cleavage Site 3 (S3) cleaved by presenilin, Notch Negative Regulatory Region (NRR) which includes the LNR motifs, Notch IntraCellular Domain (NICD) is the active fragment of Notch and mediates the interaction with the nuclear complex.


Receptor
extracellular
EGF Motifs
NRR
LNR Motifs
Site 1
Site 2
Site 3
NICD
intracellular

Subsequent cleavage events are initially prevented from occurring by the activity of the Notch negative regulatory region (NRR). The NRR is found in the vicinity of the extracellular surface of the cell membrane and functions to prevent further cleavage events, thereby preventing the signal transduction from occurring. However, upon ligand binding the NRR region of the Notch receptor is physically destabilized and leads to the site 2 (S2) cleavage region being exposed (Gordon et al., 2009; Stephenson and Avis, 2012), allowing for cleavage to occur at the S2 site by the ADAM family of metalloproteases. While the destabilization of the NRR upon ligand binding is necessary to allow for the S2 cleavage event, NRR destabilization has also been shown to occur in a ligand-independent manner at the cell surface (Gordon et al., 2009). In vivo evidence shows ligand-independent activation occurring following the endocytosis of Notch receptor into vesicles and subsequent processing during endosome maturation (Wilkin et al., 2008). This ligand-independent signal transduction has been shown to function during the patterning of the Drosophila eye disk. However, this is thought to occur independently of ADAM metalloproteases (Fortini and Bilder, 2009) and involve a separate repertoire of post-translational regulation (Cho and Fischer, 2011).

The extracellular cleavage site S2, exposed during destabilization of the NRR, is also located within the spacer region of the Notch receptor. Upon binding by a DSL-class ligand the site 2 region is cleaved by the ADAM family of metalloproteases which have numerous roles in proteolytic processing (Wen et al., 1997; Brou et al., 2000). Different ADAM family members have been implicated as the major player in site 2 cleavage of Notch in different model organisms, such as *sup-17* in *C. elegans* (Wen et al., 1997) or Kuzbanian in Drosophila (Rooke et al., 1996), while an alternative ADAM

family protein, TACE, functions in mammalian site 2 cleavage (Brou et al., 2000). Similarly, *C. elegans* employs two different ADAM metalloproteases during Notch processing. Both SUP-17 and the *C. elegans* TACE ortholog ADM-4 have been shown to be functionally redundant in some cellular contexts while also demonstrating distinct developmental roles in other cellular contexts (Jarriault and Greenwald, 2005).

The successive cleavage at site 3 (S3) of Notch involves the gamma-secretase protease complex, which is also responsible for the cleavage of the β-amyloid precursor protein (β-APP) thought to be involved in the progression of Alzheimer's disease (Jurisch-Yaksi et al., 2013). Known as Presenilin in Drosophila and Mammals, there are two presenilins in *C. elegans*, called sel-12 and hop-1. The similarity between cleavage of β-APP and Notch was demonstrated through the human homologue of sel-12 first being identified in familial early-onset Alzheimer's disease (Sherrington et al., 1995) followed by work showing sel-12 could suppress lin-12 gain-of-function mutations (Levitan and Greenwald, 1995). Afterwards, hop-1 was shown to function in a redundant manner with sel-12 (Li and Greenwald, 1997) demonstrating the presence of two presenilins that are thought to comprise the catalytic core of the γ-secretase complex (Kopan and Goate, 2000).

The canonical DSL-class (Delta/Serrate/Lag-2) ligands of the Notch pathway in *C. elegans* include ten different factors containing an amino terminal DSL motif and a number of EGF motifs. The first several ligands identified, lag-2, apx-1 and arg-1 are membrane bound ligands (Tax et al., 1994; Mello et al., 1994; Mango et al., 1994). Interestingly other ligands, such as dsl-1, were identified that contain all of the hallmark motifs of DSL-class ligands but lack a transmembrane domain, suggesting that they are

part of a secreted signal rather than a membrane-associated signal (Chen and Greenwald, 2004). Similar to the functional redundancy and biochemical similarity observed between LIN-12 and GLP-1 (Fitzgerald, 1993), experimental evidence showed that the ligand DSL-1 could substitute for the transmembrane ligand LAG-2 when placed under the lag-2 regulatory promoter, indicating that the secreted ligand can functionally replace a transmembrane ligand despite lacking a transmembrane domain itself (Chen and Greenwald, 2004).

Following ligand binding and the multistep cleavage events of the Notch receptor, the final cleavage event at S3 results in the release of the Notch intracellular domain (NICD). Upon this release, the NICD is free to shuttle to its site of activity in the nucleus and interact with other factors to affect transcription (Kopan et al., 1994). One such factor is LAG-1, the sole CSL (CBF-1, Su(H), Lag-1) class transcription factor in *C. elegans* named for its strong *lin-12* and *glp-1* phenotype (Lambie and Kimble, 1991). LAG-1 plays a critical role in Notch signal transduction and has been indicated as one of the three core components of the Notch nuclear complex. When LAG-1 is not complexed with the NICD, LAG-1 acts as a transcriptional repressor (Morel and Schweisguth, 2000). Formation of the complex with the NICD leads to the displacement of corepressors (Hsieh et al., 1996) and a corresponding association with coactivators (Jarriault et al., 1995).

One such cofactor that makes up a core component of the ternary nuclear complex of active Notch signaling is SEL-8, which is homologous to Drosophila *mastermind*. Originally named for its suppression of a *lin-12* mutant phenotype (Sundaram and Greenwald, 1993), *sel-8* was later identified in a yeast two-hybrid

screen looking for proteins that could interact with the NICD in the presence of LAG-1. The sole candidate isolated was analyzed using RNAi knockdown and found to have a phenotype of complete loss of Notch function, leading to *sel-8* also being known as *lag-3* (Petcherski and Kimble, 2000). Taken together, the NICD, LAG-1 and SEL-8 make up the core components of the ternary nuclear complex of activated Notch.

The Notch signaling pathway plays multiple roles in *C. elegans*, such as the AC/VU cell fate decision (Greenwald, 1983; Seydoux and Greenwald, 1989; Wilkinson et al., 1994), patterning of the VPCs (Sternberg, 1988; Greenwald et al., 1983; Chen and Greenwald, 2004), and establishing the dorsoventral asymmetry of the M lineage (Greenwald et al, 1983; Foehr and Liu, 2008). In particular, the LIN-12/Notch pathway is known to be required in multiple cell types during the development of the *C. elegans* egg-laying system (described more below).

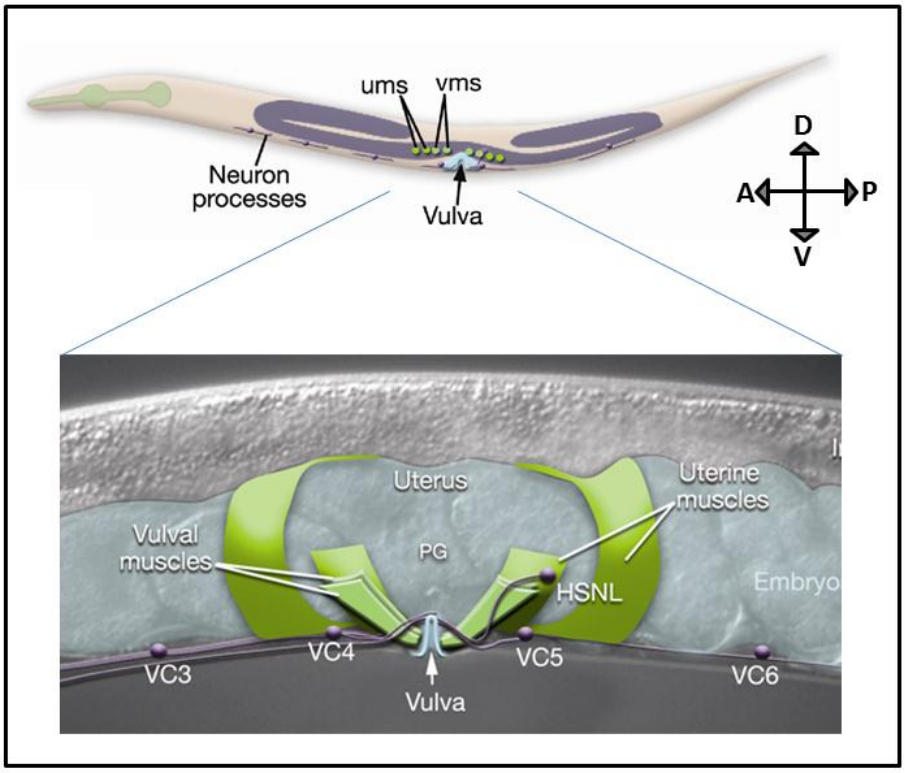
The *C. elegans* egg-laying system

The *C. elegans* egg-laying machinery is an intricate system comprised of multiple different parts, including an organization center, musculature, neurons and hypodermal structures, all of which are necessary for the proper expulsion of embryos (Figure 1.2). Each of these distinct components relies upon each other and together comprises sophisticated interconnected systems. If any of the myriad steps during the development of any of these components are disrupted it results in an animal in which the progeny will devour the adult from within in a phenotype known as “bag of worms.” The proper specification and function of the egg-laying machinery relies heavily upon

LIN-12/Notch signaling in multiple different cell types and during various stages of development.

Figure 1.2: The Hermaphrodite Egg-Laying System

The egg-laying system of the adult *C. elegans* hermaphrodite consists of multiple integrated parts, including primarily structural components such as the uterus and vulva, as well as musculature and neurons. The uterine muscles form sheets that surround the uterus and the vulval muscles attach to the opening of the vulva. A subset of the vulval muscles are directly innervated by ventral chord neurons 4 and 5 (VC4/5) and the hermaphrodite specific neurons (HSN) The remaining vulval and uterine muscles are innervated indirectly through gap junctions originating from the directly-innervated vulval muscles (Figure adapted from Wormatlas, reprinted with permission).


ums
vms
Neuron processes
Vulva
D
A
P
V
Uterus
Uterine muscles
Vulval muscles
PG
HSNL
Embryo
VC3
VC4
VC5
VC6
Vulva

LIN-12/Notch signaling in vulval development

The vulva of *C. elegans* is formed during a multistep process during development of the hermaphrodite animal. Initially, two equipotent somatic gonadal cells undergo a cell fate decision regulated by signaling events between both cells. Of these two cells, one cell will adopt the anchor cell fate and the other will become the ventral uterine precursor cell. The anchor cell functions as the organizing center of the vulval region and signals the cells of the hypodermis to differentiate into primary, secondary or tertiary vulval precursor cells. The primary and secondary cells will then further divide and terminally differentiate, giving rise to the primary structure of the vulva. The anchor cell also initiates the patterning of the uterine cells through an invasive process between the vulF cells, thereby forming the uterine seam syncytium (Reviewed in Schindler and Sherwood, 2013).

An early stage in which LIN-12/Notch signaling begins to establish the necessary components of the egg-laying system is during the cell fate decision between the Anchor Cell (AC) and the Ventral Uterine precursor cell (VU). This decision illustrates an example of the role of LIN-12/Notch in the classical model of establishing an alternating differentiation pattern, known as lateral inhibition. In this event, two adjacent equipotent cells both express the receptor *lin-12* and its ligand *lag-2*. However, only one cell can ultimately express *lin*-12 and the other only *lag*-2 by the conclusion of the cell fate specification. As the decision progresses, reciprocal feedback mechanisms restrict the expression of both genes into a mutually exclusive manner (Seydoux and Greenwald, 1989). Whichever of the two cells has a slight edge initially in LIN-12 activation will begin to downregulate its *lag*-2 expression and positively autoregulate its expression of

lin-12 (*Wilkinson et al., 1994)*. This will push the cell towards the VU cell fate while reinforcing the feedback loop. Conversely, the other cell will continue to express *lag-2* thereby activating LIN-12 in the neighboring cell, while establishing its own identity as the AC.

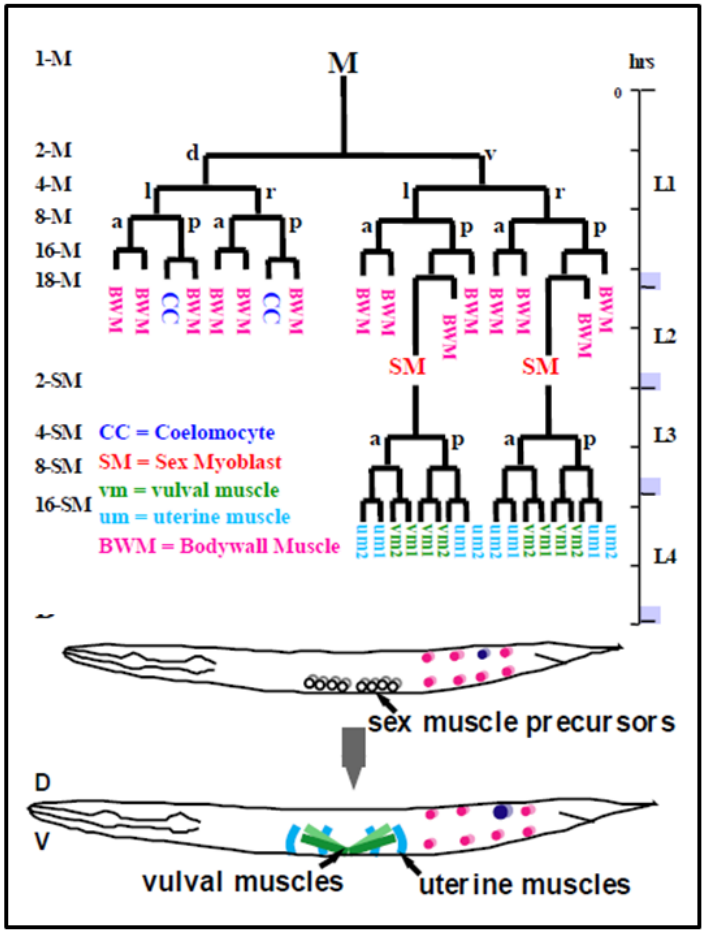
Similarly, proper specification of the AC is critical for the patterning of the Ventral uterine Precursor Cells (VPCs) during the development of the egg-laying system. The VPCs are patterned from the Pn.p cells and their specification is critical for the formation of the vulva. The AC produces a graded induction signal, lin-3, an EGF_like protein (Hill and Sternberg, 1992). This signaling activates the EGFR-Ras-ERK-like pathway through the actions of *let-23* (Ferguson et al., 1987). The P6.p cell is the most proximal cell to the AC and receives the highest dose of inductive signal, forcing the cell to adopt the 1° VPC cell fate. The 1° VPC then produces a subordinate lateral signal comprised of the DSL class (Delta/Serrate/Lag-2) ligands LAG-2, APX-1 and DSL-1. The DSL ligands activate LIN-12/Notch signaling in the adjacent P5.p and P7.p cells expressing LIN-12 which in turn specifies the 2° VPC cell fate and inhibits them from taking on the 1° VPC cell fate (Chen and Greenwald, 2004). The descendants of the 1° and 2° VPCs will then undergo morphogenesis to become the vulva.

LIN-12/Notch signaling in the development of the egg-laying muscles

The musculature that attaches to the vulva and comprises the core mechanical portion of the egg-laying machinery is descended from the M lineage (Figure 1.3).

Figure 1.3: Diagram of the M Lineage

The M lineage of the *C. elegans* hermaphrodite is born during embryogenesis from a single M mesoblast. The lineage divides dorsoventrally during postembryonic development producing a dorsal portion that gives rise to Body Wall Muscles (BWMs) and two non-muscle coelomocytes (CCs). The ventral portion gives rise to BWMs and two Sex Myoblasts (SMs). The SMs migrate to the presumptive vulval region where they further divide and differentiate giving rise to 16 non-striated muscle cells, four each of type I and type II vulval and uterine muscles. Dorsal (d), ventral (v), left (l), right (r), anterior (a) and posterior (p).


1-M
2-M
4-M
8-M
16-M
18-M
2-SM
4-SM
8-SM
16-SM
M
d
v
l
r
a
p
BWM
CC
SM
hrs
0
L1
L2
L3
L4
CC = Coelomocyte
SM = Sex Myoblast
vm = vulval muscle
um = uterine muscle
BWM = Bodywall Muscle
sex muscle precursors
D
V
vulval muscles
uterine muscles

The M lineage of *C. elegans* is mesodermal in origin and arises during embryogenesis from a single M mesoblast cell (Sulston and Horvitz, 1977). During post-embryonic development the M mesoblast undergoes a dorsoventral division to establish an asymmetrical lineage comprised of a dorsal and ventral M lineage. The dorsal M lineage then divides and differentiates into six BWM cells and two non-muscle coelomocytes (CCs) which are involved in heavy metal detoxification (Schwartz et al., 2010). The ventral M lineage divides and differentiates into eight BWMs but also generates two sex myoblasts (SMs) instead of CCs. These SMs then migrate from the posterior of the animal to the presumptive vulval region where they divide and differentiate to generate four each of the type I and type II vulval (VM1s and VM2s) and uterine muscles (UMs), respectively. These sixteen cells are all non-striated sex muscles that together form the musculature of the egg-laying apparatus of hermaphrodites. The type I and type II uterine muscles form sheets that surround the arms of the uterus. The type I and type II vulval muscles attach to the vulva and are involved in its opening during egg release. Both types of vulval muscles need to coordinately function for proper egg release; however only the type II vulval muscles are directly innervated by the egg-laying neurons (HSNs, hermaphrodite specific neurons, and VC 4/5, ventral cord neurons 4/5) and the rest of the musculature is innervated through gap junctions (White et al., 1986; Li et al., 2013).

The dorsoventral patterning of the M lineage lays the groundwork for the asymmetry of the lineage and the specification of some very different cell fates. This asymmetry is established by the action of two major pathways: the LIN-12/Notch pathway and the Sma/Mab TGFβ signaling pathway (Foehr and Liu, 2007). Specifically,

the ventral SM cell fate requires the LIN-12/Notch signaling pathway. It has been found that mutations in the LIN-12/Notch pathway result in the transformation of the ventral M lineage to the dorsal M lineage, yielding an animal that lacks SMs and subsequently lacks the terminally differentiated cells of the SM sub-lineage, namely the vulval muscles and uterine muscles. This signaling event is mediated through the ligands *lag-2*, *apx-1* and *dsl-1*, which are expressed in the ventral hypodermal cells directly adjacent to ventral M lineage cells (Greenwald et al., 1983; Foehr and Liu, 2008).

The SM sub-lineage of *C. elegans* gives rise to sixteen sex muscles, including type I and type II vulval muscles and uterine muscles, that form the musculature of the egg laying machinery. I have found (described in Chapter 2) that the specification of the type II vulval muscles (VM2s) requires the LIN-12/Notch signaling pathway. I have shown that knockdown of multiple members of the LIN-12/Notch pathway, including *lag-1, lin-12, lag-2, sel-12* and *sel-8,* results in animals that undergo cell fate transformation of type II vulval muscle into type I vulval muscle. This requirement for LIN-12/Notch signaling is independent of its role in the specification of the SMs. Additionally I have shown that the LAG-2 expression in the AC is the likely ligand source initiating this signaling event (Hale et al., 2014).

After the VM2s have been specified, the egg-laying musculature requires innervation by the HSN and VC4/5 neurons in order to be functional. The VM2s are the only muscles of the egg-laying machinery that are directly innervated while the remaining VM1s and uterine muscles are innervated indirectly through gap junctions connecting to the VM2s (White et al., 1986). This innervation requires the VM2s to form muscle arms to act as synaptic targets, a process that has been shown to require LIN-

12/Notch signaling (Li et al., 2013). This signaling event is driven by the ligand APX-1 from the secondary vulval epithelial cells and VM1s, and appears to be another role of LIN-12/Notch signaling in the formation of a functional egg-laying system (Li et al., 2013; Hale et al., 2014).

Mechanisms underlying the specification of other M lineage cells

In addition to the egg-laying muscles, the *C. elegans* M lineage provides a nice system to study cell fate specification in mesoderm development. The non-gonadal mesoderm of *C. elegans* is somewhat simpler when compared to vertebrate mesoderm. However, both are remarkably analogous. There are two distinct types of muscles, the multiple sarcomere striated muscles and the smooth muscle-like non-striated muscles, that comprise several different muscle groups. The most abundant type of muscle in *C. elegans* is the striated body wall muscles (BWMs) 81 of which are derived embryonically and 14 of which are derived postembryonically. The BWMs are necessary for locomotion and are considered analogous to human skeletal muscle (Waterston, 1988). In humans, the MyoD family of transcription factors is both necessary and sufficient to generate muscle (Tapscott, 2005). All of the members of the MyoD family of transcription factors are bHLH transcription factors, and similarly the *C. elegans* MyoD homolog, HLH-1, is required for proper development of *C. elegans* striated muscles (Chen et al., 1994). The *C. elegans* pharynx, with its rhythmic pumping necessary for food intake, is considered analogous to the human heart. *C. elegans* CEH-22, which is required for proper pharyngeal development, is a homolog of the

vertebrate homeobox gene Nkx2.5 that regulates heart development (Pilon and Mörck, 2005). Additionally, the defecation muscles are comprised of non-striated muscle in which the *C. elegans* Twist homolog, HLH-8, is required for their proper formation (Corsi et al., 2000).

The M lineage gives rise to BWMs and non-muscle CCs, in addition to the egg-laying muscles. The BWMs derived from the M lineage require expression of the MyoD homolog, *hlh-1* (Harfe et al., 1998). Without proper HLH-1 function several BWMs and the CCs of the M lineage will undergo fate transformation into SMs. The HMX homeodomain factor, MLS-2, is necessary for the expression of *hlh-1* within the M lineage (Jiang et al., 2005). Interestingly, the PBC homeodomain factor CEH-20 has been shown to activate expression of *mls-2*, which itself can regulate *hlh-1* (Jiang et al., 2008). There has also been evidence demonstrating functional redundancy within this system of establishing M-derived BWMs. FOZI-1 is a zinc-finger containing protein that when absent also results in a phenotype of some BWMs and the CCs of the M lineage undergoing fate transformation into SMs (Amin et al., 2007). The redundancy was established by the observation that neither *hlh-1* nor *fozi-1* influences the expression pattern of the other factor, and yet when mutant for both *hlh-1* and *fozi-1* the animals lose all, or nearly all, of the BWMs of the M lineage. Additionally *mab-5*, a homolog of Antennapedia, has been shown to function together with *fozi-1*, therefore also functioning redundantly with *hlh-1* in autonomously specifying the BWMs and CCs of the M lineage (Amin et al., 2007). The PBC homeodomain factor CEH-20 has been shown to regulate *fozi-1* expression in a manner independent of *mls-2* (Amin et al.,

2007; Jiang et al., 2008). This highlights the intricacies of BWM cell fate patterning within the M lineage.

The LIN-12/Notch pathway and the Sma/Mab TGFβ signaling pathway both play critical roles in establishing the dorsoventral asymmetry of the M lineage (Foehr and Liu., 2007). I have already described above the role of LIN-12/Notch signaling in regulating this dorsoventral asymmetry. I will therefore primarily focus on the roles of the TGFβ pathway here.

The Sma/Mab TGFβ signaling pathway is a member of the BMP signaling pathway superfamily. Active TGFβ signaling begins when two each of type 1 and type 2 receptors are brought together through ligand binding to form a hetero-tetrameric complex. This close interaction allows for phosphorylation of the type 1 receptor by the type 2 receptor which, in turn, then phosphorylates R-Smads. The activated R-Smads then accumulate in the nucleus and form complexes together with DNA-binding factors and transcriptional co-activators and co-repressors to regulate transcription (Massagué et al., 2005).

SMA-9 is homologous to the Drosophila Smad cofactor Schnurri and has been shown to function in the Sma/Mab TGFβ signaling pathway (Foehr et al., 2006). It was shown that mutations in *sma-9* resulted in animals with a ventralization of the dorsal portion of the M lineage, wherein the lineage specifies additional SMs in place of CCs. This results in animals that lack the M-derived CCs of the dorsal M lineage but instead develop additional VMs and UMs. Interestingly, mutations within the core components of the Sma-Mab TGFβ signaling pathway do not result in this dorsal-to-ventral fate

transformation within the M lineage, but rather they suppress the M lineage-specific *sma-9* phenotype without affecting non-M lineage defects (Foehr et al., 2006). This supports the role of SMA-9 as antagonizing the Sma/Mab TGFβ signaling pathway to promote the dorsal M lineage necessary for proper CC specification.

Interestingly, LIN-12/Notch signaling and SMA-9 along with Sma/Mab TGFβ signaling function independently to regulate dorsoventral asymmetry of the M lineage. Evidence comes from multiple double and triple mutant analyses (Foehr and Liu, 2008). In particular, in *lin-12* and *sma-9* double mutants the polarity of the M lineage is completely reversed, meaning the normally dorsal cells are born in the ventral lineage and the ventral cells are born in the dorsal lineage. These animals still give rise to the M-derived CCs, however they remain in a ventral location rather than the WT dorsal location. In these mutant animals the SMs are specified from the dorsal lineage but still properly migrate to the presumptive vulval region and give rise to the correct sex muscles as in WT animals. Taken together with the evidence for SMA-9 as an antagonist for TGFβ signaling, this supports a model wherein LIN-12/Notch is required for the ventral cell fates of the M lineage, and the antagonism of TGFβ signaling by SMA-9 is independently required for the dorsal cell fates of the M lineage (Foehr and Liu, 2008).

The decision between BWM or CC fates is regulated by the actions of the SIX homeodomain factor CEH-34 and its cofactor EYA-1 (Amin et al., 2009). Expression of *ceh-34* and *eya-1* in the presumptive CCs requires a functional Wnt/β-catenin asymmetry pathway (Amin et al., 2009). The proper specification of CCs additionally requires the same players common to the M-derived BWMs, namely *hlh-1*, *fozi-1* and

mab-5. These factors act to up-regulate the expression of the transcription factor LET-381, thereby promoting the CC fate (Amin et al., 2010). Distinctly, the specification of the SMs relies upon the SoxC factor SEM-2 (Tian et al., 2011). It has been shown that *sem-2* is both necessary and sufficient in specifying SMs instead of BWMs. Additionally, the expression of *sem-2* is regulated by the Hox factors LIN-39, MAB-5 and CEH-20 (Tian et al., 2011). Together, these findings improve our understanding of the factors critical to the cell fate decisions of the early M lineage.

In contrast, very little is known about how the non-striated sex muscles are specified. Previous studies have identified two highly conserved transcription factors critical for sex muscle fate specification. The T-box transcription factor MLS-1/TBX1 acts as a fate determinant of uterine muscles (Kostas and Fire, 2002): loss of mls-1 leads to the fate transformation of UMs to VMs, while forced expression of mls-1 throughout the M lineage can convert other M lineage cells to adopt the fate of UMs. The TALE homeodomain protein UNC-62/MEIS also plays a role in sex muscle specification (Jiang et al., 2009): while knocking down *unc-62* causes early M lineage defects, a partial loss-of-function allele of unc-62, ku234, leads to the conversion of UMs and VM2s to VM1s. How these two factors function to specify the particular types of sex muscles is not well understood. Also not known is whether additional factors play a role in this process. My thesis research focuses on the specification of these non-striated sex muscles.

Chapter 2: A role of the LIN-12/Notch signaling pathway in diversifying the non-striated egg-laying muscles in *C. elegans*[1]

2.1 INTRODUCTION

One of the fascinating questions in developmental biology is how functional organs form from cell types of diverse origins. The *C. elegans* hermaphrodite egg-laying system requires the proper specification, differentiation and functional integration of four major components: the uterus, the vulva, the egg-laying muscles attached to the vulva, and the egg-laying neurons that innervate the vulval muscles (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; White et al., 1986; Li and Chalfie, 1990). The proper formation and function of the egg-laying systems allow eggs that are stored in the uterus to be released through the vulva by the contraction of the egg-laying muscles, which are innervated by the egg-laying neurons. The molecular mechanisms underlying the development of the vulva, the uterus, and the egg-laying neurons have been relatively well understood (Sternberg, 2005; Gupta et al., 2012; Schafer, 2005). In contrast, very little is known about how the different types of egg-laying muscles are specified.

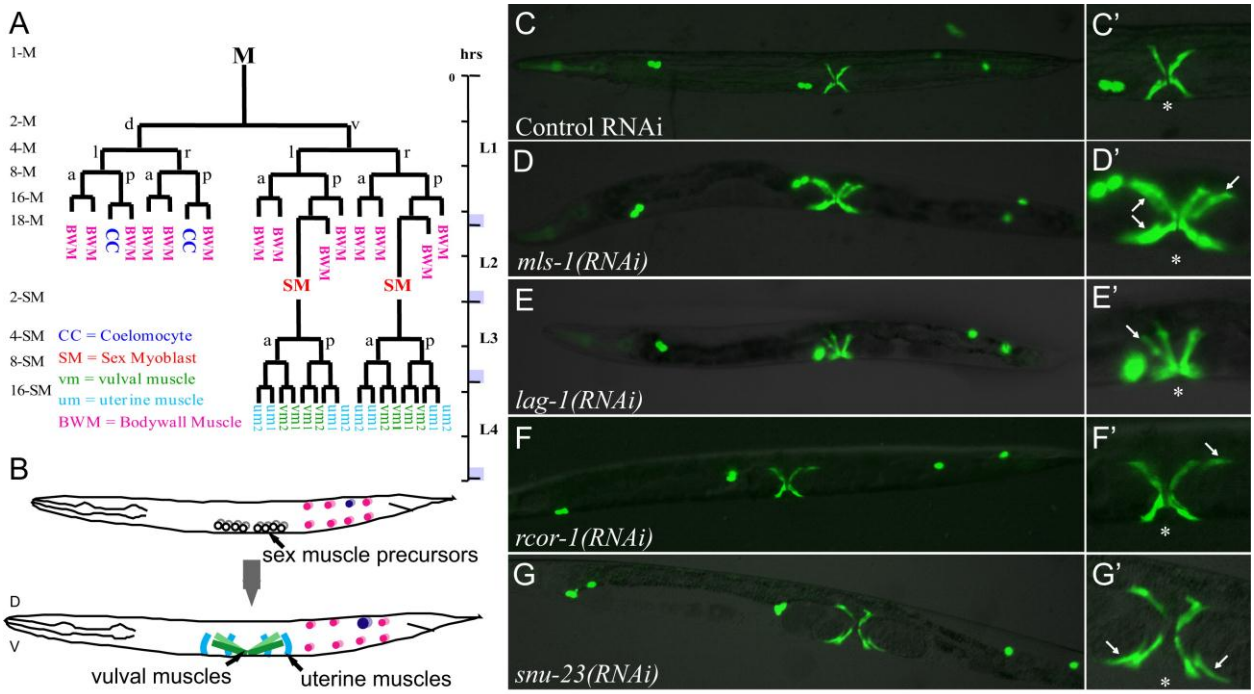
The egg-laying muscles are descendants of the multipotent sex myoblasts (SMs), which are derived from the postembryonic mesoderm lineage, the M lineage (Sulston

[1]This chapter was published in *Developmental Biology* (Hale, J.J., Amin, N.M., George, C., Via, Z., Shi, H., and Liu, J. (2014). A role of the LIN-12/Notch signaling pathway in diversifying the non-striated egg-laying muscles in C. elegans. Dev. Biol. *389*, 137–148.) and is reprinted with permission. RNAi screening and validation was performed by Nirav Amin, Carolyn George and Zachary Via. Molecular cloning and injections were performed by Herong Shi and Jun Liu.

and Horvitz, 1977, Figure 2.1A-C). The M lineage arises during embryogenesis from a single M mesoblast cell. During hermaphrodite post-embryonic development the M mesoblast undergoes two waves of proliferation. The first wave of proliferation occurs during the L1 larval stage, where the M cell produces fourteen striated body wall muscle (BWMs) cells, two non-muscle coelomocytes (CCs) on the dorsal side, and two SMs on the ventral side. The SMs then migrate to the future vulval region, where they undergo another wave of proliferation during the L3 larval stage to produce sixteen non-striated sex muscles or egg-laying muscles of four different types: four type I and four type II vulval muscles (VM1s, VM2s), as well as four type I and four type II uterine muscles (UM1s, UM2s). The sixteen non-striated sex muscles exhibit different morphology, location and function. The eight uterine muscles (UMs) wrap around the uterus, while the eight vulval muscles (VMs) are associated with the vulva and organized into two layers: the four VM1s are attached to the body wall subventrally, while the VM2s are attached to the body wall more ventrally. Only VM2s are directly innervated by the HSNs (hermaphrodite specific neurons) and VC4/5 (ventral type C 4/5) egg-laying neurons, while the remaining VM1s and UMs are connected together with VM2s by gap junction (White et al., 1986). Defects in the proper specification and development of the sex muscles do not affect the viability of the hermaphrodite as embryos can hatch inside the mother and continue to develop and reproduce, making it possible to study the mechanisms involved in the specification of these cells via various genetic manipulations.

Figure 2.1: An RNAi screen identified factors important for sex muscle development in *C. elegans*.

(A) The *C. elegans* postembryonic mesodermal lineage, the M lineage, in wild-type hermaphrodites. Indicated on the left are the corresponding stages of M lineage development referenced within the text. Bar on the right indicates larval stages and shading indicates molt. a. anterior, p. posterior, d. dorsal, v. ventral., l. left, r. right. (B) Schematics of L4 and adult hermaphrodites with their M lineage cells highlighted. (C–G) Adult hermaphrodites visualized with an intrinsic *CC::gfp* and *egl-15::gfp*, with the corresponding region expressing *egl-15::gfp* shown in (C′–G′). Asterisks indicate the presumptive vulval region. Arrows indicate additional*egl-15::gfp*-expressing type I vulval muscle-like cells. Control RNAi with empty vector L4440 (C), *mls-1(RNAi)* (D), *lag-1(RNAi)* (E), *rcor-1(RNAi)* (F), and *snu-23(RNAi)* (G).


A
1-M
2-M
4-M
8-M
16-M
18-M
2-SM
4-SM
8-SM
16-SM
M
d
v
l
r
a
p
BWM
CC
SM
hrs
0
L1
L2
L3
L4
CC = Coelomocyte
SM = Sex Myoblast
vm = vulval muscle
um = uterine muscle
BWM = Bodywall Muscle
B
sex muscle precursors
D
V
vulval muscles
uterine muscles
C
Control RNAi
C'
D
mls-1(RNAi)
D'
E
lag-1(RNAi)
E'
F
rcor-1(RNAi)
F'
G
snu-23(RNAi)
G'

Previous studies have identified two highly conserved transcription factors critical for sex muscle fate specification. The T-box transcription factor MLS-1/TBX1 acts as a fate determinant of uterine muscles (Kostas and Fire, 2002): loss of mls-1 leads to the fate transformation of UMs to VMs, while forced expression of mls-1 throughout the M lineage can convert other M lineage cells to adopt the fate of UMs. The TALE homeodomain protein UNC-62/MEIS also plays a role in sex muscle specification (Jiang et al., 2009): while knocking down *unc-62* causes early M lineage defects, a partial loss-of-function allele of unc-62, ku234, leads to the conversion of UMs and VM2s to VM1s. How these two factors function to specify the particular types of sex muscles is not well understood.

We set out to identify additional factors important in sex muscle specification by interrogating the putative transcription factors in the *C. elegans* genome using RNAi. By screening through over 70% of the predicted transcription factors, we identified a number of factors not previously known to function in sex muscle specification. In particular, we uncovered a role for the key effector and the sole CSL (CBF1, Suppressor of Hairless, LAG-1) protein of the Notch pathway in VM2 fate specification.

2.2 MATERIALS AND METHODS

2.2.1 C. elegans strains

Strains were cultured and handled as described by Brenner (Brenner, 1974). All analyses were conducted at 20°C, unless stated otherwise. The reference wild-type strain was LW0081 [ccIs4438(*intrinsic CC::gfp*) III; ayIs2(*egl-15::gfp*) IV; ayIs6(hlh-

8p::gfp) X] (Jiang et al., 2005). The M lineage-specific reporters used include arg-1p::gfp(ccIs4443) that labels both type I and type II vulval muscles (Kostas and Fire, 2002), rgs-2p::gfp(vsIs4) that preferentially labels uterine muscles (Dong et al., 2000), egl-15::gfp(ayIs2) that labels type I vulval muscles (Harfe et al., 1998a), hlh-29p::gfp(TLM908) that labels type II vulval muscles (McMiller et al., 2007, kindly provided by Dr. Casonya Johnson), hlh-8::gfp(ayIs6) that labels all undifferentiated M lineage cells (Harfe et al., 1998b) and an intrinsic CC::gfp(ccIs4438) that has a nuclear-localized GFP under the control of a coelomocyte-specific enhancer from the hlh-8 upstream region (Harfe et al., 1998b; Kostas and Fire, 2002). Additional transcriptional reporters used include arIs88[apx-1p::lacZ, dpy-20(+), ceh-22::gfp], arEx481[dsl-1p::lacZ, dpy-20(+), ceh-22::gfp] (Chen and Greenwald, 2004), and asIs131[lag-2p::2xnls-yfp::unc-54 3'UTR] (Zhang and Greenwald, 2011), which were all kindly provided by Dr. Iva Greenwald. The strain LW0683 [rrf-3(pk1426); ayIs6(hlh-8::gfp); ayIs2(egl-15::gfp); ccIs4438 (intrinsic CC::gfp)] (Amin et al., 2009) was used to visualize M lineage cells in the RNAi screen. Mutant alleles included LG III, lin-12(n676n930ts), *lin-12(wy750);* LG IV, dsl-1(ok810); LG V, unc-62(ku234), lag-2(q420ts), apx-1(zu183), *apx-1(wy755),* LG X, sel-12(ar171).

2.2.2 RNAi library construction and screening

RNAi clones targeting 934 putative transcription factors identified by Reece-Hoyes et al. (Reece-Hoyes et al., 2005) were retrieved from commercially available RNAi feeding libraries (Kamath et al., 2003, Rual et al., 2004) and organized into 96-well plates. Clones were retrieved from two RNAi libraries, with preference given to clones from the *C. elegans* ORF-RNAi library v1.1 (Rual et al., 2004). In total, 630

clones were retrieved from this library, with some overlap of target genes for these clones. For genes not found in the ORF-RNAi library, clones (267 clones) were retrieved from the Ahringer RNAi feeding library (Kamath et al., 2003). 91 genes had no RNAi clones in either library. To test the accuracy of these clones, a random set of clones retrieved from each library was sequenced. A fraction of the clones tested, 11.1% (n=81) and 12.82% (n=78) from the ORF-RNAi library and Ahringer RNAi library, respectively, did not match their predicted sequences.

RNAi plates were prepared as previously described (Kamath and Ahringer, 2003), with some minor changes. Briefly, bacteria was grown in LB with 12.5μg/ml Tetracycline and 75μg/ml Ampicillin for 12-14 hours at 37˚C with shaking and then seeded onto NGM agar plates (Brenner, 1974) with 25μg/ml carbenicillin and 4 mM IPTG. dsRNA production was induced at room temperature overnight and these RNAi plates were used within one week of induction. To observe the effects of RNAi during postembryonic development, we performed feeding RNAi using the strain LW0683 by plating 100-200 synchronous L1 larvae in duplicate on prepared RNAi plates for genes of interest. RNAi was performed at 25˚C and animals were scored for M lineage phenotypes around 24-48 hours after plating. For each round of screening, L4440 was used as a negative control and pNMA49 (fozi-1), pNMA50 (mab-5) and pNMA51 (hlh-1) were used as positive controls. RNAi clones that caused M lineage phenotypes were verified via sequencing, re-transformed into HT115(DE3) bacterial cells and re-tested for M lineage phenotypes.

Additional plasmids used in the RNAi experiments were: pMLF38 (dsl-1), pMLF47 (lag-2), pMLF48 (apx-1), all described in Foehr et al., (2008). For RNAi feeding

with temperature sensitive animals, the procedure was as described above except the animals were allowed to grow at the permissive 16°C until being shifted to restrictive 25°C at the appropriate time point.

2.2.3 Temperature shift experiments

Two temperature sensitive strains were used in the temperature shift experiments: LW1994: lin-12(n676n930ts) unc-32(e189) ccIs4438 (intrinsic CC::gfp) III; ayIs2 (egl-15::gfp) IV; ayIs6(hlh-8p::gfp) X and LW861: ccIs4438 (intrinsic CC::gfp) III; ayIs2 (egl-15::gfp) IV; lag-2(q420ts) V; ayIs6(hlh-8p::gfp) X. Gravid adults grown at 16˚C were collected, lysed with hypochlorite and the resulting embryos were kept in M9 buffer at 16˚C until hatching. Synchronized L1s were plated onto NGM plates seeded with *E. coli* OP50 bacteria at 16˚C. The stage of M lineage development was monitored by hlh-8p::gfp, which labels all undifferentiated cells of the M lineage (Harfe et al., 1998b). Temperature downshift was performed by picking desired staged animals that have been developing at the restrictive 25˚C to the permissive 16˚C, where they continued to develop. Similarly, temperature upshift was performed by picking desired staged animals that have been developing at the permissive 16˚C to the restrictive 25˚C. The animals were scored at young adult stage for their M lineage phenotypes.

2.2.4 lag-2 rescuing constructs

The following plasmids were used to perform rescuing experiments in the LW861 [ccIs4438 (intrinsic CC::gfp) III; ayIs2 (egl-15::gfp) IV; lag-2(q420ts) V; ayIs6(hlh-8p::gfp) X] background:

pJKL1027: lag-2p(-7142…-1)::lag-2 cDNA::unc-54 3’UTR, derived from p859 and p866.

pJKL1028: lag-2p(Δ-5674…-5567)::lag-2 cDNA::unc-54 3'UTR, derived from p862 and p866.

p866: lag-2p(-5674…-5567)::lag-2 cDNA::unc-54 3'UTR

p859, p862 and p866 are described in Zhang and Greenwald (2011) and kindly provided by Dr. Iva Greenwald.

2.2.5 Antibodies, immunostaining and microscopy

For immunostaining, animals were fixed according to the protocol of Amin et al (2007). Goat anti-GFP antibody (Rockland Immunochemicals) and Rabbit anti-HLH-2 antibody (Krause et al., 1997, kindly provided by Drs. Ann Corsi and Mike Krause) were both used at a 1:1000 dilution. Mouse anti-β-galactosidase antibody (Promega) was used at a 1:50 dilution. Secondary antibodies from Jackson Immunoresearch Laboratories were used at 1:50 to 1:200 dilutions. Differential interference contrast and epifluorescence microscopy were conducted using a Leica DMRA2 compound microscope. Image capture was performed using a Hamamatsu Orca-ER camera with the Openlab software (Improvision). Subsequent image processing was performed using Adobe Photoshop CS and Adobe Illustrator CS.

2.3 RESULTS

2.3.1 Identification of transcription factors involved in proper development of the C. elegans hermaphrodite egg-laying muscles

To identify transcription factors important for the proper development of the egg-laying muscles, which are derived from the M lineage, we screened through approximately 730 of the 934 putative transcription factors in *C. elegans* (Reece-Hoyes et al., 2005) via post-embryonic feeding RNAi (see Materials and Methods. In this study, RNAi refers to post-embryonic feeding RNAi beginning at the L1 larval stage. This screen identified 17 genes that reproducibly caused M lineage phenotypes when knocked down (Table 2.1). Among these 17 genes, six have been previously shown to play a role in the M lineage. These include mls-1, hlh-1, fozi-1, mab-5, mls-2 and unc-62 (Harfe et al., 1998a, 1998b; Liu and Fire, 2000; Kostas and Fire, 2002; Jiang et al., 2005, 2008, 2009; Amin et al., 2007, Figure 1D). These genes served as internal controls and validated the efficacy of the RNAi screen. Moreover, we have also identified and reported the functional requirement of *ceh-34* and *let-381* in a feed-forward manner to regulate CC fate specification and differentiation (Table 2.1, Amin et al., 2009, 2010).

RNAi knockdown of the remaining nine genes, lag-1, icd-2, snu-23, unc-130, pha-4, rcor-1, dct-13, pat-9 and syd-9, primarily resulted in an extra type I vulval muscle (VM1) phenotype (Table 2.1, Figure 2.1C-G). Using cell type specific markers of the M lineage (see Materials and methods), we found that these extra VM1s likely do not arise from supernumerary SMs, but from either extra proliferation of the SMs or fate transformation within the SM sub-lineage (data not shown). Thus we have identified a set of nine new factors not previously known to be required for proper patterning and cell fate specification of SM descendants.

Table 2.1: An RNAi screen identified putative transcription factors important for M lineage development

For all the genes listed in this table, initial RNAi screening was carried out as described in Materials and Methods. RNAi clones that caused M lineage phenotypes were verified via sequencing, re-transformed into HT115(DE3) bacterial cells and re-tested for M lineage phenotypes. Terminal M lineage phenotypes were determined using a coelomocyte specific marker *cc::gfp* and a VM1-specific maker *egl-15::gfp* (see Materials and Methods). Only clones that gave M lineage phenotypes in at least three rounds of re-test were included in this table.

* Genes previously known to function in M lineage development: *hlh-1* (Harfe et al., 1998a), *fozi-1* (Amin et al., 2007), *mab-5* (Harfe et al., 1998b, Liu and Fire, 2000), *mls-2* (Jiang et al., 2005, 2008), *unc-62* (Jiang et al., 2009), *mls-1* (Kostas and Fire, 2001).

** Genes identified from the same RNAi screen described here; but we have already published their analysis: *ceh-34* (Amin et al., 2009), *let-381* (Amin et al., 2010).

Gene name	Transcription factor type	M lineage terminal phenotype	RNAi Penetrance	N
*hlh-1**	MyoD, bHLH	fewer CCs, extra SMs	82.46%	57
*fozi-1**	C2H2 Zinc Finger	fewer CCs, extra SMs	83.18%	107
*mab-5**	UBX/Antp, Hox	fewer CCs, extra SMs	64.95%	97
*mls-2**	Nkx/Hmx homeodomain	fewer CCs, extra SMs	41.82%	55
*unc-62**	Meis, TALE homeodomain	fewer or no CCs and SMs	100%	100
*mls-1**	Tbx1, Tbox	extra vm1s	100%	64
*ceh-34***	Six2 homeodomain	fewer CCs, extra BWMs	82.20%	107
*let-381***	FoxF, Forkhead	fewer CCs, extra SMs	95.20%	125
lag-1	CSL	extra vm1s	59.7%	62
icd-2	NAC, TS-N	extra vm1s	85.14%	101
snu-23	C2H2 zinc finger	extra vm1s	34.4%	61
unc-130	FoxD, Forkhead	extra vm1s	32.4%	71
pha-4	FoxA, Forkhead	extra vm1s	29.7%	64
rcor-1	SANT domain, CoREST	extra vm1s	26.67%	60
dct-13	CCCH tandem zinc finger	extra vm1s	12%	50
pat-9	C2H2 zinc finger	extra vm1s/CCs (mixed fate)	50%	58
syd-9	C2H2 zinc finger	extra vm1s	17.24%	58
		small/unattached vm1s	51.72%	

2.3.2 LIN-12/Notch signaling plays two independent roles during M lineage development: dorsoventral (D/V) patterning and sex muscle specification

Among the nine genes we identified to have a role in the M lineage, we decided to focus on *lag-1*. LAG-1 is the sole CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1) transcription factor mediating LIN-12/Notch signaling in *C. elegans* (Christensen et al., 1996). Using a combination of hlh-8::gfp, which labels all undifferentiated cells in the M lineage (including the SMs and SM descendants), cc::gfp, which labels the coelomocytes (CCs), and egl-15::gfp, which labels the type I vulval muscles (VM1s), we followed the M lineage development of lag-1(RNAi) animals. Like wild-type animals, lag-1(RNAi) animals had two SMs that divided three times to produce 16 sex muscle precursors (n=53). Unlike wild-type animals in which four of these 16 cells differentiated to VM1s, 59.7% of lag-1(RNAi) animals (n=62) had five to seven VM1-like cells that express egl-15::gfp (Figure 1E). These observations suggest that lag-1(RNAi) resulted in extra VM1-like cells due to a fate transformation of other types of sex muscles to VM1s in the SM sub-lineage.

This extra VM1 phenotype in lag-1(RNAi) animals was rather intriguing as it was an unexpected phenotype given the role of LIN-12/Notch signaling in the M lineage: previous studies have shown that LIN-12/Notch signaling is required for dorsoventral patterning of the M lineage and that loss of LIN-12/Notch pathway function results in a transformation of the ventral SM fate to the dorsal CC fate (Greenwald et al., 1983; Foehr and Liu, 2008). The loss of SMs will result in the loss of all SM descendants, including the VM1s, an opposite phenotype from the lag-1(RNAi) phenotype shown in Table 2.1 and Figure 2.1. To reconcile this discrepancy, we hypothesized that LIN-

12/Notch signaling may have two separate functions during M lineage development, first in dorsoventral patterning in the early M lineage, and second in proper fate specification in the SM sub-lineage. It is possible that *lag-1(RNAi)* via feeding does not fully attenuate LIN-12/Notch signaling during early M lineage development, allowing for proper dorsoventral patterning and formation of the SMs, after which comes another role for LIN-12/Notch in the SM sublineage, which is compromised by *lag-1(RNAi)*.

To test the above hypothesis, we used a lin-12 temperature sensitive allele n676n930ts (Sundaram and Greenwald, 1993) to specifically attenuate LIN-12/Notch signaling at various time points during M lineage development. Consistent with our previous findings (Foehr and Liu, 2008), lin-12 activity is required during early M lineage development for proper D/V patterning of the M lineage (Table 2.2A). However when n676n930ts animals were shifted from permissive (16˚C) to restrictive (25˚C) temperature after this initial critical period, they did not exhibit any early D/V patterning defects, rather an extra VM1 phenotype (Table 2.2A, Figure 2.2A). Both temperature up-shift and down-shift experiments further defined the critical period to be between the 2-SM and the 4-SM stage when LIN-12 function is required for proper sex muscle specification (Table 2.2B). Similar temperature up-shift experiments using the temperature sensitive allele *q420ts* of *lag-2*, which encodes one of the DSL-ligands (Henderson et al., 1994), also resulted in an extra VM1 phenotype (Figure 2.2B). These results are consistent with the hypothesis that LIN-12/Notch signaling has two independent roles in the M lineage. It is required in the early M lineage for proper D/V patterning, and it functions in the SM sub-lineage for proper sex muscle fate specification. As mentioned above, our *lag-1(RNAi)* feeding condition appears to bypass

Table 2.2. LIN-12 functions at two distinct stages during M lineage development for proper D/V patterning and proper specification of sex muscles.

2.2A. The function of LIN-12/Notch in sex muscle fate specification is independent from its function in D/V patterning in the M lineage.

Time of shift*	Normal M lineage 4 VM1s, 2 M-CCs	D/V defect <4 VM1s, >2 M-CCs	Sex muscle defect >4 VM1s, 2 M-CCs	N
1-M	20%	80%	0%	51
2-M	27%	46%	27%	22
4-M	67%	6%	28%	172
2-SM	84%	0%	16%	74

2.2B. LIN-12/Notch is required in late M or early SM lineage for proper VM1 numbers.

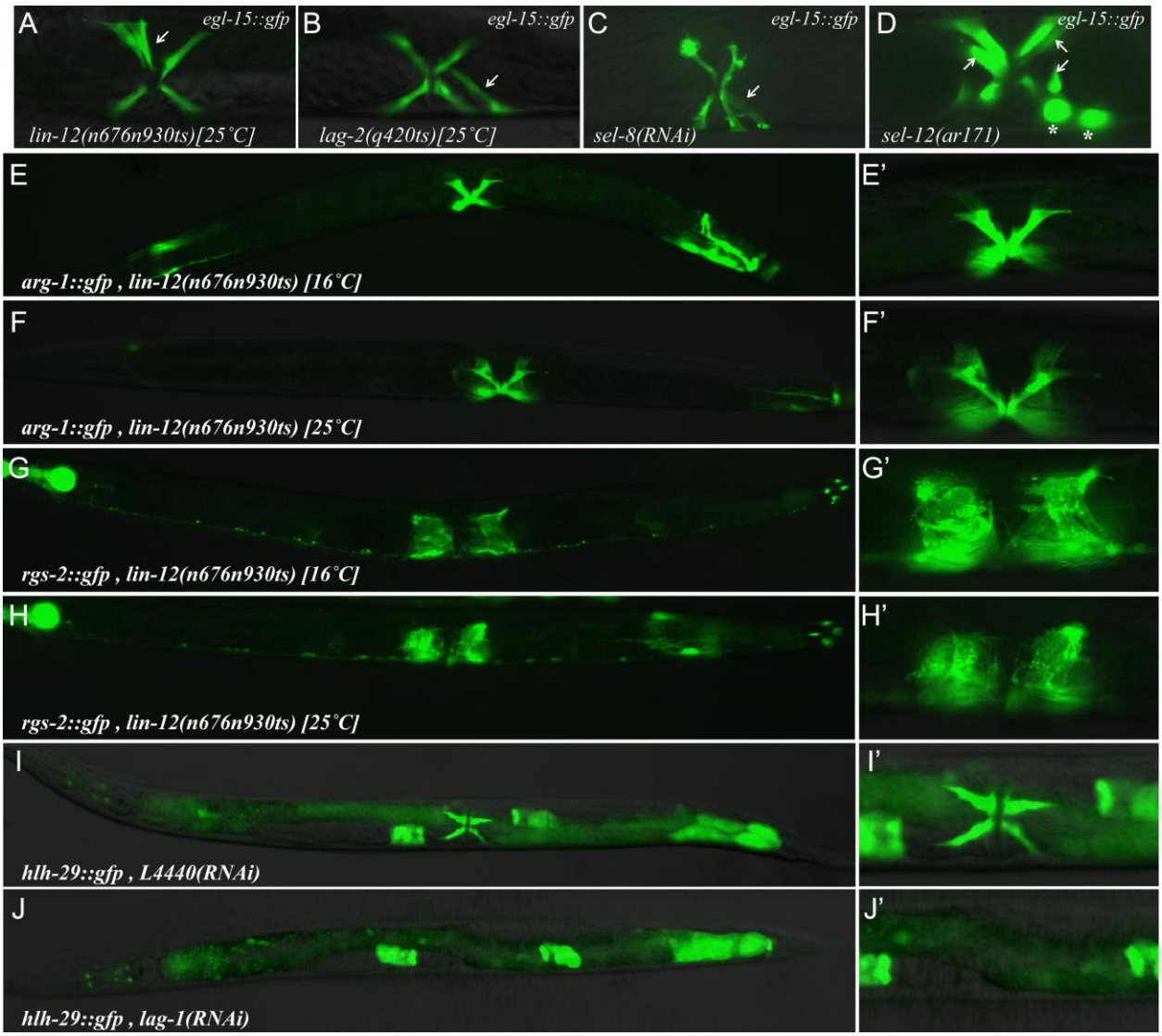
Time of shift*	Upshift 16°C → 25°C	Downshift 25°C → 16°C
8-M	5/11	ND
16-M	8/14	ND
2-SM	6/16	0/13
4-SM	0/25	1/11
8-SM	0/15	1/11
16-SM	ND	4/10

The number of animals with extra VM1/total number of animals scored from either upshift or downshift experiments is shown in Table 2.2B. ND: Not determined.

* Stage of M lineage development in both 2.2A and 2.2B.

Fig. 2.2: LIN-12/Notch signaling is required for specifying the fate of the type II vulval muscles (VM2s).

(A-D) *egl-15::gfp* (labels VM1s) reporter expression in *lin-12(n676n930ts)* (A) and *lag-2(q420ts)* (B) animals at the restrictive temperature, in *sel-8(RNAi)* (C) and *sel-12(ar171)* (D) animals. Arrows in A‑D mark the extra *egl-15::gfp*-expressing VM1-like cells. Asterisks in D mark the two embryonically-derived coelomocyte (CC) cells near the vulva. (E‑H) *arg-1::gfp* (labels both VM1s and VM2s) (E-F), *rgs-2::gfp* (labels UMs) (G-H) reporter expression in *lin-12(n676n930ts)* animals at the permissive (E, G) or restrictive (F, H) temperature. (I,J) *hlh-29::gfp*(labels VM2s) expression in control L4440 (I) or *lag-1(RNAi)* (J) animals. (E′‑J′) Corresponding magnified images from the left panels. Note that VM2-specific *hlh-29::gfp* expression is completely lost in 98% (*n*=111) of the *lag-1(RNAi)* animals examined (J,J′), indicating its high apparent sensitivity to knockdown of LIN-12 signaling relative to other markers, such as *egl-15*::*gfp*.


A
egl-15::gfp
lin-12(n676n930ts)[25˚C]
B
egl-15::gfp
lag-2(q420ts)[25˚C]
C
egl-15::gfp
sel-8(RNAi)
D
egl-15::gfp
sel-12(ar171)
E
arg-1::gfp , lin-12(n676n930ts) [16˚C]
E'
F
arg-1::gfp , lin-12(n676n930ts) [25˚C]
F'
G
rgs-2::gfp , lin-12(n676n930ts) [16˚C]
G'
H
rgs-2::gfp , lin-12(n676n930ts) [25˚C]
H'
I
hlh-29::gfp , L4440(RNAi)
I'
J
hlh-29::gfp , lag-1(RNAi)
J'

the early D/V patterning defect and result in the presence of extra VM1-like cells. Consistently, knocking down LAG-2 (data not shown) and the LAG-1 cofactor SEL-8 (Petcherski and Kimble 2000; Doyle et al., 2000) by RNAi feeding also resulted in the presence of extra VM1-like cells (45%, N=31, Figure 2.2C). It is worth noting that our previous report (Foehr and Liu, 2008) on *lag-1(RNAi)* and *sel-8(RNAi)* causing D/V patterning defect of the M lineage is likely because the RNAi conditions used in those earlier experiments allows for more efficient knockdown of these genes during early M lineage development. In those experiments, the RNAi defective *rde-1(0)* mutant hermaphrodites were first injected with dsRNA against *lag-1* or *sel-8* and then mated with wild-type males, and their resulting cross progeny exhibited D/V patterning defect in the M lineage (Foehr and Liu, 2008).

In addition to core members of the LIN-12/Notch pathway, we also examined a genetic null allele of sel-12(ar171), which encodes a positive modulator of LIN-12/Notch signaling (Kitagawa et al., 2003). We found that sel-12(ar171) animals do not exhibit any D/V defect in the M lineage (data not shown), but have a 37% penetrance (n=41) of the extra VM1 phenotype (Figure 2.2D). This result implies that proper sex muscle specification in the SM sublineage may require a higher threshold level of LIN-12/Notch signaling than proper D/V patterning of the early M lineage.

2.3.3 LIN-12/Notch signaling is required for specifying the type II vulval muscles

To address the nature of the role of LIN-12/Notch signaling in sex muscle fate specification in the SM sub-lineage, we determined the source of the extra type I vulval

muscle-like cells (VM1s) in animals with reduced LIN-12/Notch signaling. As described above, we observed no additional SMs or SM descendants in lag-1(RNAi) animals by following M lineage development using hlh-8::gfp. Similarly, lin-12(n676n930ts) animals growing at the restrictive temperature after the 4-M stage did not have extra SMs or SM descendants (data not shown). Three lines of evidence suggested that the extra VM1-like cells in animals with reduced LIN-12/Notch signaling are a result of VM2-to-VM1 fate transformation. First, we examined the expression pattern of arg-1::gfp, which is expressed in both type I vulval muscles (VM1s) and type II vulval muscles (VM2s) (Kostas and Fire, 2002, Figure 2.2E-F), and that of rgs-2::gfp, which is primarily expressed in uterine muscles (UMs) (Kostas and Fire, 2002, Figure 2.2G-H), in lin-12(n676n930ts) animals that were shifted to the restrictive temperature at the 4-M stage. As shown in Figure 2.2 and Table 2.3, these animals exhibited a wild-type pattern of expression of arg-1::gfp (n=68) and rgs-2::gfp (n=75). Second, we examined the expression pattern of the VM2-specific marker hlh-29p::gfp in lin-12 pathway mutants (Figure 2.2I-J). hlh-29 is a target of the LIN-12/Notch pathway and shows strong and specific expression in the VM2 cells of the M lineage (McMiller et al., 2007, Li et al., 2013). We found that in 98% (n=111) of the lag-1(RNAi) animals examined, hlh-29p::gfp expression was undetectable specifically in the VM2s, but its expression outside of the M lineage was not affected (Figure 2.2F, Table 2.3). A similar pattern of *hlh-29p::gfp* expression was also detected in *lag-2(q420ts)* animals at 25˚C (95%, n=125, Table 2.3). The higher penetrance of the loss of hlh-29p::gfp phenotype (98%, Table 2.3) compared to that of the gain of egl-15::gfp phenotype (59.7%, Table 2.1) upon lag-1(RNAi) may be due to the incomplete conversion of VM2s to VM1s in these animals, where the cells

Table 2.3. Type II vulval muscles (VM2s) are the source of additional type I vulval muscles (VM1s) in LIN-12 pathway loss-of-function mutants.

Genotypes	Sex muscle markers	Phenotype* penetrance	N
lin-12(n676n930ts) [25°C]	*egl-15::gfp*	35% with more VM1s	65**
lin-12(n676n930ts) [16°C]	*egl-15::gfp*	1% with more VM1s	83
lin-12(n676n930ts) [25°C]	*arg-1::gfp*	0% with more VMs	68**
lin-12(n676n930ts) [16°C]	*arg-1::gfp*	0% with more VMs	73
lin-12(n676n930ts) [25°C]	*rgs-2::gfp*	0% with fewer UMs	75**
lin-12(n676n930ts) [16°C]	*rgs-2::gfp*	3% with fewer UMs	78
lag-2(q420ts) [25°C]	*hlh-29::gfp*	95% with fewer VM2s	125**
lag-2(q420ts) [16°C]	*hlh-29::gfp*	5% with fewer VM2s	74
lag-1(RNAi) [25°C]	*hlh-29::gfp*	98% with fewer VM2s	111
L4440(RNAi) [25°C]	*hlh-29::gfp*	1% with fewer VM2s	79

The *lin-12(n676n930ts)* and *lag-2(q420ts)* animals were shifted to 25˚C at the 4-M stage.

* Phenotypes were scored based on the cell type specific sex muscle GFP markers used.

** Animals with an early D/V defect were excluded from the table.

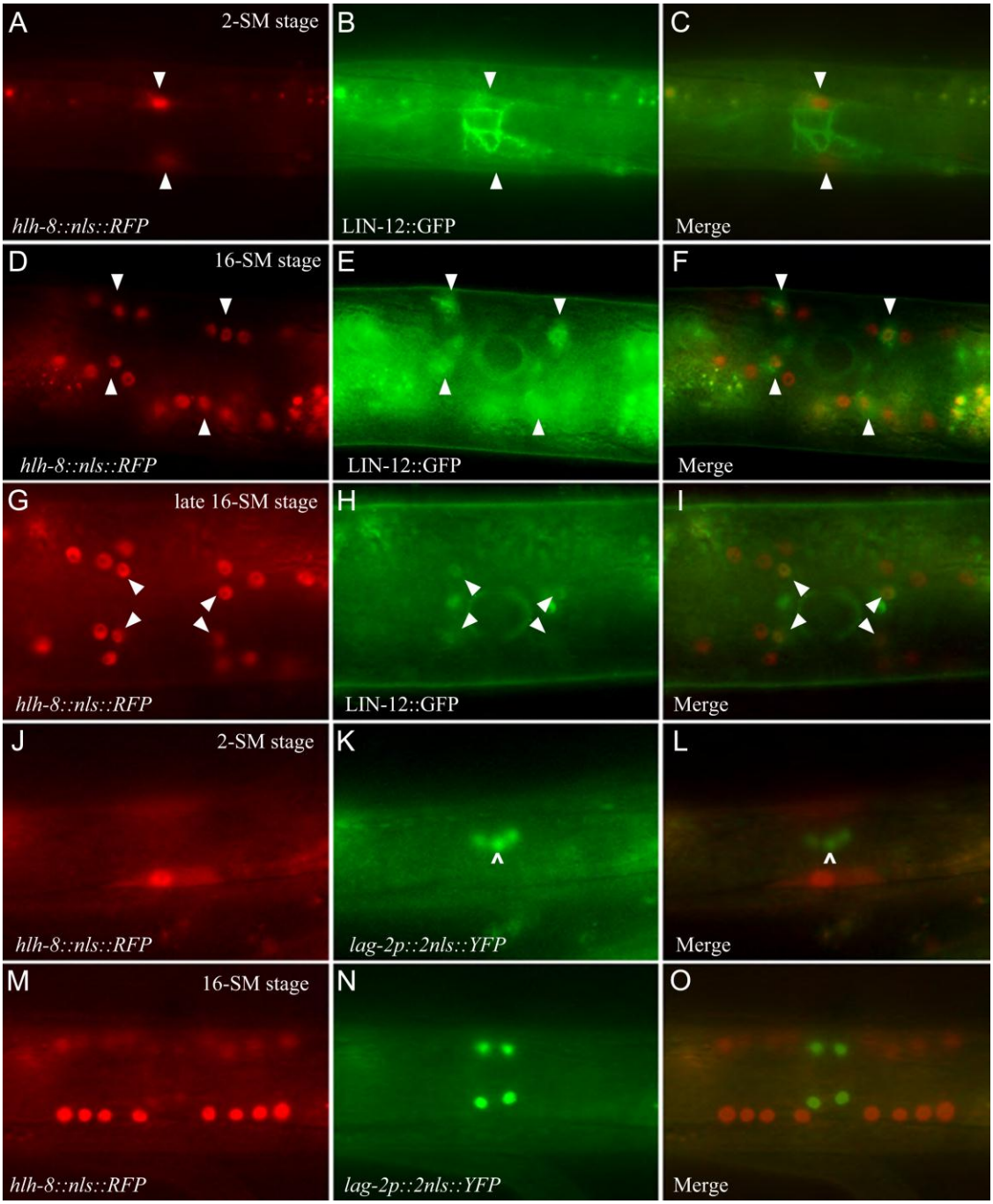
lost their VM2 identity and *hlh-29p::gfp* expression without fully converting to VM1s that express *egl-15::gfp*. Because *hlh-29* is a known downstream target of LIN-12/Notch signaling (McMiller et al., 2007, Li et al., 2013), *hlh-29p::gfp* expression might be more sensitive to the level of LIN-12/Notch signaling activity in the sex muscles.

Finally, the expression pattern of a functional translational LIN-12::GFP reporter (Levitan and Greenwald, 1998) in the SM sub-lineage is consistent with the notion that LIN-12/Notch signaling is required for VM2 specification. LIN-12::GFP is present and membrane-localized in all SM sub-lineage cells from the 2-SM stage through the 8-SM stage (Figure 3.3A-C, data not shown). At the 16-SM stage the expression of LIN-12::GFP becomes restricted to the four SM descendants that are fated to become VM2s (Figure 3D-F). As these cells begin to differentiate into VM2s, LIN-12::GFP becomes nuclear localized (Figure 3.3G-I). Thus LIN-12 is present and activated in cells fated to become VM2s.

Taken together, results from analysis of cell type specific reporters and the expression and localization patterns of the functional LIN-12::GFP suggest that LIN-12/Notch signaling is required for specifying the type II vulval muscles (VM2s).

Fig. 2.3: LIN-12 is expressed throughout the SM sub-lineage but becomes restricted to the nuclei of the undifferentiated VM2s at the 16-SM stage.

(A-I) Expression of LIN-12::GFP during SM lineage development. (A, D, G) *hlh-8::nls::mRFP*, which labels all undifferentiated SM lineage cells. (B,E,H) LIN-12::GFP. (C, F, I) corresponding merged images. LIN-12::GFP is expressed and localizes to the cell membrane in all SM-lineage cells from the 2-SM stage (A‑C) until the 8-SM stage (data not shown). At the early 16-SM stage (D‑F) LIN-12::GFP is restricted to cells fated to become VM2s (solid arrowheads). By the late 16-SM stage (G‑I) LIN-12::GFP becomes nuclear localized in the undifferentiated VM2s. (J‑O) Expression of *lag-2p::2nls::YFP* during SM lineage development. (J, M) *hlh-8::nls::mRFP*. (K, N) *lag-2p::2nls::YFP*. (L, O) corresponding merged images. *lag-2p::2nls::YFP* is expressed in the anchor cell (open arrowhead) and the P6.px cells from the 2-SM stage (J‑L) until the 16-SM stage (M‑O), when the AC has fused with the uterine seam syncytium and is out of the focal plane.


A
2-SM stage
hlh-8::nls::RFP
B
LIN-12::GFP
C
Merge
D
16-SM stage
hlh-8::nls::RFP
E
LIN-12::GFP
F
Merge
G
late 16-SM stage
hlh-8::nls::RFP
H
LIN-12::GFP
I
Merge
J
2-SM stage
hlh-8::nls::RFP
K
lag-2p::2nls::YFP
L
Merge
M
16-SM stage
hlh-8::nls::RFP
N
lag-2p::2nls::YFP
O
Merge

2.3.4 LAG-2 is the primary DSL ligand involved in the specification of type II vulval muscles

We have previously shown that the DSL ligands LAG-2, APX-1 and DSL-1 function redundantly for D/V patterning of the M lineage (Foehr and Liu, 2008). To determine the possible involvement of these ligands in VM2 specification, we first knocked down the functions of these three ligands either individually or together and examined the consequences on VM2 specification. As shown in Figure 2.2B and Table 2.3, shifting the temperature-sensitive lag-2(q420ts) allele to the restrictive temperature resulted in a phenotype of extra VM1-like cells with a penetrance of 67% (n=131). However, knocking down apx-1 or dsl-1 individually by RNAi did not cause any significant increase in the number of VM1-like cells (Table 2.). Similarly, knocking down each one in the lag-2(q420ts) background did not cause a significant increase of the penetrance of the extra VM1 phenotype (Table 2.4). We also examined *apx-1(wy755)* animals, which have been shown to exhibit dramatic reduction of muscle arms in VM2s (Li et al., 2013), and found that these mutant animals did not have any extra *egl-15::gfp*-expressing VM1-like cells (data not shown). These results suggest that LAG-2 is the primary ligand for LIN-12/Notch signaling in specifying the VM2s.

To determine the source of LAG-2 in VM2 specification, we first examined the expression patterns of lag-2 using a recently generated transcriptional reporter (Zhang and Greenwald, 2011). As shown in Figure 3, lag-2 expression was not detected within the SM sub-lineage. Rather, beginning at the L3 stage, the lag-2 reporter showed expression in the anchor cell (AC) and in P6.p and its descendants (Figure 2.3J-O).

Table 2.4. LAG-2 is the major ligand for LIN-12/Notch in specifying the type II vulval muscles (VM2s).

Genotypes*	**Percent with >4 VM1s**
Wild-type *[25°C]*	1% (N=101)
*lag-2(q420ts) [25°C]**	67% (N=131)
L4440(RNAi) [25°C]	3% (N=80)
apx-1(RNAi) [25°C]	3% (N=70)
dsl-1(RNAi) [25°C]	4% (N=82)
*lag-2(q420ts); L4440(RNAi) [25°C]***	56% (N=41)
*lag-2(q420ts); apx-1(RNAi) [25°C]***	49% (N=57)
*lag-2(q420ts); dsl-1(RNAi) [25°C]***	58% (N=83)

* All worm strains described in this table carry *cc::gfp* and *egl-15::gfp*.

** The *lag-2(q420ts)* animals were shifted to 25˚C at the 4-M stage.

Both the AC and the P6.px cells are in close proximity to the undifferentiated VM2s and VM2 precursors.

To further determine which of these two cell types are the source(s) of LAG-2 for VM2 specification, we took advantage of two lag-2 promoter deletion constructs generated by the Greenwald lab, one allows for P6.px expression and the other, AC expression, among other cells (Zhang and Geenwald, 2011). We used each of these promoter/enhancer elements to drive the expression of lag-2 cDNA in lag-2(q420ts) mutants, and examined the sex muscle phenotypes of the transgenic animals at the restrictive temperature using egl-15::gfp. The lag-2 full promoter driving lag-2 cDNA was used as a control. As shown in Table 2.5, the transgene lacking lag-2 expression in the AC (p866) failed to rescue the sex muscle phenotype of lag-2(q420ts) animals at the restrictive temperature. In contrast, transgenes allowing lag-2 expression in the AC (both pJKL1027, which has *lag-2* cDNA under the control of the full length *lag-2* promoter, and pJKL1028, which has *lag-2* cDNA under the control of the *lag-2* promoter with the P6.px enhancer deleted) rescued the sex muscle phenotype of lag-2(q420ts) animals at the restrictive temperature. Taken together the expression pattern of lag-2 during SM lineage development (Figure 2.3J-O), our results suggest that the AC is likely a source of LAG-2 for LIN-12/Notch that is required for proper VM2 specification.

Table 5. The anchor cell is likely a source of LAG-2 for proper specification of the type II vulval muscles (VM2s).

Plasmid (transgenic line)	LAG-2 expression		Control animals*	Experimental animals**
	AC	P6.px	Percent with >4 VM1s	Percent with >4 VM1s
pJKL1027 (line 1)	Yes	Yes	36% (N=148)	16% (N=98)
pJKL1027 (line 2)	Yes	Yes	38% (N=121)	15% (N=103)
pJKL1028 (line 1)	Yes	No	33% (N=139)	17% (N=90)
p866 (line 1)	No	Yes	37% (N=160)	32% (N=105)
p866 (line 2)	No	Yes	40% (N=120)	37% (N=106)

The *lag-2(q420ts)* animals were shifted to 25°C at the 4-M stage.

* Non-transgenic siblings served as control animals.

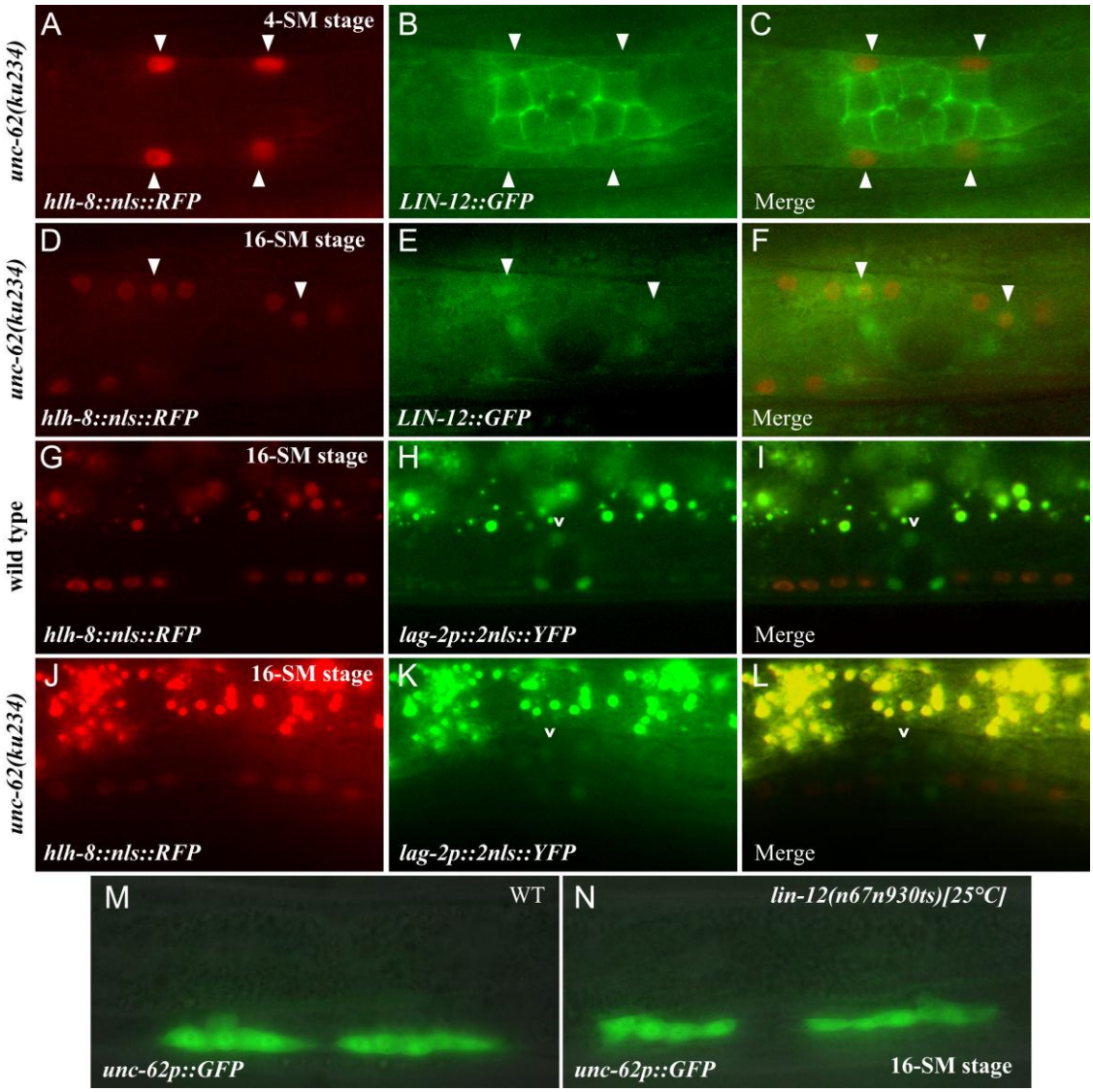
** Transgenic siblings express the specified *lag-2* rescuing construct.

2.3.5 Assessing potential interactions between LIN-12/Notch signaling and two other factors required for sex muscle specification: MLS-1/TBX1 and UNC-62/Meis

Two transcription factors have been previously shown to function in sex muscle specification in the SM sub-lineage. One such factor is the TALE homeodomain protein UNC-62/Meis, where a partial loss of function allele ku234 has been shown to cause a fate transformation of both uterine muscles (UMs) and VM2s into VM1s (Jiang et al., 2009). The other factor is the T box transcription factor MLS-1, which acts as an UM fate determinant (Kostas and Fire, 2002). We determined their relationship with LIN-12/Notch signaling using molecular epistasis analysis. We found that neither LIN-12::GFP expression nor *lag-2p::yfp* expression was altered in unc-62(ku234) mutant animals (Figure 2.4A-F, 2.4G-L). Similarly, the M lineage expression of an unc-62 transcriptional reporter (Jiang et al., 2009) was not altered in lin-12(n676n930ts) mutants at the restrictive temperature (Figure 2.4M-N). We also did not observe any change of expression pattern of a functional mls-1::LacZ reporter upon lag-2(RNAi) knockdown (data not shown). These observations suggest that LIN-12/Notch signaling and UNC-62/Meis or MLS-1/TBX1 do not exhibit reciprocal regulatory interactions regarding their respective expression during SM lineage development. They do not, however, rule out the possibility that UNC-62/Meis and/or MLS-1/TBX1 may function as cofactors of LAG-1/CSL in sex muscle specification.

Fig. 2.4: LIN-12/Notch signaling and UNC-62 do not appear to regulate each other's expression during SM lineage development.

(A–F) LIN-12::GFP expression is not altered in the SM lineage of *unc-62(ku234)* animals. (A, D) SM lineage cells marked by*hlh-8::nls::RFP*. (B, E) LIN-12::GFP. (C, F) Corresponding merged images. (A–C) at the 4-SM stage, (D–F) at the 16-SM stage. Arrowheads point to LIN-12::GFP-expressing cells. (G–L) *lag-2p::2nls::YFP* expression during SM lineage development is similar between wild-type (G–I) and *unc-62(ku234)* animals (J–L). Shown are animals at the 16-SM stage. (G, J) SM lineage cells marked by *hlh-8::nls::RFP*. (B, K) LIN-12::GFP. (C, F) Corresponding merged images. Open arrowheads mark the anchor cell. (M,N) *unc-62p::gfp* expression at the 16-SM stage (one focal plane shown) is identical between wild-type (M) and *lin-12(n676n930ts)* animals at the restrictive temperature (N).


unc-62(ku234)
A
4-SM stage
hlh-8::nls::RFP
B
LIN-12::GFP
C
Merge
unc-62(ku234)
D
16-SM stage
hlh-8::nls::RFP
E
LIN-12::GFP
F
Merge
wild type
G
16-SM stage
hlh-8::nls::RFP
H
lag-2p::2nls::YFP
I
Merge
unc-62(ku234)
J
16-SM stage
hlh-8::nls::RFP
K
lag-2p::2nls::YFP
L
Merge
M
WT
unc-62p::GFP
N
lin-12(n67n930ts)[25°C]
unc-62p::GFP
16-SM stage

2.4 DISCUSSION

2.4.1 The LIN-12/Notch signaling pathway is used repeatedly for the proper formation and function of the hermaphrodite egg-laying system

In this study, we described a feeding RNAi screen that led to the identification of the LIN-12/Notch pathway in proper specification of the non-striated egg-laying muscles, specifically the type II vulval muscles (VM2s), in *C. elegans*. LIN-12/Notch signaling has been shown to play multiple roles during the development of the hermaphrodite egg-laying system. The necessary patterning of the vulval region depends upon multiple finely tuned processes organized largely by the anchor cell (AC) (Sternberg, 2005). Generation of the AC arises from two equipotent cells during the L2 stage of development. Through lateral signaling between LAG-2/DSL and LIN-12/Notch an initially small imbalance between both cells is reinforced through reciprocal feedback loops driving one cell to adopt the AC fate and the other to adopt the ventral uterine (VU) precursor cell fate (Greenwald, 1983; Seydoux and Greenwald, 1989; Wilkinson et al., 1994). The AC then patterns the vulva by specifying the vulval precursor cells (VPCs) through inductive signaling mediated by LET-23/EGFR (Sternberg and Horvitz, 1989; Aroian et al., 1990). LIN-12/Notch-mediated lateral signaling between the P5.p, P6.p and P7.p VPC cells allow the proper patterning and specification of these cells into 1° cell fate (P6.p) vs. 2° cell fate (P5.p and P7.p) respectively (Sternberg, 1988; Greenwald et al., 1983; Chen and Greenwald, 2004). Three DSL ligands, LAG-2, APX-1 and DSL-1, have been shown to function redundantly in this process (Chen and Greenwald, 2004). The AC also induces a particular VU intermediate precursor fate (pi) via LIN-12/Notch signaling (Newman et al., 1995). In addition to its roles in the

development of the vulva and the uterus, LIN-12/Notch signaling also plays multiple roles in the specification and differentiation of the sex muscles involved in egg-laying. The three DSL ligands, LAG-2, APX-1 and DSL-1, and their receptor LIN-12/Notch, are required for establishing the dorsoventral asymmetry of the early M lineage and the production of the egg-laying muscle precursor cells, the SMs (Greenwald et al, 1983; Foehr and Liu, 2008). Here, we showed that after the establishment of the D/V asymmetry in the M lineage, LAG-2/DSL and LIN-12/Notch–mediated signaling are required in the SM lineage for the proper specification of the VM2s, likely through direct induction.

Reducing the activity of LIN-12/Notch signaling by postembryonic RNAi (for *lag-2, lag-1* and *sel-8*) or by using hypomorphic temperature sensitive (*ts*) alleles (for *lag-2* and *lin-12*) all resulted in the presence of extra *egl-15::gfp*-expressing VM1-like cells. We showed that these extra VM1-like cells are not a result of extra SMs or extra cell proliferation in the SM lineage. Instead, they are a result of a fate transformation from VM2s to VM1s. Intriguingly, the penetrance of extra *egl-15::gfp*-expressing VM1-like cells in the RNAi knockdown or the hypomorphic *ts* animals never reached 100% in any of our experiments (Tables 2.1-2.4), and we rarely observed any animal with four extra VM1-like cells (except for 1 out of 41 sel-12(ar171) animals examined). Meanwhile, close to 100% of *lag-1(RNAi)* animals or *lag-2(q420ts)* animals at the restrictive temperature lost the expression of the VM2-specific *hlh-29p::gfp* (Table 2.3). This incomplete fate transformation could be due to 1) the partial loss-of-function nature of RNAi and the hypomorphic alleles used, and/or, 2) proper specification of VM2s requires both LIN-12/Notch signaling and additional redundant mechanism(s). At

present, we cannot distinguish between these two possibilities, because critical requirement of LIN-12/Notch signaling in the early M lineage for specifying the VM progenitor cells, the SMs (Greenwald et al., 1983; Foehr and Liu, 2008), prevents us from using complete loss-of-function mutations in core members of this pathway for our studies.

Our results indicated that the AC cell is likely the source of the LAG-2/DSL ligand for the proper specification of VM2s. Both VM1 and VM2 precursor cells are located in close proximity to the AC cell. Each of the four VM1-VM2 pairs shares a common progenitor/mother cell, M.vlpaaap, M.vlpaapa, M.vrpaaap and M.vrpaapa, respectively (Figure 2.1), and LIN-12::GFP is present in all of these four progenitor cells (Figures 2.3-2.4 and data not shown). How VM2, but not the VM1, precursor cells can respond to LIN-12/Notch signaling is not known. Additional molecular asymmetries might exist between the two types of precursor cells, thus affecting the competence of these cells to respond to LIN-12/Notch signaling. Because of the anterior-posterior location of each VM1-VM2 cell pairs (Figure 2.1), one likely candidate for the molecular asymmetry is the nuclear POP-1/SYS-1 asymmetry. Nuclear POP-1/SYS-1 asymmetry has been observed and shown to be critical in multiple anterior-posterior cell fate decisions during *C. elegans* embryonic and postembryonic development, including in the early M lineage (for example, see Lin et al., 1995; 1998; Huang et al., 2007; Green et al., 2008; Amin et al., 2009). Other candidate(s) may be among the factor(s) that we have identified from our RNAi screen (see below). Future studies on these factors and on possible roles of POP-1 and SYS-1 in sex muscle specification will help unravel this mystery.

Once specified, proper differentiation of the VM2s again requires LIN-12/Notch signaling. Differentiated VM2s are synaptic targets of the egg-laying HSN and VC4/5 neurons (White et al., 1986). Li and colleagues recently showed that LIN-12/Notch signaling is required for the proper differentiation of VM2s, specifically the formation of muscle arms that act as synaptic targets for the egg-laying neurons (Li et al., 2013). Several lines of evidence suggest that this function of LIN-12/Notch signaling in VM2 differentiation is both spatiotemporally and molecularly separable from its function in VM2 specification. First, our temperature shift experiments showed that LIN-12/Notch signaling is required in the early SM lineage prior to the 8-SM stage for proper specification of VM2 (Table 2.2), while its function in VM2 muscle arm formation is required in mid-L4 stage when all 16 sex muscle precursors have already been born (Li et al., 2013). Second, we found that two alleles that cause VM2 muscle arm formation defects, *lin-12(wy750)* and *apx-1 (wy755)* (Li et al., 2013), did not exhibit any VM2 specification (i.e., extra *egl-15::gfp*-expressing VM1s) defect. Similar findings were reported for *lin-12(wy750)* by Li and colleagues (Li et al., 2013). Finally, we showed that LAG-2 produced by the AC is likely the primary ligand for LIN-12/Notch signaling in VM2 specification, while Li and colleagues found that APX-1 expressed in the secondary vulval epithelial cells and the VM1s is the ligand for LIN-12/Notch signaling in VM2 postsynaptic muscle arm formation (Li et al., 2013). Previous studies have shown that APX-1 can substitute for LAG-2 function (Fitzgerald and Greenwald, 1995; Gao and Kimble, 1995), and that APX-1 and LAG-2, together with another DSL ligand, DSL-1, function redundantly during larval development and early M lineage development (Chen and Greeenwald, 2004; Foehr and Liu, 2008). The switch from LAG-2 produced by the

AC to APX-1 produced by the secondary vulval epithelia cells and VM1s in VM2 development may be due to the complex and well orchestrated cell rearrangements that occur during somatic gonad organogenesis during late larval development (Gupta et al., 2012), which place APX-1-expressing cells that do not express LAG-2 in closer proximity to the VM2s while they differentiate. Thus, LIN-12/Notch signaling plays two roles in VM2 development, first in their specification, and then in their differentiation. These two sequential functions of LIN-12/Notch signaling in VM2 development are likely the reason why we detected exclusive nuclear enrichment of the C-terminally tagged LIN-12::GFP (Levitan and Greenwald, 1998) in the differentiated VM2s (Figure 2.3), an indicator of high level of LIN-12/Notch activation (Greenwald, 2012; Kopan, 2012).

Thus LIN-12/Notch signaling is required in multiple cell types at multiple stages for the proper formation and function of the hermaphrodite egg-laying system. A single cell, the AC, functions to coordinate multiple aspects of this process: induction of the VPCs, specification of the VU precursor pi cell fate, and specification of the VM2s. The specificity of the output of LIN-12/Notch signaling appears to be due to a combination of the specific ligands involved and the functions of additional lineage intrinsic factor(s) in a specific cell type. These lineage intrinsic factors may include UNC-60/Meis, MLS-1/TBX1, and/or other factor(s) that we have identified from our RNAi screen (see below).

2.4.2 Additional factors are required for the proper specification of the egg-laying muscles

The mesodermal germ layer gives rise to a variety of functionally important cell types, including striated and non-striated muscles as well as non-muscle cells. Specification of the striated/skeletal and cardiac muscles has been extensively studied: myogenic regulatory factors (MRFs) such as MyoD, and the Nkx homeodomain protein Nkx2.5, are key players in striated muscle and cardiac muscle specification, respectively, both in vertebrates (Mok and Sweetman, 2011; McCulley and Black, 2012) and in invertebrates such as Drosophila and *C. elegans* (Krause and Liu, 2012; Wei et al., 2007; Zaffran et al., 2002; Haun et al., 1998). Much less is known about how different types of non-striated muscles are specified, due to the complexity of the various types of non-striated muscles present in vertebrates (for example, Fisher, 2010). In this study, we have identified a role of LIN-12/Notch signaling in the proper specification of the non-striated type II vulval muscles in *C. elegans*. In addition, our RNAi screen identified eight new factors whose knockdown led to abnormal development of the egg-laying muscles: *pha-4, unc-130, syd-9, pat-9, icd-2, rcor-1, snu-23*, and *dct-13* (Table 2.1). Among these factors, PHA-4, UNC-130, SYD-9, PAT-9 and ICD-2 have been previously studied in *C. elegans*. PHA-4, a FoxA transcription factor, has been shown to be critical for pharynx and foregut development as well as play a role in diet-restriction induced longevity (Horner et al., 1998; Kalb et al., 1998; Panowski et al., 2007). UNC-130 is another forkhead transcription factor that is important in male tail morphogenesis, in the specification of the chemosensory neurons, and in axon guidance by regulating the proper expression of the TGF-β guidance factor UNC-129 (Nash et al., 2000; Sarafi-Reinach and Sengupta, 2000). SYD-9 is a C2H2 zinc finger protein that functions as a post-transcriptional regulator of synaptic vesicle endocytosis

(Wang et al., 2006). PAT-9, another C2H2 zinc finger protein, is a nuclear protein that is important for the assembly of body wall muscle attachment during embryogenesis (Liu et al., 2012). ICD-2, a nascent polypeptide-associated complex (NAC) subunit, has recently been shown to play a role in protein folding and localization during translation (Arsenovic et al., 2012). Our findings that these factors are also required for proper patterning and specification of the egg-laying muscles added new roles to these factors during development.

The developmental functions of the remaining three factors identified in our screen, RCOR-1, SNU-23 and DCT-13, have not been elucidated in *C. elegans*. RCOR-1 is a SANT domain (SWI3, ADA2, NCoR, TFIIIB B" subunit) containing protein that shows homology to *C. elegans* SPR-1 and human RCOR proteins, which are components of the transcriptional corepressor CoREST complex (Jarriault and Greenwald, 2002). Jarriault and Greenwald (2002) showed that SPR-1, but not RCOR-1, plays a negative role in regulating LIN-12/Notch signaling in multiple cell fate decisions, suggesting that RCOR-1 might not be a CoREST component involved in LIN-12/Notch signaling. Thus either RCOR-1 functions in LIN-12/Notch-independent processes or acts as a modulator of LIN-12/Notch signaling in a SM lineage specific manner. SNU-23 is a C2H2 zinc finger protein with homology to the *S. cerevisiae* small ribonucleoprotein SNU23, which has been shown in yeast to be critical for proper pre-mRNA splicing (Stevens et al., 2001). DCT-13 is a CCCH zinc finger protein homologous to the RNA-binding proteins TIS11 in *S. cerevisiae* (Puig et al., 2005) and MEX-5 (Schubert et al., 2000) and POS-1 (Tabara et al., 1999) in *C. elegans*. It is likely that both SNU-23 and

DCT-13 are acting as RNA-binding proteins involved in regulating sex-muscle fate diversification.

All these new factors with potential roles in the proper development of the egg-laying muscles are evolutionarily conserved. The M lineage and specifically the SM lineage-derived egg-laying muscles thus provide us a unique model system for determining the functions of these factors as well as for uncovering new roles for factors previously known to function in other developmental processes. Because of the established or predicted roles of these factors in regulating transcriptional, post-transcriptional, or translational processes, further studying the roles of these factors in M lineage and SM lineage development will also allow us to decipher the specific contributions of transcriptional, post-transcriptional or translational mechanisms involved in cell fate specification. Future research will be geared towards determining how these factors function in sex muscle development and their relationship among each other and with other factors/pathways known to function in the SM lineage, such as LIN-12/Notch signaling, UNC-62/Meis and MLS-1/TBX1.

2.5 ACKNOWLEDGMENTS

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Chapter 3: Conclusion and Perspectives

This work establishes a role of LIN-12/Notch in specifying the type II vulval muscles of the *C. elegans* egg-laying system. It provides insights into a core component of the egg-laying machinery and illustrates how diversity is established within the non-striated musculature. Despite this additional knowledge regarding the relatively little studied non-striated muscle, there remain questions to be addressed.

Are there additional ligands functioning redundantly with LAG-2?

I have proposed in this work that expression of the DSL-class ligand LAG-2 in the AC provides the major signal for the specification of the VM2s. RNAi knockdown of *lag-2* showed VM2 to VM1 cell-fate transformation, however, the phenotype is not completely penetrant. Knockdown of additional ligands such as *apx-1* and *dsl-1* did not show the cell-fate change. More importantly, performing a double knockdown through the use of a temperature sensitive *lag-2* mutant in combination with either *apx-1* or *dsl-1* RNAi knockdown did not increase the penetrance of the VM2 to VM1 fate transformation phenotype. This result does not rule out the possibility that these additional ligands or others may be functioning redundantly with LAG-2.

In order to test for potential redundancy a more complete knockdown of DSL-class ligands would be useful. One way to accomplish this is to perform triple knockdowns, but RNAi knockdown of multiple genes has been shown to be ineffective.

Instead, a *lag-2; dsl-1* double mutant could be generated and used in combination with *apx-1* RNAi to assay for an increased penetrance and potential redundancy. This experiment can then be expanded to include the other, less well-studied, DSL-class ligands (Chen and Greenwald, 2004).

What roles do the newly identified factors play in M lineage development?

The post-embryonic RNAi feeding screen presented in Chapter 2 identified eight new candidates that upon knockdown, presented a similar extra-VM1 phenotype and have yet to be exhaustively studied. I have begun preliminary analysis on several of these candidate genes to try and cluster the factors into similar groups based upon reporter analysis. However, in order to properly study *icd-2, snu-23, unc-130, pha-4, rcor-1, dct-13, pat-9 and syd-9* the development of several other tools would speed the analysis and likely provide a clear explanation as to the details of their respective phenotypes. For example, generating lines with multiple reporters such as *egl-15::gfp(ayIs2); hlh-29p::gfp(TLM908)* would allow for a clear distinction if cell-fate transformations similar to LIN-12/Notch pathway knockdown were occurring.

Final Thoughts and Reflection

C. elegans is an organism that expertly sits upon the fine line between complexity and accessibility. It is an organism with numerous cell-types, but with a remarkably well defined cellular lineage. It is an excellent model for studying the

intricate processes critical to organogenesis, cell signaling and disease yet has a simple body plan and clear structure lending itself to visualization. It is overall an excellent model for studying genes of interest in higher eukaryotes with its many conserved homologous genes, yet is conducive to genetic manipulation due to such traits as the ability to self-fertilize, maintain extra-chromosomal arrays and its ease of RNAi knockdown. The M lineage is likewise a fantastic model to study cell-fate specification such as those events likely controlled by the genes identified in the RNAi screen. It is my hope that this work will contribute a small part to the understanding of non-striated muscle and aid the Liu lab and the scientific community in future studies.

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