Rowan University

Rowan Digital Works

8-2022

Dual Mechanisms Implemented by LIN-28 for Positive Regulation OF HBL-1 Are Necessary for Proper Development of Distinct Tissues in Caenorhabditis elegans

Madeleine Minutillo
Rowan University

Follow this and additional works at: https://rdw.rowan.edu/gsbs_etd

Part of the Cell Biology Commons, Genetic Processes Commons, Laboratory and Basic Science
Research Commons, Medical Cell Biology Commons, Molecular Biology Commons, and the Molecular
Genetics Commons

DUAL MECHANISMS IMPLEMENTED BY *LIN-28* FOR POSITIVE REGULATION OF *HBL-1* ARE NECESSARY FOR PROPER DEVELOPMENT OF DISTINCT TISSUES IN *CAENORHABDITIS ELEGANS*

Madeleine Minutillo, B.S.

A Dissertation submitted to the

Graduate School of Biomedical Sciences,

Rowan University

in partial fulfillment of the requirements for the Ph.D. Degree

Stratford, New Jersey 08084

August 2022

Table of Contents

Acknowledgements

I would like to thank the man who will forget more science than I will ever know, Dr. Moss. Thank you for riding this crazy rollercoaster with me. Thank you for teaching me how to develop as a scientist. Thank you for teaching me that there is no loss in science there is only experience and knowledge to be gained. Thank you for continually reminding me that the unexpected is the fun in science and to take satisfaction in doing science well. Thank you for all your support throughout this project, for providing either help or a push depending on what I needed. Most of all, thank you for providing an environment that taught me how to speak with confidence.

I would like to thank my committee members. Thank you for the comments and questions that were always constructive and prompted me to find different approaches to overcome the obstacles in this project. Thank you for your support as my project evolved. I would especially like to thank Dr. Ellis. Thank you for your perpetual positivity and encouragement, and for being an invaluable resource for my education in the *C. elegans* somatic gonad.

I would like to thank Maria Ivanova. Thank you for all your help in making and using the degron strain. Thank you for all the observation swapping and in depth discussions we had that helped me refine my project.

I would like to thank Kevin Kemper. Thank you for all your help with everything involved in lab upkeep, I could have never done it on my own. Thank you for taking the time to teach me how to perform many of the techniques I used for this project.

Thank you for lending your ears and insight to troubleshoot with me when I hit a snag. Thank you for your patience and for never audibly sighing when answering the same question for the hundredth time (we'll pretend it was just one). Thank you for always being available to help regardless of the time or your location. Thank you for injecting my days with some truly epic humor.

I would like to thank Jon Harbin. Thank you for talking shop with me and always offering helpful advice. Thank you for fixing lab equipment adding years back to my life. Thank you for all the impromptu discussions about anything and everything that always made my day better. Thank you for providing me with support and perspective when times were rough. Thank you for always getting things from the top shelf for me. Thank you for all the laughs. Stay hard my friend.

I would like to thank my family. Thank you for encouraging me to pursue my interests and thank you for your unwavering support through this journey.

Abstract

In *Caenorhabditis elegans*, the heterochronic pathway is comprised of a hierarchy of genes that control the proper timing of developmental events. *hbl-1* (Hunchback Like-1) encodes an Ikaros family zinc-finger transcription factor that promotes the L2 stage cell fate events of the hypodermis. The downregulation of *hbl-1* is a crucial step for the transition from the L2 to the L3 stage. There are two known processes through which negative regulation of *hbl-1* occurs: suppression of *hbl-1* expression by *3 let-7* miRNAs through the *hbl-1* 3'UTR and inhibition of HBL-1 activity by LIN-46. The mechanisms by which *hbl-1* is positively regulated have not yet been full defined. Currently, this positive regulation seems to be the responsibility of the conserved developmental regulator *lin-28*. *lin-28* is purported to oppose the activities of the *3 let-7* miRNAs and the *Caenorhabditis* specific heterochronic gene *lin-46*. Here I demonstrate the removal of *3 let-7* miRNA binding sites in 3'UTR of *hbl-1* does not abolish negative regulation of *hbl-1* in seam cells. I find *lin-28* negatively regulates *lin-46* expression by direct binding of the 5'UTR of *lin-46*. I report a novel sterilely phenotype due to the loss of HBL-1 activity in post-embryonic development. Due to the increased sensitivity of the somatic gonad to HBL-1 protein levels, I utilize the development of this tissue as an alternate means to study the genetic relationships between *lin-28*, *lin-46* and *hbl-1*. My results suggest *lin-28* acts through a branched pathway, partially bypassing *lin-46* to positively regulate *hbl-1* either through its 3'UTR or by targeting a third unknown factor.

Chapter 1: Introduction

Caenorhabditis elegans

The round worm *Caenorhabditis elegans* is a free-living species of nematode that is predominantly hermaphroditic (Brenner, 1974). *C. elegans* is a small, transparent, multicellular organism with a simple anatomy. It is these characteristics that allowed Dr. John Sulston to map the entire cell lineage of the nematode. He found that the cell division patterns of the wild type animal are invariant and the timing of these division patterns consistent (Brenner, 1974; Sulston and Horvitz, 1977; Sulston et al., 1980; Sulston et al., 1983). With this knowledge, *C. elegans* has become a powerful model organism for studying the regulation of cell fates in developmental timing.

The life cycle of *C. elegans* from embryo to a sexually mature adult in continuous development spans three days. After fertilization the animal undergoes embryogenesis and hatching, followed by four post-embryonic developmental stages (L1, L2, L3, L4) distinguished by subsequent molts before maturing to adulthood. In unfavorable conditions developing larvae can execute alternate developmental programs until conditions are again favorable. Embryos that hatch in the absence of food are able to undergo L1 arrest, a cellular quiescence which temporarily halts development. L2 larvae are able to enter into an alternate developmental pathway in order to undergo dauer arrest. Dauer arrest is an alternative developmental state that larvae can remain in for months (Cassada and Russell, 1975; Sulston and Horvitz,

1977; Golden and Riddle, 1984; Kostal, 2006; Angelo and Van Gilst, 2009, Baugh, 2013).

Heterochrony in *C. elegans*

The evolution of multicellular organisms has been the result of mutations affecting cellular division, spacial patterns, and timing. Some physical variations seen within related species are proposed to be the result of differences caused by changes in developmental timing also known as heterochrony (Ambros and Horvitz, 1984). Two types of heterochrony have been defined: sequence heterochrony in which the order of events changes, and growth heterochrony in which developmental size relative to shape changes (Moss, 2007; Smith, 2002). In *C. elegans* genes that control temporal cell fate programs were identified by comparing cell lineages of mutant and wild type animals. Many of the first heterochronic mutants were identified as cell lineage defects in the post-embryonic stages of development that produced physical characteristics deviating from the normal, simple anatomy (Ambros and Horvitz, 1984).

In *C. elegans*, the heterochronic pathway is comprised of a phenotypic hierarchy of genes that control the proper progression of developmental events (Ambros and Horvitz, 1984; Ambros, 1989). The expression patterns of heterochronic genes in the hypodermis follow the model of protein activity during specific stages of larval development. The up or down regulation of protein and microRNA expression

corresponds to transitions in developmental time (Tennessen et al., 2006). The hypodermis is one of the predominant tissues employed to study heterochronic genes due to the characteristic periodic activity of seam cells; cells that run laterally along the left and right sides of the worm (Sulston and Horvitz, 1977; Resnick et al., 2010). These seam cells display stem cell like characteristics and divide at every larval molt (**Fig. 1.1**). The anterior daughters differentiate and enter the hypodermal syncytium while the posterior daughters remain separate to repeat this pattern in the next stage of development. This asymmetric division occurs at every larval molt. The L2 stage is unique in that the seam cells undergo two rounds of division. The first round is a symmetric division increasing the overall number of seam cells from 10 to 16. The second round is then the conventional asymmetric division. At the L4 molt, or the L4-to-adult transition, the seam cells exit the cell cycle, differentiate, fuse to form a syncytium, and secrete an adult specific hypodermal structure called alae (Sulston and Horvitz, 1977; Ambros, 1989; Rougvie and Moss, 2013; Abbott et al. 2005). Heterochronic mutants have been studied by observing changes in the timing and division patterns of these seam cells. Mutations in genes involved in the heterochronic pathway have also been found to affect the development of other tissues including the vulva, intestine, nervous system, and gonad. (Chalfie at al., 1981; Liu and Ambros, 1989; Ruvkun and Giusto, 1989; Antebi et al., 1998; Hallam and Jin, 1998; Ollson-Carter and Slack, 2010; Choi and Ambros, 2019).
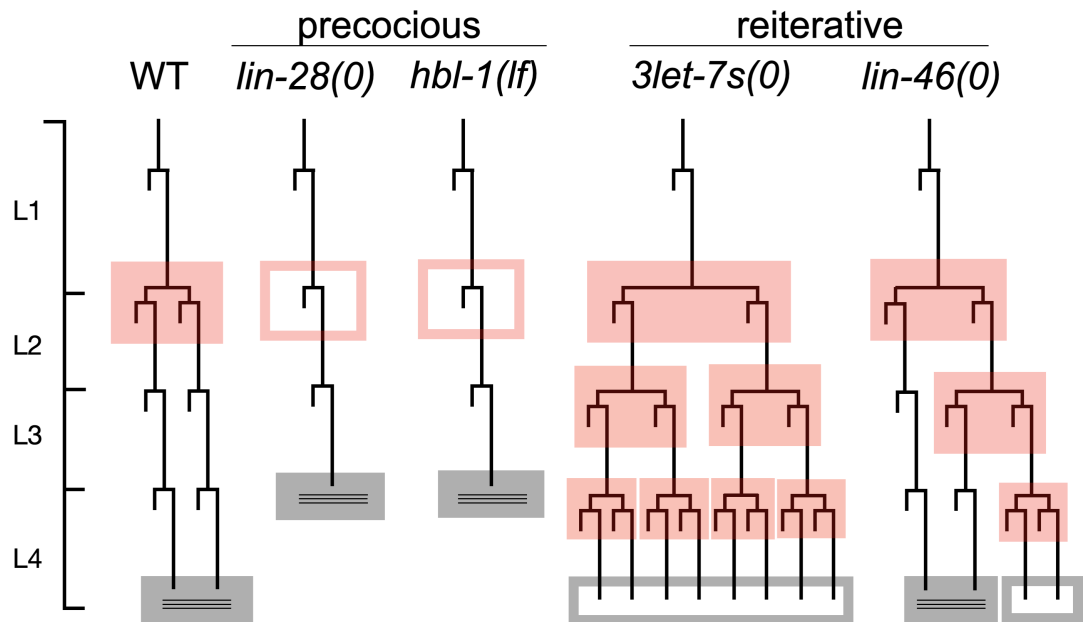
Fig. 1.1 Seam cell lineages of wild-type and heterochronic mutant *C. elegans.* At **left** is the lineage of a typical wild-type seam cell (5 of 8 seam cells on each side of the animal) as it develops through the 4 larval stages (L1-L4). **Pink solid box** indicates the double-cell division characteristic of the L2 for this lineage. The **grey box** indicates differentiation of these cells and the formation of adult alae (three cuticle ridges running the length of the body). To the **right**, differences in these lineages are shown for two precocious mutants and two reiterative mutants (specific genes and alleles are discussed below). In the precocious mutants, the double cell division does not occur and a single division occurs in its place (**pink open box**), reducing the total number of seam cells at adulthood. Detailed analysis of multiple heterochronic mutants indicates that the L3 pattern has occurred in place of the normal L2 pattern (Ambros and Horvitz, 1984). Differentiation of the seam cells occurs one stage early, at the end of the L3. In the reiterative mutants, the double cell division characteristic of the L2 occurs again in all or some later stages and sublineages. The **open grey box** indicates that all or some seam cells fail to differentiate at adulthood. In these cases, larval fates occurred in the place of normal adult fates (Ambros and Horvitz, 1984).

Two classes of heterochronic mutants have been reported. Precocious mutants skip stage specific developmental events causing subsequent developmental events to occur earlier relative to normal development. Reiterative mutants repeat stage specific

developmental events causing a delay in subsequent developmental events relative to normal development (Ambros and Horvitz, 1984; Moss et al.,1997; Resnick et al., 2010). One result of skipping developmental cell fate events is the characteristic premature display of the adult-specific cuticle structure alae by precocious mutants. Those precocious mutants that skip the double division cell fate event of the L2 stage, display a lower number of seam cells at adulthood than wild type animals (**Fig. 1.1**). Antithetical to precocious mutants, reiterative mutants display alae later than their wild type counterpart. Reiterative mutants that repeat the L2 stage, thus undergoing the double division event more than once, will display a higher number of seam cells at adulthood (**Fig. 1.1**) (Ambros and Horvitz, 1984; Rougvie and Moss, 2013).

Defective developmental timing of the hypodermis results in its desynchronization with other tissues. In precocious mutants the early formation of the vulva by the hypodermis prevents proper convergence with the somatic gonad resulting in the terminal phenotype of a protruded vulva (Pvl). In reiterative mutants the development of the vulva is delayed resulting in a lesion in the hypodermis, which causes the contents of the gonad to spill out of the body. This phenomenon is known as the bursting terminal phenotype (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1985; Seydoux et al., 1993; Abbott et al., 2005, Ecsedi et al., 2015).

Gonad development

Formation of the reproductive system occurs entirely in the post embryonic

stages of development (**Fig. 1.2**). For the reproductive system to form properly,

temporal and spacial developmental events must be strictly regulated and

synchronized. This system is made up of cells that have arisen from multiple cell

lineages that are limited in variability. The reproductive system of the hermaphrodite

is comprised of three distinct parts: the somatic gonad, the egg laying apparatus, and

the germ line (Sulston and Horvitz, 1977; Sulston et al. 1983). The somatic gonad is

composed of the gonadal sheath, the distal tip cells (DTCs), the spermatheca and the

spermathecae-uterine valve (Sp-Ut), and the uterus (Kimble and White, 1981;

McCarter et al., 1997; Hall at al., 1999). The egg laying apparatus is composed of

uterine cells as well as uterine muscles, the vulva and vulval muscles, and neurons

that mediate egg laying (Li and Chalfie, 1990; Thomas et al., 1990). Germ cells are

distinct from the somatic cells of the reproductive system. While the proximal germ

cells are surrounded by sheath cells of the somatic gonad, the germ cells located at the

distal ends of the gonad are not. These distal germ cells have incomplete borders and

are connected to each other by a central canal called the rachis (Hirsh et al., 1976).

The hermaphrodite germ line is able to produces both male and female gametes;

producing only sperm during the L4 stage of development and producing only

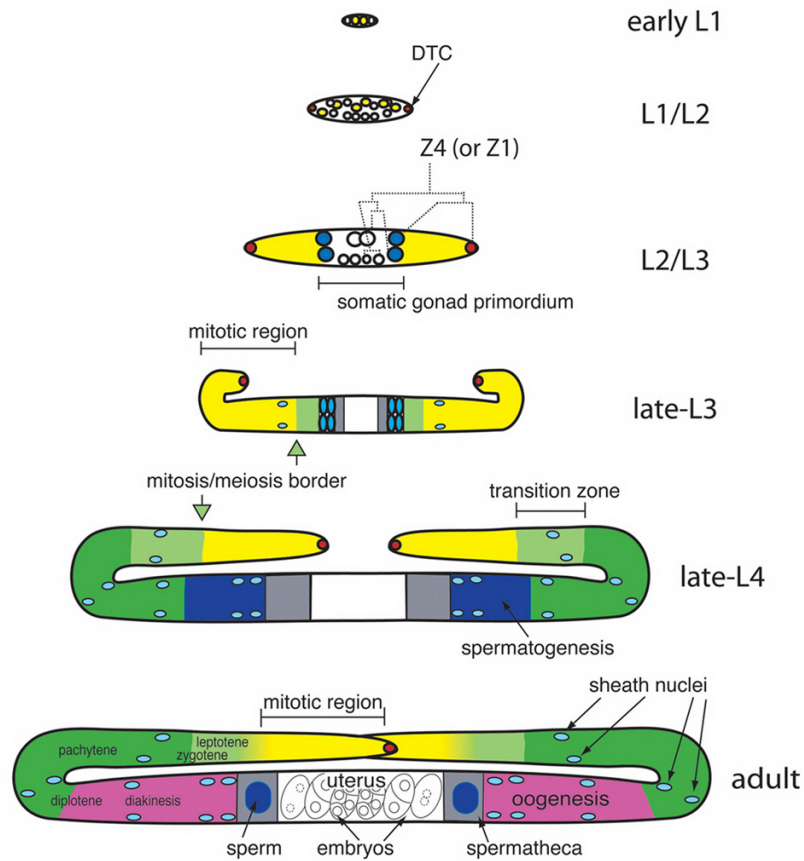oocytes during adulthood (Kimble and Hirsh, 1979).

Fig. 1.2 Gonad development in *C. elegans*. A schematic of the progression of gonad growth and development through the 4 larval stages (L1-L4). Germline color scheme: **yellow** mitotic region, **light green** transition (early prophase of meiosis I), **dark green** pachytene, **dark blue** spermatogenesis, and **pink** oogenesis. The mitosis/meiosis border is indicated in the late-L3 and late-L4 by **light green arrows**. In the adult, the mitosis/meiosis border is not sharp (mitotic and meiotic nuclei are interspersed at the border) as indicated here by a **yellow/green color gradient**. Somatic gonad color scheme: **red** Distal Tip Cells (DTCs), **blue** sheath/spermatheca precursor cells, **light blue** sheath nuclei, **grey** spermatheca, and **white** uterus. Germline nuclei and their surrounding cytoplasm are often referred to as "germ cells", though they are open to a core of shared cytoplasm (rachis) during much of their development. (Adapted from Hubbard and Greenstein, 2005).

The hermaphrodite adult gonad, consisting of both somatic gonad and germ line cells, has two U shaped arms that traverse the body wall. The gonad originates from a cluster of four cells located in the center of an L1 larvae called the gonad

primordium. Two of these cells are somatic gonadal precursor (SGPs) cells and the remaining two are germ line precursor cells (Kimble and Hirsh, 1979). In the L2 the gonad begins to elongate, the two SGPs divide to create 12 cells: two DTCs, nine somatic cells and the anchor cell (AC). These cells rearrange to produce a DTC at each end of the gonad. In the L3 and L4 the somatic cells divide to generate 140 cells. These cells differentiate into the five structures of the somatic gonad: the anterior and posterior sheath cells, the anterior and posterior spermathecae, and the uterus (Kimble and Hirsh, 1979).

The DTCs play two important roles in the gonad development: migration for gonad arm extension during larval development and promoting mitosis of the germ cells both during and after development (see red circles in **Fig. 1.2**). The DTCs act as leader cells and in the L2, migrate laterally in opposite directions toward the anterior and posterior ends of the larva. In the L3 the DTCs execute two turns, migrating to the dorsal side of the animal and then begin migration back toward the center of the animal. In the L4 the DTCs continue to migrate toward the center of the animal. At the end of the L4 stage the DTCs meet at the center of the animal and arrest their migration, settling opposite the vulva (Kimble and White, 1981; McCarter et al., 1997; Hall at al., 1999).

The DTCs produce a metalloprotease which remodels the basal laminae of the body wall and muscles (Blelloch and Kimble, 1999; Blelloch et al., 1999). Further, these cells are able to discern multiple developmental signals. The DTCs of animals

undergoing dauer arrest halt migration until the proper signals of post-dauer development are received. Mutations in some heterochronic genes such as *daf-12* (Abnormal <u>da</u>uer <u>f</u>ormation) are known to affect DTC migration which cause a delay in migration in L3 and L4 stages (Antebi et al., 1998).

The DTCs have also been shown to be necessary and sufficient to control germ line proliferation during larval development, as well as promote mitosis in germ cells at adulthood. This is controlled through Notch signaling which utilizes the LAG-2 (*lin-12* and *glp-1*) signaling ligand and the GLP-1(<u>G</u>erm <u>L</u>ine <u>P</u>roliferation) receptor. The DTCs express the LAG-2 ligand throughout development as well as throughout adulthood (Henderson et al., 1994; Tax et al., 1994). During adulthood, the DTCs are located at the distal ends of the gonad and have multiple thin cytoplasmic arms extending proximally to surround the germ cells nearby. The germ cells in contact with the DTC processes receive the LAG-2 signaling ligand via the GLP-1 receptors. This signals the germ cells to stay in a mitotic phase (Kimble and Crittenden, 2007). There is a physical range within which the LAG-2 signal can act. As the germ cells migrate proximally down the gonad arm, further from the DTCs, they enter what is called the transition zone (Austin and Kimble, 1987; Henderson et al., 1994). This is where the germ cells begin to express the protein GLD-1(<u>G</u>erm <u>L</u>ine <u>D</u>evelopment), which represses the translation of the GLP-1 receptor resulting in the promotion of meiosis instead of mitosis. As the germ cells migrate proximally, traversing the loop of the gonad arm, they undergo meiosis. From the loop and into

the proximal gonad arm, the germ cells mature into individual oocytes ready to be fertilized (Marin and Evans, 2003).

The spermathecae are accordion like tubes that are the sites of fertilization for oocytes (see grey areas in **Fig. 1.2**). In the late L4 all 24 cells that make up the spermathecae are present. The spermathecal cells develop in a spiral pattern executing a left-handed twist along the longitudinal axis (Kimble and Hirsh, 1979). In late L4 spermatogenesis also occurs, creating male gametes residing in the sheath lumen until ovulation. Hermaphrodites produce approximately 300 sperm (Kimble and Hirsh, 1979; Singson, 2001). Oogenesis does not occur until the hermaphrodite has fully developed into an adult animal. The spermatheca does not achieve its adult form until the first ovulation, when the passage of the first oocyte pushes the sperm into the spermatheca. After the oocyte has been fertilized, the embryo passes through a tube connecting the spermatheca to the animal's uterus. This spermathecal-uterine (Sp-Ut) valve is made up of 6 cells and, like the spermatheca, requires the passage of an embryo for maturation into the adult structure. Finally, the embryo passes into the uterus, a two lobed structure that meets at a central chamber that connects with the hypodermal vulva. Here a protective calcium shell forms around the embryo in preparation for its expulsion through the vulva (Kimble and Hirsh, 1979; McCarter et al., 1997). If the hermaphrodite remains unmated throughout adulthood, it will use all available sperm and produce around 300 progeny. If the hermaphrodite mates with a

male during adulthood, it will preferentially use that male derived sperm and produce up to 1400 progeny (Singson, 2001).

At a large metabolic cost, hermaphrodites supply their embryos with large quantities of yolk (Van Nostrand et al., 2013; Perez and Lehner, 2019). While yolk appears dispensable for embryogenesis, it plays a key role in supporting post-embryonic survival and development (Sharrock, 1983). Yolk proteins are secreted from the intestine of the hermaphrodite in the late L4 stage and synthesis increases as the animal gets older (Kimble and Sharrock, 1983; Perez et al., 2017; Sornda et al., 2019). The yolk is secreted into the pseudocoelom (body cavity) from whence it passes through both the gonadal basal lamina and sheath pores of the somatic gonad. It is then taken up through receptor-mediated endocytosis by the three most proximal oocytes to the spermatheca (Grant and Hirsh, 1999; Hall et al., 1999). The yolk comprises approximately 37% of total protein present in the embryo (Kimble and Sharrock, 1983). In a wild type animal yolk will accumulate in the pseudocoelom of the hermaphrodite as the self fertile reproductive period comes to a close; this is due to the reduction in yolk uptake by the embryos (Perez et al., 2017; Sornda et al., 2019). Adult hermaphrodites with mutations that disrupt the transfer of yolk to embryos (such as a mutation affecting the yolk receptor RME-2 (Receptor-Mediated Endocytosis) can be identified by the display of this unused yolk pooling within the pseudocoelom (Grant and Hirsh, 1999). Correspondingly, this trait is also observed in animals with defective gonad development in cases where oocytes are never formed.

Vitellogenins are the predominant yolk proteins by which egg laying animals supply nutrients to support development of their progeny. Vitellogenins are large lipo-glyco-phosphoproteins expressed in somatic tissues (Perez and Lehner, 2019; Romano et al., 2004). In *C. elegans,* the hypodermis is necessary for inducing vitellogenesis in the intestine at the end of the L4 stage. The *alg-1* (Argonaute Like Gene) gene encodes an Argonaute protein that is an essential cofactor for miRNA biogenesis. The *alg-1* gene was found to be a regulator of the *vit-3* (Vitellogenin) promotor (Dowen et al., 2016; Perez and Lehner, 2019). The requirement of *alg-1* in vitellogenesis initiation suggested a role for miRNAs in vitellogenesis as well. A study into miRNA regulation of vitellogenesis led to the identification of both *lin-4* (Lineage abnormal) and *let-7* (Lethal) heterochronic miRNAs as having a prominent role in vitellogenesis. Animals with loss-of-function alleles in either gene fail vitellogenesis initiation. The expression of *let-7* under a hypodermal promotor but not an intestinal promotor was able to rescue vitellogenesis in a *let-7* mutant. The heterochronic gene *lin-29* is not expressed in either *lin-4* or *let-7* mutants. It was found that in *lin-29* null mutants, the expression of *lin-29* under a hypodermal or an intestinal promotor was able to rescue the vitellogenesis initiation defect. This indicated LIN-29 is able to signal the intestine to begin vitellogenesis from within the hypodermis (Dowen et al., 2016).

Vulval Development

The vulva of the *C. elegans* hermaphrodite develops from epidermal precursors on the ventral side of the animal. In wild type animals vulval development is carried out in steps (**Fig. 1.3**): First, in the L1 and L2, six vulval precursor cells (VPCs) are specified among particular ventral epidermal cells and remain quiescent until the L3. Second, during the L3, signals from the gonad anchor cell (AC) and neighboring epidermal cells induce three of the VPCs to produce vulval cells. Both induced and uninduced cells are organized in a specific pattern along the ventral axis of the animal. Third, the induced precursors divide and differentiate to produce the cells that will make up the adult vulva. These resulting 22 cells will differentiate into one of seven types. The AC prompts cell invasion by inducing breakdown of the basement membrane separating the AC from the VPCs. This creates a hole in the epidermis. The AC then extends basolateral membrane processes through this hole in the basement membrane to attach to the descendants of the 1º lineage (p6.pap and p6.ppa). The AC then moves through this hole and inserts itself between p6.pap and p6.ppa. The vulval cells then invaginate and fuse, forming seven toroid rings that create the vulval lumen and attach to the uterus. Finally at the L4/Adult switch the vulva partially everts (Sulston and Horvitz, 1977; Sharma-Kishore et al., 1999; Sherwood and Sternberg, 2003; Sternberg, 2005; Sapir et al., 2007; Schindler and Sherwood, 2013).
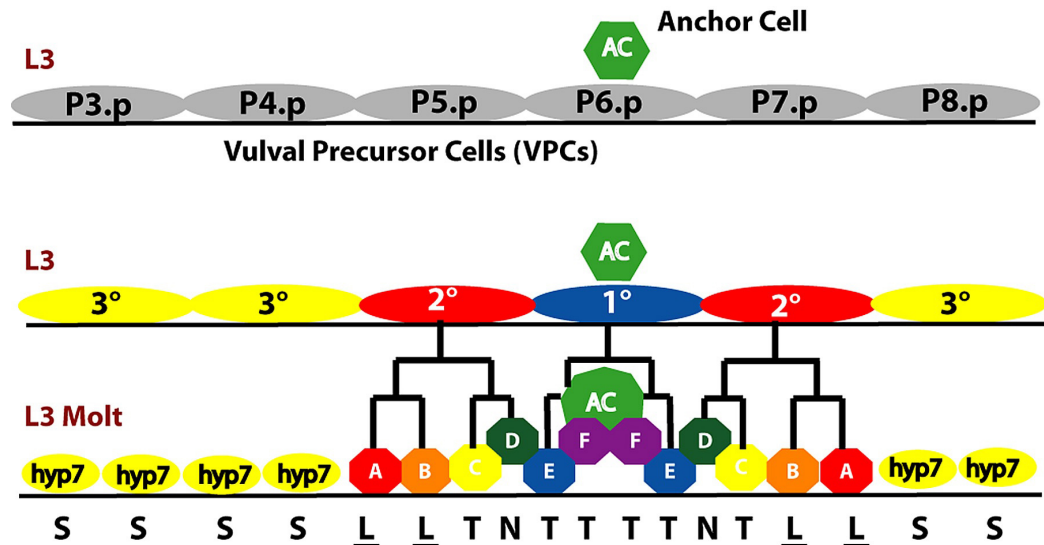
Fig. 1.3 Vulval development in *C. elegans.* A schematic of the progression of vulva induction and development during the L3. **Top**, the ventral hypodermal cells are not yet specified as vulval precursor cells (VPCs) and the anchor cell (AC) is positioned above the cell P6.p. **Middle**, inductive signals from the AC, as well as signals among the VPCs and the surrounding hypodermis, induce three cells nearest the AC, while the remaining VPCs are uninduced. **Bottom**, the primary and secondary VPCs divide and differentiate into the 22 cells of the vulva, which invaginates, undergoes morphogenesis and cell fusion, and opens the uterus to the outside. (Adapted from Sternberg, 2005).

The VPCs adopt one of three lineage types, induced VPCs will adopt either the 1º or 2º lineage while uninduced VPCs will adopt 3º lineage (**Fig. 1.3**). The pattern adopted by these cells involves signaling from multiple cells: the dorsal AC, the lateral VPCs, and the hyp 7 syncytial epidermis cell. In normal VPC patterning the AC signals, through the LET-23 protein, to promote the 1º lineage to three VPCs: P5p, P6p, and P7p. These three VPCs also signal each other laterally, through the LIN-12 protein, to both prevent adjacent VPCs from adopting 1º lineage as well as induce the VPCs on either side of a 1º VPC to adopt the 2º lineage. The resulting

spacial pattern is that of: 3º-3º-2º-1º-2º-3º. As a result, in wild type animals the P6p

cell adopts the 1º lineage and the P5p and P7p cells take on the 2º lineage. Mutations

in genes controlling these signaling pathways result in VPCs adopting different

lineage patterns resulting in either no vulval formation or a multivulva (Muv)

phenotype, in which a functional vulva as well as nonfunctioning pseudovulvas are

formed. Loss of LIN-12 activity results in more than one VPC adopting the 1º lineage

resulting in a multivulva phenotype (Sulston and Horvitz, 1977; Greenwald et al.,

1983; Sternberg and Horvitz, 1989; Katz et al., 1995).

The specification of vulval cell lineage results in distinct cell cycle regulation.

Heterochronic genes *lin-4*, *lin-14* and *lin-28* are known to control the transition of the

G1 phase to the S phase in the 1º and 2º VPCs. CKI-1 (<u>C</u>yclin-dependent <u>K</u>inase

<u>I</u>nhibitor) is expressed in VPCs in the G1 phase and inactivation of CKI-1 results in

initiation of VPC division in the L2 stage in development. *lin-4*, *lin-14* and *lin-28*

have been shown to target target *cki-1* expression (Hong et al., 1998). Loss of *lin-4*

causes delayed VPC divisions. Conversely, loss of *lin-14* or *lin-28* causes the VPCs to

divide precociously (Euling and Ambros, 1996a; Euling and Ambros, 1996b). As its

name indicates, *lin-28* (<u>Lin</u>eage abnormal) also plays a role in the cell lineage of the

VPCs. In wild type animals, the division patterns of the 2º vulval cells P5p and P7p

are mirror images of each other. The loss of LIN-28 activity causes variable

disregulation of P5p and P7p orientation resulting in a multivulval phenotype (Euling

and Ambros, 1996a). It is worth noting that hypomorphic alleles of *hbl-1* display this

multivulval phenotype as well, however in these mutants vulva morphogenesis ceases and the vulva is never fully developed (Abrahante et al., 2003; Fay et al., 1999).

The Dauer Developmental Pathway

Under ideal growth conditions *C. elegans* larvae develop continuously through four larval stages to adulthood. However under unfavorable conditions such as overcrowding, high temperature, or lack of food, the larvae may switch to an alternative pathway in which development can be arrested for a long period of time. Near the time of the L1 molt, larvae in stressful conditions will make a developmental choice: to either continue normal, rapid development to adulthood, or enter into a separate developmental program called L2d. The dauer-capable L2d larvae still undergo continuous development, but the progression of development is slower. The L2d program extends the time interval between the L1 and L2 molts delaying L3 cell fates. Larvae undergoing this L2d program are bipotent, it is during this extended window of time that the larvae assess their environment to commit to one of two developmental programs. If conditions become favorable for growth and reproduction, the dauer-capable L2d larvae execute L3 cell fates resuming continuous development to adulthood. If conditions remain unfavorable, the dauer-capable L2d larvae interrupt development and enter into an arrested state called dauer diapause (Cassada and Russell, 1975; Sulston and Horvitz, 1977; Golden and Riddle, 1984; Liu and Ambros, 1989; Karp and Ambros, 2012; Baugh, 2013; Avery, 2014; Ilbay and

Ambrose, 2019a). Depending on if their environment changes, larvae can stay in this alternate stage for months, which is well past the lifespan of a continuously developing animal; a continuously developing hermaphrodite will only live up to a few weeks (Fielenbach and Antebi, 2008).

This alternate stage has allowed for insights into the relationships among heterochronic genes regulating development. Studies have determined that the developmental defects in the hypodermis and vulva caused by mutations in early acting heterochronic genes are suppressed when the larvae exit from the alternate developmental pathway of dauer (Euling and Ambros, 1996b). An example is the loss of LIN-28 activity, which while important in continuous development, is not needed to enter the dauer stage nor for post dauer development. Thus, when *lin-28* null mutants enter post-dauer development they will develop normally to adulthood (Liu and Ambros, 1991). Dauer arrest provides a developmental pause allowing for stage specific transcription of certain genes to be reset. As stated above, larvae must be undergoing the L2d developmental program in order to enter dauer diapause. This is partially due to the required alternate *hbl-1* downregulation program that occurs when the commitment to arrest development is made. Initiation of post-dauer development results in the reprogramming of the hypodermal seam cells as well as the precocious vulval cells which revert back into VPCs. This reversal of cell fate determination in multiple tissues suggests the ability of the different developmental pathways to have a body-wide influence on temporal identity, which guides the development of post-

dauer larvae (Liu and Ambros, 1991; Euling and Ambros 1996b). Additionally, it was found that in this alternate post-dauer developmental pathway the influence of *lin-4*, *mir-84* (one of the *3 let-7* miRNAs) and *lin-46* differ from that in continuous development, becoming more important for regulation of stage specific cell fates (Karp and Ambros, 2012).

The heterochronic pathway

As stated above, the up or down regulation of heterochronic genes in the hypodermis correspond to transitions in developmental time. Many of the first heterochronic genes were identified by cell lineage defects that cause abnormal physical characteristics. In addition to cell lineage defects in the hypodermis, defects have been observed in other tissues including muscle tissue, the somatic gonad and specific neurons (Ambros and Moss, 1994; Antebi et al. 1998; Hallam and Jin, 1998; Slack et al., 2000; Choi and Ambros, 2019). The study of heterochronic gene activity in different tissues has allowed for better resolution of the genetic relationships among themselves and other genes.

The relationships among the known heterochronic regulators have been determined by genetic epistasis and molecular analysis (Rougvie and Moss, 2013). One version of the pathway based on current information is shown in **Fig. 1.4**. In general, the protein factors are expressed at the beginning of larval development and then down-regulated by miRNAs expressed as development progresses (Rougvie and
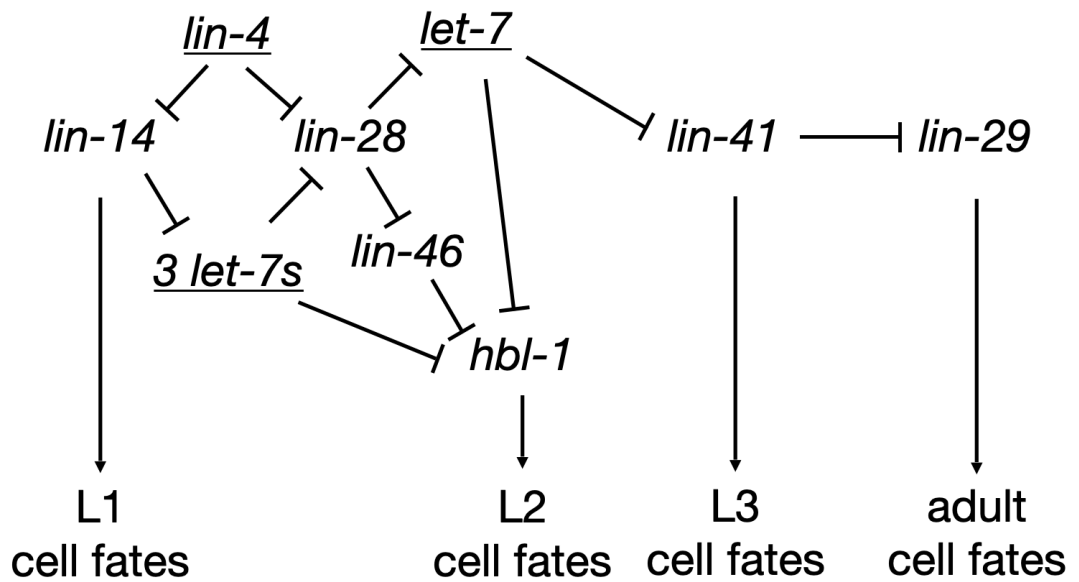
Fig. 1.4 The heterochronic gene pathway of *C. elegans.* Selected heterochronic genes are shown with their regulatory relationships to stage-specific cell fates and to each other. Time of each gene's activity is roughly shown from hatching at **left** to adulthood at **right**. The miRNA genes are **underlined**. This diagram reflects results presented this dissertation.

Moss, 2013). The first miRNA expressed is *lin-4*, followed by *mir-48*, *mir-84*, and *mir-241*, three redundantly-acting *let-7*-family members (henceforth called *3 let-7s*) (Chalfie et al., 1981; Abbott et al., 2005). The third miRNA expressed is *let-7* itself (Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003). Protein factors promote stage-specific cell fates while in certain instances inhibiting later events by regulating the miRNAs either transcriptionally or post-transcriptionally (Rougvie and Moss, 2013). Several important heterochronic genes are not indicated in this pathway either because they regulate miRNA expression levels or their roles are not precisely known. These include *daf-12*, *lin-42*, and *ztf-16*. Several heterochronic genes relevant to this study are discussed below.

Table 1.1 Selected heterochronic regulators

Gene	Product	Phenotype	Stage of action	Conservation
lin-28	LIN28-family RNA binding protein	Precocious	L2/L3	Orthologs in many animals
let-7	miRNA	Reiterative	L3	Orthologs in many animals
3 let-7s	miRNAs	Reiterative	L2	Broad conservation of family
lin-14	Unique transcription factor	Precocious	L1/L2	*Caenorhabditis*-specific
hbl-1	Ikaros-family transcription factor	Precocious	L2/L3	Broad conservation of family
lin-46	Unique regulatory protein	Reiterative	L2/L3	*Caenorhabditis*-specific member of a family
daf-12	Nuclear hormone receptor	Reiterative	L2	Broad conservation of family
lin-42	Period-like protein	Precocious	L2, all	Broad conservation of family
ztf-16	Ikaros-family transcription factor	Precocious	L2, all	Broad conservation of family

lin-28

A number of developmental timing regulators were found in *C. elegans* and several of these regulators are conserved in other species including humans (**Table 1.1**). A well studied member of these conserved regulators is *lin-28* (Lineage abnormal) (Ambros and Horvitz, 1984; Moss et al., 1997; Moss and Tang, 2003). The LIN-28 protein has been found in the neuronal stem cells of mammals and has been shown to be one factor in combination with Sox2, Oct4, and Nanog that can cause the reprogramming of a differentiated cell back into a pluripotent stem cell (Yu et al., 2003; Hanna et al., 2009). The heterochronic gene *lin-28* encodes an RNA binding

protein unique in structure, containing both a cold shock domain and a CCHC zinc

knuckle motif (Moss et al., 1997). Cold shock domains have been shown to bind

several different ligands including nucleic acids, metal ions and proteins (Mihailovich

et al., 2010). Due to the similarity to motifs found on retroviral nucleocapsid proteins,

the CCHC motif on the LIN-28 protein is predicted to bind RNA (Yu et al., 2003;

Ramboarina et al., 2004).

The gene *lin-28* has the characteristic expression profile of being up regulated

in early development and down regulated in late stages of development. (Moss et al.,

1997; Moss and Tang, 2003; Faas et al., 2013; Tsialikas and Romer-Seibert, 2015). In

C.elegans lin-28 is an early acting developmental regulator controlling the L2 to L3

transition (Ambros and Horviz, 1984; Ambros, 1989; Moss et al., 1997). The *lin-28*

gene is expressed in late embryos and in the L1 and L2 stages. In the L2 stage LIN-28

levels begin to drop as expression is repressed and by the L3 stage LIN-28 is barely

detectable (Moss et al., 1997; Seggerson et al., 2002; Morita and Han, 2006).

Expression of *lin-28* is regulated post-transcriptionally by miRNAs *lin-4* and the *3*

let-7 miRNAs through its 3'UTR. Levels of *lin-28* mRNA are reduced in the L3 when

compared to the L1 stage (Moss et al., 1997, Seggerson et al., 2002; Morita and Han,

2006).

The better known activity of LIN-28 is that in the L1 and L2 stages in which it

blocks the maturation of the microRNA *let-7*, another highly conserved

developmental regulator (Newman et al., 2008; Viswanathan et al., 2008). Whereas in

mammals LIN-28 binds both primary and precursor *let-7* miRNAs, in *C.elegans*

LIN-28 only binds *let-7* primary transcripts, blocking their processing by Drosha

(Lehrbach et al., 2009; Van Wynsberghe et al., 2011). As a result of this activity, when

lin-28 expression is high, the mature form of the *let-7* miRNA is absent. It is through

this inhibition of *let-7* processing that *lin-28* prevents L3 cell fates from occurring. As

development progresses the expression of *lin-28* is down regulated and mature *let-7* is

produced (Newman et al., 2008; Balzer et al., 2010; Vadla et al., 2012). Additionally,

LIN-28 has a second less known *let-7* independent activity. Vadla et al. (2012) found

that *lin-28* positively regulates *hbl-1* expression by blocking the down regulation of

hbl-1 by the *3 let-7* miRNAs. This earlier activity of LIN-28 promotes the L2 the cell

fates.

The activity of LIN-28 promotes L2 cell fates in multiple tissues including

seam cells and vulval precursor cells (VPCs). Null mutants of *lin-28* skip L2 specific

cell fate events and prematurely execute L3 specific cell fate events. As mentioned

above, the second larval stage of development is unique in that hypodermal seam

cells divide twice, first dividing symmetrically increasing the number of seam cells

from 10 to 16 and then dividing again asymmetrically. At the end of larval

development seam cells terminally differentiate and produce an adult-specific cuticle

structure called alae. Animals with null alleles of *lin-28* display a precocious

phenotype characterized by a reduced number of hypodermal seam cells as well as

the production of alae a stage early. Null mutants of *lin-28* undergo only three larval

molts instead of four. Further, some seam cells exit the cell cycle earlier than their sisters resulting in an animal displaying patches of adult alae as early as the L2 molt (Ambros and Horvitz, 1981; Ambros and Horvitz, 1987). In a *lin-28* null mutant the vulval precursor cells (VPCs) initiate divisions in the L2 stage, a stage earlier than wild type (Euling and Ambros, 1996a; Euling and Ambros, 1996b). This causes a desynchronization in timing of development between the gonadal tissue and the vulval precursor cells. As a result, *lin-28* mutants also display a terminal phenotype of a protruding vulva (Pvl).

The desynchronization of the normally developing gonad and the advanced (or delayed) development of the vulva, results in a hermaphrodite incapable of normal egg laying, reducing the overall number of progeny produced by the animal. In *lin-28* mutants, the lack of a vulval-uterine connection prevents the fertilized embryos from exiting the uterus through the vulva, rendering the vulva ineffective. Therefore, a limitation in progeny number is imposed by how much the uterus is able to extend within the hermaphrodite. Analysis of somatic and germ line gonadal nuclei as well as gonadal migration throughout development led to the long held belief that gonad development was normal in *lin-28* mutants (Ambros and Horvitz, 1984). Taken together, it was reasoned that the reduced progeny number of *lin-28* mutants was solely due to the inability to lay eggs, however recent studies have challenged this idea. Choi and Ambros (2019) found that the loss of *lin-28* activity results in abnormal development of the spermatheca-uterine (Sp-Ut) valve. This abnormal Sp-

Ut valve morphology was found to cause both defective ovulation as well as defective

exit from the spermatheca resulting in a reduction in progeny number. Additionally,

they found that the delay in spermathecal exit negatively affects eggshell integrity

which in turn causes embryonic lethality (Choi and Ambros, 2019).

let-7 miRNA

The *let-7* (Lethal) gene encodes a 21 nucleotide microRNA that plays an

important role in controlling developmental timing. The sequence of *let-7* as well as

the temporal expression pattern (expressed late in development) is conserved in

multiple species. This suggests that *let-7*'s role in cell fate patterning is also

conserved among species (Pasquinelli et al., 2000). In humans *let-7* has been found to

function in cell differentiation and cell cycle regulation, accordingly, reduction of

let-7 activity is associated with certain cancer types (Kloosterman and Plasterk, 2006;

Shi et al., 2008).

In *C. elegans*, *let-7* controls the timing of the L4 larval-to-adult switch by

negatively regulating the expression of the heterochronic genes *lin-28*, *hbl-1* and

lin-41 (Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003). Negative

regulation of *let-7* upon these three genes allows for the expression of a later acting

heterochronic gene *lin-29,* which is responsible for specifying adult fates (Ambros

and Horvitz, 1984; Bettinger et al., 1997; Rougvie and Moss, 2013; Azzi et al., 2020).

Mutants with null *let-7* alleles display a reiterative phenotype, where cell fate division

patterns are repeated. While the hypodermal seam cells divide correctly during the four stages of development, the seam cells of *let-7* mutants then incorrectly repeat the seam cell division pattern of the L4 stage. As a result, these animals do not secrete adult-specific alae and exhibit defects in the expression of adult-specific genes. This delay in hypodermal development interferes with the concurrent development of the gonad resulting in a terminal bursting vulva phenotype (Reinhart et al., 2000).

The mature *let-7* miRNA has the characteristic expression profile of being suppressed early in development to then be up regulated in late stages of development. The immature Pri-let-7 mRNA is detectable in the L1, however the expression of mature *let-7* is not seen until the L3. This indicates both a strict transcriptional and post transcriptional regulation of *let-7* expression (Lehrbach et al., 2009; Van Wynsberghe et al., 2011). The expression of *let-7* starts in the L3 stage and gradually increases to maximum expression by the L4 stage and adulthood. Multiple early acting heterochronic genes are involved in the regulation of *let-7* expression. The best known heterochronic gene, *lin-28,* encodes a protein that binds the *let-7* miRNA precursor and blocks its conversion to mature miRNA. Another heterochronic gene, *hbl-1*, encodes an Ikaros family zinc-finger transcription factor that has been shown to delay *let-7* expression by blocking its transcription (Johnson et al., 2003; Roush and Slack, 2009). Another heterochronic gene, *daf-12*, has also been implicated as a negative regulator of *let-7*, it too repressing transcription; however, whether *let-7* is a direct target of DAF-12 activity is unknown (Johnson et al., 2003;

Esquela-Kerscher et al., 2005). It is important to note that, conversely, *lin-28*, *hbl-1*

and *daf-12* are all targets of negative regulation by *let-7*.

3 let-7 **miRNAs**

MicroRNAs are categorized into families based on a shared seed sequence at

their 5' end. There are at least nine members of the *C.elegans let-7* miRNA family. In

addition to *let-7* itself, there are three other miRNAs grouped into the *let-7* family that

act in the heterochronic pathway: *mir-48*, *mir-84* and *mir-241*. Currently, nothing is

known about the expression and regulation of the other five members: *mir-793*,

mir-794, *mir-795*, *mir-1821* and *mir-265* (Roush and Slack, 2008). While the *3 let-7*

miRNAs are part of the same family as *let-7*, they act at a separate time and have no

known overlapping activity with *let-7*. The *3 let-7s* act earlier in the pathway than

let-7, to control the timing of the L2 to the L3 transition (Abbott et al., 2005). The

expression of the *3 let-7s* begins in the L2 stage and act to repress the expression of

lin-28 and *hbl-1*. This down regulation of *lin-28* and *hbl-1* in the L2 stage allows for

developmental progression to the L3 stage.

The *mir-48*, *mir-84* and *mir-241* miRNAs function together to control the L2

to the L3 transition. Animals that are lacking one of the *3 let-7s* have a wild type

phenotype, indicating a functional redundancy in the pathway. Animals lacking two

or three of the *3 let-7* miRNAs display a reiterative phenotype indicating the miRNAs

act cooperatively (Abbot et al., 2005; Vadla et al., 2012; Tsialikas et al., 2017).

Because these miRNAs act in the L2 stage, the characteristic double seam cell

division event that takes place at that stage is repeated resulting in an increased

number of seam cells. Additionally, the majority of *3 let-7s* mutants fail to display

adult specific alae and present a bursting vulva phenotype (Abbot et al., 2005). The

reiterative phenotype displayed by these triple mutants requires HBL-1 activity

(Abbot et al., 2005; Vadla et al., 2012). Most known binding sites of miRNAs reside

in the 3'UTR's of target genes, and both *lin-28* and *hbl-1* contain *let-7*

complementary sites (LeCSs) in their 3'UTR's. To control the events of the second

larval stage *lin-28* acts to block the negative regulation of the *3 let-7s* upon the

3'UTR of *hbl-1* (Vadla et al., 2012).

lin-14

The heterochronic gene *lin-14* (<u>Lin</u>eage abnormal) encodes a DNA binding

transcription factor that promotes the L1 cell fate events as well as the L1 to L2

transition. Ambros and Horvitz (1987) demonstrated that while high levels of LIN-14

were necessary for L1 specific cell fates, low levels of LIN-14 were also needed for

the later developmental stages. These separate activities are genetically separable and

are termed *lin-14a* and *lin-14b*. At the beginning of the L1 stage the high levels of

LIN-14 carry out *lin-14a* activity, promoting the L1 specific cell fates while

preventing L2 specific cell fates. Towards the end of the L1 stage the low levels of

LIN-14 carry out *lin-14b* activity, specifying the double seam cell divisions that occur

in the L2 and preventing the precocious execution of L3 specific cell fates. As the L2 stage progresses, remaining LIN-14 protein is exhausted and it is the absence of the LIN-14 protein that promotes the L3 cell fates (Ambros and Horvitz, 1987).

Animals with null mutations of *lin-14* are precocious, skipping the cell fate developmental events of the L1 stage. *lin-14* null mutants display adult-specific alae a stage early and exhibit a protruding vulva phenotype. Gain-of-function *(gf)* mutations of *lin-14* result in abnormally high LIN-14 protein levels. Animals with *lin-14(gf)* mutations display a reiterative phenotype, repeating the L1 developmental stage. As a result, mutant adults display a reduced number of hypodermal seam cells. Further, these mutants display a cuticle structure similar to that of wild type L1 to L4 larvae rendering these animals unable to lay eggs. Animals with *lin-14(gf)* mutations are also seen to execute supernumerary fifth and sixth molts as well as delayed formation of gapped alae (Ambros Horvitz, 1984; Wightman et al., 1993).

Because specific activities of LIN-14 are dependent on distinct protein levels, the control of *lin-14* expression is highly regulated (Ambros and Horvitz, 1987). At the beginning of the L1 stage the level of LIN-14 protein is high, this elevation is in part due to the activity of *lin-28* (Seggerson et al., 2002). Multiple studies have confirmed that *lin-28* and *lin-14* act in a positive feedback loop. In a *lin-4* null background the removal of either *lin-14* or *lin-28* results in a drop in expression level of the other gene. (Ambros and Horvitz, 1987; Arasu et al., 1991; Moss et al., 1997; Seggerson et al., 2002). As the L1 stage progresses the LIN-14 protein is rapidly

depleted. This reduction in protein level is due to negative regulation exerted by *lin-4*, which represses the translation of *lin-14* through *lin-14*'s 3'UTR (Ruvkun et al., 1989; Ruvkun and Giusto, 1989; Arasu et al., 1991; Ruvkun et al.,1991; Wightman et al.,1991). Concurrently, *lin-4* also post-transcriptionally represses *lin-28* through *lin-28*'s 3'UTR, compounding its effect on *lin-14*. It is this reduced level of LIN-14 that allows for the L2 specific cell fate events to proceed (Arasu et al., 1991; Moss et al., 1997; Seggerson et al., 2002; Wightman et al., 1991; Ruvkun et al., 1991).

hbl-1

The gene *hbl-1* (Hunchback Like-1) encodes an Ikaros family zinc-finger transcription factor, that is homologues to the Drosophila protein *hunchback* (Fay et al., 1999). A zinc-finger is a small peptide domain that contains a secondary structure stabilized by a zinc ion bound to cystine and histidine residues. Zinc-fingers enable proteins to bind specific DNA sequences. The HBL-1 protein is classified as a multiple-adjacent-C2H2-finger protein. This protein class can bind both DNA and proteins (Iuchi, 2001). There are nine zinc-fingers within HBL-1. Six of the nine zinc-fingers of HBL-1 are conserved, four are grouped together in the center of the protein and two are located at the C-terminal end of the protein (Abrahante et al., 2003). It is likely the four clustered zinc-fingers contained within HBL-1 are responsible for binding DNA as is the case for other Ikaros family members. However, HBL-1 is unique to the Ikaros family of proteins in that despite the presence of C-terminal zinc-

fingers it does not bind to itself to form a homodimer (Iuchi, 2001; Kevin Kemper, unpublished).

In the heterochronic pathway, *hbl-1* has been shown to fulfill multiple roles throughout development. In the early stages of development *hbl-1* controls the L2 to L3 transition by promoting L2 cell fates and preventing L3 cell fates. Through positive regulation by *lin-28*, *hbl-1* acts to control the L2 stage double seam cell division event in the hypodermis (Abrahante et al., 2003; Vadla et al., 2012). The *let-7* miRNA promotor contains three highly conserved HBL-1 binding sites. In the hypodermis HBL-1 represses the expression of *let-7* by preventing its transcription in the L1, L2 and early L3 stages (Abrahante et al., 2003; Lin et al., 2003; Roush and Slack, 2009). Additionally, it is in the early part of the L3 stage that *hbl-1* also represses the expression of *lin-29*, a gene required for the larva/adult switch (Ambros and Horvitz, 1984; Bettinger et al., 1997; Rougvie and Moss, 2013; Azzi et al., 2020).

While *hbl-1* plays a significant role in the regulation of post-embryonic developmental timing, it is indispensable for late embryonic development. Embryos lacking HBL-1 activity in late embryogenesis arrest in development; thus null mutations of *hbl-1* are embryonic lethal (Fay et al., 1999). It follows that all animals containing mutant alleles of *hbl-1* that are able to progress though embryogenesis and the four larval stages of development have reduced, but not eliminated, HBL-1 activity.

Both the regulation of *hbl-1* expression and the expression pattern of *hbl-1* is

different in distinct tissues. The expression of *hbl-1* is purported to be up regulated in

the mid-embryonic stage. This coincides with the first observable stages of

morphogenesis of the embryo: dorsal hypodermal intercalation and ventral

hypodermal closure. The expression of *hbl-1* is highest in the 1.5 fold (or tadpole) and

threefold embryonic stages (Fay et al.,1999; Lin et al., 2003). Expression of *hbl-1* in

seam cells is down regulated in the mid-L1 larval stage. However, *hbl-1* expression in

the hypodermis occurs through the L1 and L2 stages, after which it is down regulated

and undetectable by the L4 stage. This downregulation of *hbl-1* in the hypodermis is

mediated post-transcriptionally thorough its 3'UTR which contains negative

regulatory binding sites for the *3 let-7s*. Downregulation of *hbl-1* in other tissues,

such the seam cells, does not rely upon the 3'UTR, indicating an alternate form of

regulation (Fay et al.,1999; Abrahante et al., 2003; Lin et al., 2003; Abbott et al.,

2005; Vadla et al., 2012).

The downregulation of *hbl-1* expression is not only crucial for the transition to

the L3 stage but also for larval entry into the alternate state of dauer diapause. The

mechanism through which this downregulation occurs in continuous development is

different from the mechanism utilized when entering the dauer pathway. Continuous

development relies mainly on the *3 let-7*s targeting the 3'UTR of *hbl-1* for down

regulation of expression (Fay et al.,1999; Abrahante et al., 2003; Lin et al., 2003;

Abbott et al., 2005; Vadla et al., 2012; Ilbay and Ambros, 2019a). Dauer entry relies

on an altered downregulation program in which the influence of the *3 let-7s* is reduced, instead assigning larger roles to *lin-46*, *lin-4* and *nhl-2* (Hammell et al., 2009; Karp and Ambros, 2012; Ilbay and Ambros, 2019a).

In contrast to wild type larvae, larvae grown on *hbl-1* RNAi are paralyzed or nearly paralyzed and have a shorter body length. Larvae grown on *hbl-1* RNAi also display a reduced number of seam cells suggesting some cells skip the double division cell fate pattern. Further, these seam cells are disorganized in that they are unevenly spaced, asymmetrical, and within different focal planes (Fay et al., 1999; Abrahante et al., 2003). Unlike *lin-28* null mutants that skip the L2 double seam cell division event, most seam cells in animals containing *hbl-1* hypomorphic alleles execute this division pattern. Like *lin-28* null mutants, the seam cells of some *hbl-1* hypomorphic allele mutants exit the cell cycle, differentiate and fuse in the L3 molt, resulting in adult alae formation occurring a stage earlier. However, unlike *lin-28* null mutants, the seam cells in *hbl-1* hypomorphic mutants divide again prior to the L4 molt and both sisters remain in the syncytium. At the L4 molt the seam cells then, for a second time, exit the cell cycle, differentiate and fuse to form alae (Abrahante et al., 2003; Lin et al., 2003). Taken together *hbl-1* seems to be required for the organization, specification, and differentiation of seam cells.

lin-46

The clustering of a protein at certain locations can facilitate the permission or repression of its activity. In *C. elegans* scaffolding proteins such as SYD-2 (<u>Sy</u>napse <u>D</u>efective) and UNC-16 (<u>Unc</u>oordinated) are needed for polarized distribution of signaling vesicles along the axons of neurons (Byrd et al., 2001; Goodwin and Juo, 2013). In epithelial cells, proteins LIN-2, LIN-7 and LIN-10 are known to form a complex to bind LET-23 (<u>Let</u>hal) receptor tyrosine kinase, mediating the basolateral localization of LET-23 (Kaech et al., 1998).

The heterochronic gene *lin-46* (<u>Lin</u>eage abnormal) is unique to the *Caenorhabditis* genus of nematode and is predicted to encode a scaffolding protein homologous to proteins such as MoeA in bacteria and Gephyrin in mammals. As mentioned above, heterochronic genes regulate the division pattern of seam cells in the hypodermis. In the L2 stage seam cells undergo a symmetric division followed by an asymmetric division. This switch from symmetric to asymmetric division requires the polarized localization of a protein called POP-1 (<u>Po</u>sterior <u>P</u>harynx defect) to the anterior daughter cells (Lin et al., 1998). The positive regulation of POP-1 activity is in part carried out by LIN-46 (Harandi and Ambros, 2015). Despite its sequence homology, it is not yet known whether LIN-46 possesses MoeA- related enzymatic activity or a scaffolding function (Ilbay and Ambros, 2019b).

The protein LIN-46 is believed to act in the L2, L3 and L4 stages. Null mutations of *lin-46* were found to suppress the precocious heterochronic phenotypes

caused by *lin-28*. *lin-46* null mutants display a weak retarded heterochronic phenotype. Mutants lacking LIN-46 will reiterate the L2 specific double seam cell division event, resulting in an increased number of seam cells; however this reiteration is a cold sensitive phenotype, occurring only at 15ºC. *lin-46* null mutants also display gaps in adult-specific alae due to some seam cells undergoing another round of division instead of differentiation. This gapped alae phenotype is cold sensitive as well, penetrance significantly increasing when grown at 15ºC (Pepper et al., 2004). Together this evidence implicates *lin-46* as a regulator of the L2 to L3 transition.

Very recent studies have shown a negative regulatory relationship between *lin-46* and *hbl-1*. Ilbay and Ambros (2019b) demonstrated that LIN-46 reduced HBL-1 accumulation in the nucleus, although the mechanism of action was not confirmed. Further, they showed *lin-28* is required for the nuclear accumulation of HBL-1, complimenting the additional finding that *lin-28* negatively regulates *lin-46* on a post-translational level (Ilbay and Ambros., 2019b; Ilbay et al., 2021).

daf-12

The *daf-12* (Abnormal <u>da</u>uer <u>f</u>ormation) gene encodes a nuclear receptor and is another heterochronic gene with two separable activities. Through the presence or absence of specific steroidal ligands, *daf-12* is able to either promote and regulate continuous development through the larval stages, or promote entry into the

alternative developmental program of dauer formation (Antebi et al., 1998; Antebi et al., 2000; Motola et al., 2006). In continuous development *daf-12* plays a role in the L2 to L3 cell fate transition. When the ligand dafachronric acid (DA) binds the DAF-12 receptor it terminates the interaction between DAF-12 and the co-repressor DIN-1. DAF-12 is then able to inhibit dauer formation by acting as a transcriptional activator. In continuous development DAF-12 and DA control the L2 to L3 transition by activating transcription of two of the *3 let-7s: mir-84* and *mir241* (Bethke at al., 2009; Hammell at al., 2009). The expression of *daf-12* and the *3 let-7s* is coordinated through a feedback loop, the *3 let-7s* in turn exerting negative regulation of *daf-12* expression. Commitment to the alternative dauer pathway requires the accumulation of a sufficient quantity of DAF-12 that is not bound to ligand. When the majority of DAF-12 receptors are free of ligand DAF-12 remains bound to the DIN-1 co-repressor. DAF-12 then is able to promote dauer formation by acting as a transcriptional repressor (Gerish and Antebi, 2004; Shostack et al., 2004; Antebi et al., 1998; Antebi et al., 2000; Ludewig et al., 2004; Motola et al., 2006; Bethke et al., 2009). In the period before commitment is made, DAF-12 represses the expression of the *3 let-7* miRNAs as they would inhibit the further accumulation of DAF-12. When commitment is finally made DAF-12 acts with DAF-3 and DAF-16 to carry out the alternative *hbl-1* downregulation pathway (Ilbay and Ambros, 2019a).

Mutants of *daf-12* are divided into six classes based on their heterochronic and dauer formation phenotypes. Animals with *daf-12* gain-of-function *(gf)* mutations

display a reiterative phenotype in that the seam cells repeat the second larval stage double division event. Additionally, some of these seam cells then repeat the fourth larval stage division patterns instead of the division pattern consistent with the larval-to-adult switch. This results in the appearance of gaps in the adult-specific alae. These reiterative phenotypes indicated that *daf-12* acts to suppress L2 developmental events and promote L3 events in continuous development. This is further supported by an enhancement of these heterochronic defects in *daf-12; lin-46* double mutants (Antebi et al., 1998; Antebi et al., 2000; Hammell et al., 2009).

The *daf-12* gene also plays a role in gonad development. In the third larval stage *daf-12(gf)* mutants display delayed division of the gonadoblasts which may be a contributing factor to the resulting low brood size characteristic of the mutant. It has also been observed that in the L3 stage, the DTCs of these *daf-12(gf)* mutants deviate from the normal migration pattern (Antebi et al., 1998).

lin-42

The gene *lin-42* is a member of the heterochronic pathway that encodes the worm homologue of the Period (Per) family of proteins which controls circadian rhythm in mammals as well as flies. Unlike the majority of the other heterochronic genes, the expression of *lin-42* is not confined to a particular time in development. While other heterochronic regulators have their expression increased or decreased as development progresses, *lin-42* expression mirrors that of the Per proteins by

oscillating during every larval stage (Jeon et al., 1999). Further, there is an increase in the abundance of mature miRNAs in *lin-42* mutants indicating a role in regulating miRNA biogenesis (Van Wynsberghe et al., 2014). Null *lin-42* mutants display normal seam cell division patterns but at the L3 molt, the seam cells exit the cell cycle, differentiate and produce adult-specific alae precociously. The appearance of precocious alae indicates that *lin-42* promotes L3 cell fates in the hypodermis. However, the oscillatory nature of *lin-42* expression signifies multiple roles for *lin-42* throughout development. Indeed, LIN-42 accumulates in multiple cell types including the vulval precursor cells (VPCs) of the hypodermis and the distal tip cells (DTCs) of the somatic gonad. A portion of animals that lack LIN-42 activity display precocious divisions of the VPCs resulting in a protruding vulval phenotype. Animals grown on *lin-42* RNAi carry out the DTC migration pattern normally executed in the L3 stage precociously in the L2 stage. Epistasis experiments have placed *lin-42* downstream to *lin-46*, parallel to *daf-12* (Tennessen et al., 2006).

ztf-16

The heterochronic gene *ztf-16* (Zinc-finger Transcription Factor) encodes a transcription factor. Often *ztf-16* is referred to as a *hunchback/Ikaros*-like (HIL) gene. HIL genes are C2H2 zinc-finger genes that share organization characteristics similar to those of the hunchback and Ikaros families (Large and Mathies, 2010). Members of the Ikaros family are classified by their unique arrangement of zinc-fingers,

containing four C2H2 zinc-fingers at the center or N-terminus of the protein

important for binding DNA, and two C2H2 zinc-fingers at the C-terminus allowing

for protein-protein interactions (McCarty et al., 2003; Molnar and Georgopoulos,

1994; Sun et al., 1996). In larvae undergoing the alternative developmental pathway

of dauer, *ztf-16* is required for remodeling glia cells associated with the amphid

chemosensory organs (Albert and Riddle, 1983; Procko et al., 2012). The *ztf-16*

mRNA is alternatively spliced to create two protein isoforms, differing only in the

presence or absence of the C-terminal zinc-fingers. Interestingly, ZTF-16 activity in

glia remodeling does not require the C-terminal zinc-fingers (Procko et al., 2012).

The gene *ztf-16* also plays a secondary role in controlling somatic gonadal

precursor (SGP) development. Another HIL gene, *ehn-3* (Enhancer of Hand) and

ztf-16, carry out overlapping roles in the development of SGPs. Both proteins have

functionally distinct zinc-finger domains, and functional differences between the two

are contingent on the N-terminal zinc-fingers. The only gonadal defect observed in

ztf-16 single mutants is an abnormal DTC migration pattern. However, loss of *ztf-16*

enhances defects seen in *ehn-3* mutants. These *ehn-3; ztf-16* double mutants often

lack one or both gonad arms. Further, if the animal does develop a gonadal arm, the

DTCs often have abnormal migration patterns. Some *ehn-3; ztf-16* larvae will display

SGPs with abnormal positions within the gonad primordium. Interestingly, some

ehn-3; ztf-16 larvae with normal SGP positioning still fail to develop two proper

gonad arms. Finally *ehn-3; ztf-16* double mutants containing a GFP reporter specific

for the spermatheca, occasionally only express this reporter in one patch of

spermathecal tissue and this patch would be the same size or smaller than a wild type

spermatheca (Large and Mathies, 2010).

Rationale

  This project began as an effort to understand the genetic and molecular relationship between *lin-28* and *hbl-1*, the two genes with overlapping but distinct roles in *C. elegans* post-embryonic developmental timing. Specific miRNAs and *lin-46* also have roles in this relationship, but at the outset those roles were not clear. In the first part of the project, I attempted to follow up on previous studies that suggested post-transcriptional regulation of *hbl-1* by *lin-28*, possibly through the inhibition of miRNA activity (discussed in Chapter 4). Along the way, while attempting to understand the role *lin-46* plays, I found an unexpected function for these genes in gonad morphogenesis (discussed in Chapter 3). By exploring this new-found phenotype, I was able return to my original question and provide insight on the complex organization of the heterochronic pathway.

Chapter 2: Materials and Methods

Worm strains and culture conditions

Nematodes were grown under standard conditions at 20ºC unless otherwise indicated (Sulston and Horvitz, 1977). Strains with multiple allelic mutations were generated by following lesions via PCR and confirmed through DNA sequencing. Most strains contain the *wIs78* transgene, a derivative of *wIs1*, which contains seam cell nuclei (s*cm::GFP*) and seam cell junction (*ajm-1::GFP*) markers to identify and quantify lateral hypodermal seam cells (Terns et al., 1997). Males for gonad analysis were produced by growing strains on *E.coli* bacteria containing the RNAi plasmid pLT651 (Timmons et al. 2014).

Strains used:

- N2 Wild Type

- RG733: *wIs78 (ajm-1::GFP & scm::GFP)*

- ME414 : *wIs78*; *hbl-1(ae23) X*

- ME418 : *wIs78*; *lin-28(ga54) I*; *hbl-1(ae21) X*

- ME419 : *wIs78*; *lin-28(ga54) I*

- ME420 : *wIs78*; *hbl-1(ae21) X*

- ME423 : *wIs78*; *lin-28(ga54) I*; *hbl-1(ae26) X*

- ME429 : *wIs78*; *hbl-1(ae26) X*

- ME432 : *wIs78*; *lin-28(ga54) I*; *hbl-1(ae29 OLLAS) X*

- ME433 : *wIs78; lin-28(ga54) I; lin-46(ma174) V; hbl-1(ae23) X*

- ME437 : *wIs78; hbl-1(ae29 OLLAS) X*

- ME438 : *wIs78; lin-46(ae30) V*

- ME443 : *wIs78; lin-28(ga54) I; hbl-1(ae23)/tmC30[myo-2p::Venus]X*

- ME452 : *wIs78; lin-28(ga54) I; hbl-1(ve18)/tmC30[myo-2p::Venus]*X

- ME455 : *wIs78; lin-28(ga54) I; qIs56[lag-2p::GFP + unc-119(+)] V; hbl-1(ae23)/ tmC30[myo-2p::Venus]X*

- ME456 : *wIs78; lin-28(ga54) I; ezIs2[fkh-6::GFP + unc-119(+)] III; hbl-1(ae23)/ tmC30[myo-2p::Venus]X*

- ME481 : *wIs78; lin-28(ga54) I; qIs56[lag-2p::GFP + unc-119(+)] V*

- ME485 : *wIs78; lin-28(ga54) I; ezIs2[fkh-6::GFP + unc-119(+)] III*

- ME502 : *cshIs140 [rps-28pro::TIR-1(F79G)_P2A mCherry-His-11; Cbunc-119(+)] II; HBL-1-AID (hbl-1 (aeIS8 (12.4), HBL-1::AID)); wIs78 (pDP#MM016B (unc-119) + pJS191 (AJM-1::GFP + pMF1 (SCM::GFP) + F58E10)*

- ME509 : *wIs78; lin-28(ga54) I; arTi145 [ckb-3p::mCherry::his-58::unc-54 3'UTR] II; hbl-1(ve18)/tmC30[myo-2p::Venus]X*

- ME521: *wIs78; lin-28(ga54) I; cshIs140 [rps-28pro::TIR-1(F79G)_P2A mCherry-His-11; Cbunc-119(+)] II; HBL-1-AID (hbl-1 (aeIS8 (12.4), HBL-1::AID))*

- ME522 : *wIs78; lin-28(ga54) I; lin-46(ma174) V; cshIs140 [rps-28pro::TIR-1(F79G)_P2A mCherry-His-11; Cbunc-119(+)] II; HBL-1-AID (hbl-1 (aeIS8 (12.4), HBL-1::AID))*

- ME524 : *hbl-1(ve18)X; ezIs2[fkh-6::GFP + unc-119(+)] III*

- ME525 : *hbl-1(ve18) X; qIs56[lag-2p::GFP + unc-119(+)] V*

- ME542 : *wIs78; lin-46(ae30) V; hbl-1(ae23) X*

- ME543 : *wIs78; lin-46(ae30) V; hbl-1(ve18) X*

- ME551 : *wIs78; lin-28(ga54) I; lin-46(ma174) V; hbl-1(ve18) X*

Microscopy and phenotype analysis

Nomarski DIC and fluorescence microscopy were used to count seam cell nuclei and score adult alae. Developmental stage was assessed by the extent of the gonad and germ line development. Images were taken with a 100x or 63x objective on a Zeiss Axioplan2 microscope.

Strain Constructions

New alleles of *lin-28*, *lin-46* and *hbl-1* were created using the CRISPR/Cas9 method of genome editing according to standard protocols (Dickinson and Goldstein, 2016). Injections were carried out using the Cas-9 expression vector pDD162 (Goldstein lab, Addgene). The Q5 Site Directed Mutagenesis Kit (New England Biolabs) was employed to insert the guide RNA sequences (Dickinson and Goldstein, 2016). The co-CRISPR technique was used to screen for successful edits (Dickinson

and Goldstein, 2016; El Mouridi et al., 2017). In our application, a second plasmid containing sequence to target the *dpy-5* locus was co-injected with plasmids containing sequence targeting either the *lin-28, lin-46,* or *hbl-1* endogenous loci. F1s were screened for the dumpy and/or heterochronic phenotype. Animals were then cloned and their progeny screened for mutations in *lin-28, lin-46,* or *hbl-1* using PCR and then confirmed through sequencing.

RNA interference

Bacterial-mediated RNA-interference was performed as previously described (Timmons et al., 2001, Sturm et al., 2018). The RNAi vectors used contain a 3.5kb region of *hbl-1* genomic sequence in the T444T backbone (Sturm et al., 2018). Animals were grown on dsRNA expressing bacteria. Bacteria were induced in culture and seeded on nematode growth media (NGM) plates containing 1mM ITPG (GoldBio) and 50μg/ml ampicillin (VWR Life Science) and 12.5 μg/ml tetracycline (FisherBiotech) (Timmons et al., 2001, Sturm et al., 2018).

Auxin Inducible Degron (AID)

The AID-tagged *hbl-1* allele was constructed using the CRISPR/Cas9 system as described above. The gRNA was synthesized using Invitrogen™, MEGAshortscript™ T7 Transcription Kit and the repair template was generated using forward primer dn1439:

ATCAGGCTTTCAATGAGCTAAGCTTTGCTCTCCACATGTAtCAgGCgcGtCAtC

Agatgcctaaagatccagccaaacc

and reverse primer dn1440:

TTAACGAGGACGTCCTCATTATTGGTGTCTGGCTTGGTACTTActtcacgaacgccg

ccgc

The repair templates were mixed and heated to 96ºC for 5 minutes and then placed on

ice (Dokshin et al., 2018). The sequence targeted the 3' end of the *hbl-1* protein

coding region. The guide RNA sequence used targeted the top strand:

CATGTACCAAGCCAGACACC. 3-Indoleacetic acid (5-Ph-IAA) was resuspended

in DMSO to make 10mM stocks (Hills-Muckey et al., 2021). Animals were grown on

two variations of NGM plates. Plates treated with 1μM 5-Ph-IAA (Sigma-Aldrich), or

plates treated with: 1μM 5-Ph-IAA, 1mM ITPG (GoldBio), 50μg/ml ampicillin

(VWR Life Science), and 12.5 μg/ml tetracycline (FisherBiotech).

Yeast Two-Hybrid

Yeast two-hybrid assays were performed as described previously (Golemis et

al., 2001). The *lin-46* open reading frame was fused to the DNA-binding domain in

pMW103. Portions of the *hbl-1* open reading frame were fused to the activation

domain in pJG4-5. These plasmids were co-transformed with the *lacZ* reporter. X-gal

overlays were assessed after 6 hours and overnight.

Yeast Three-Hybrid

Yeast three-hybrid assays were performed using the YBZ-1 strain as described previously (Hook et al., 2005). The *lin-28* open reading frame was fused to the activation domain sequence in pACT2, and experimental RNAs were fused to the MS2 stem loop sequence in pIIIA/MS2-2. X-gal overlays were assessed after 6 hours and overnight.

Chapter 3: Somatic gonad morphogenesis in *C. elegans* requires the heterochronic pathway acting through HBL-1

Abstract

Heterochronic genes determine the timing of cell fates during larval development. The activities of these timing regulators have considerable reach, controlling and synchronizing developmental events occurring in several different tissues. Despite this, the hypodermis has been the fundamental tissue in which the heterochronic pathway has been studied. Consequently, far less is known about the prominence and function of these genes in other tissues. Multiple heterochronic mutants have been shown to influence the development of the somatic gonad. Generally, the individual effects of mutant heterochronic genes on gonad development are minor and often imperceivable during phenotypic analysis. Nevertheless, heterochronic mutants can have a synergistic effect when combined with other mutants known for defective gonad development. Here I show loss of LIN-28 activity with reduced HBL-1 activity causes defective DTC migration as well as defective spermathecal morphogenesis which results in sterility. I demonstrate that the abnormal gonad morphology and novel sterility phenotype is ultimately due to loss of HBL-1 activity in larval development. Moreover, through the enhancement and suppression of this sterility phenotype I was able to define the genetic relationship between *lin-28*, *lin-46* and *hbl-1* in the gonad. I propose that my findings in tandem with the currently accepted relationship between *lin-28*, *lin-46* and *hbl-1* in

the hypodermis collectively establish the somatic gonad as another powerful system with which the heterochronic pathway can be studied.

Introduction

Because *lin-28* and *hbl-1* both control L2 stage-specific events, I attempted to determine whether *lin-28* and *hbl-1* act in a linear pathway to control the timing of hypodermal development or whether the pathway branches. Previous work suggested that *lin-28* positively regulates *hbl-1* expression via *hbl-1*'s 3'UTR (discussed further in Chapter 4). Other results suggested these genes might regulate each other as indicated in **Fig. 1.3**, with *lin-28* repressing *lin-46*, which in turn inhibits *hbl-1*. However, not all existing evidence is consistent with a simple linear pathway which suggests *lin-28* influences L2 events independently of *lin-46*. To deconstruct this pathway a series of double and triple mutants were created. One such strain constructed contained a *lin-28 null* allele and a *hbl-1* hypomorphic allele. This double mutant displayed severe developmental defects of the somatic gonad never before seen in heterochronic mutants. Because heterochronic genes have been found to regulate developmental events in different tissues through the hypodermis, it was plausible the relationship between *lin-28*, *lin-46*, and *hbl-1* could be determined by epistasis analysis of somatic gonad defects.

Results

Both LIN-28 and HBL-1 activity are required for fertility

The genes *lin-28* and *hbl-1* are required for normal L2 development, with

mutations in each causing animals to skip L2-specific events in the hypodermis (Moss

et al., 1997; Abrahante et al., 2003). Previous studies have shown mutations in *lin-28*

cause abnormal development of certain structures of the somatic gonad (Choi and

Ambros, 2019). Mutations in genes causing structural abnormalities that affect

ovulation, fertilization, spermathecal exit and egg laying reduce the overall fertility of

an animal (Kovacevic and Cram, 2010; Choi and Ambros, 2019). To address the

relationship between these two regulators, I constructed a double mutant between a

null allele of *lin-28* and a hypomorphic allele of *hbl-1* that shows only a heterochronic

phenotype (*ve18*) (Abrahante et al., 2003). Animals homozygous for both alleles were

sterile, displayed a "dumpy" morphology, and lacked the characteristic protruding

vulva of each mutant alone (**Fig. 3.1**). Occasionally, some double mutants were able

to produce offspring, but the progeny number was severely reduced relative to the

single mutants alone (**Table 3.1**). However, I could maintain a balanced strain

containing both alleles when *lin-28(ga54)* was homozygous and *hbl-1(ve18)* was

heterozygous. All double mutants characterized were offspring of balanced strains.

Like the *lin-28(ga54)* single mutant, the *lin-28(ga54); hbl-1(ve18)* animals

show a precocious heterochronic phenotype characterized by a reduced number of

seam cells and formation of adult alae at the end of the L3 (**Table 3.1**). The number of
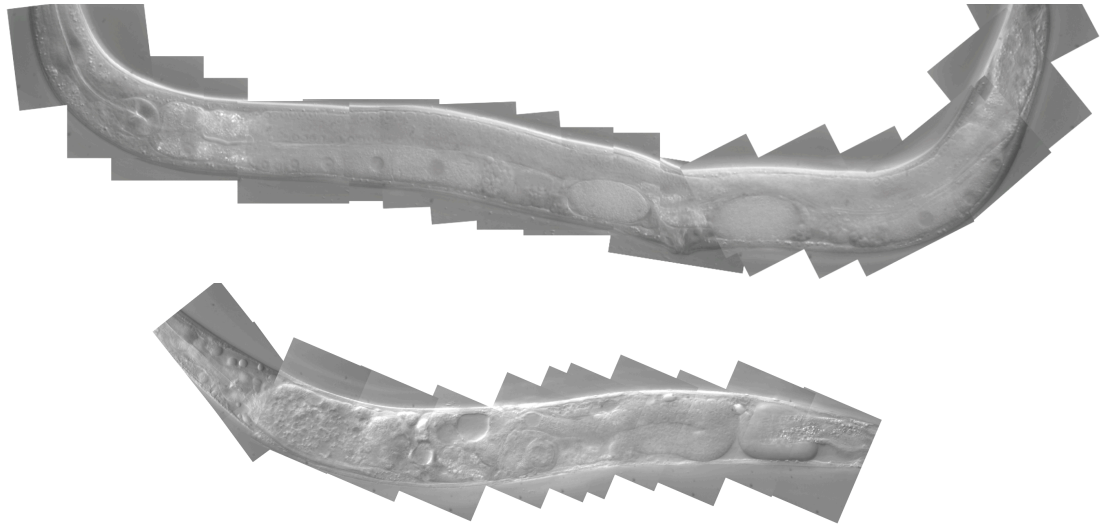
Fig. 3.1 Gross terminal phenotype of *lin-28; hbl-1* double mutant of *C. elegans.* Top, Wildtype *C. elegans* adult with mature oocytes and two eggs in the uterus. Anterior to the left and dorsal up. **Bottom,** a similarly-aged *lin-28(null); hbl-1(hypomorph)* double mutant, displaying an overall "dumpy" morphology, apparent lack of vulva, disorganized gonad, vacuoles, yolk deposits, and sterility.

seam cells in the double mutant is not appreciably reduced compared to either single mutant (**Table 3.1**). Similarly, the precocious alae of the double mutant are the same as that of the single mutant: patches of precocious alae appearing at the L2 molt and full-length adult alae at the L3 molt (**Fig 3.2**). Yet, unlike single mutants the double mutant displays molting defects, becoming trapped in the unshed cuticle. Occasionally, when a few progeny are produced they can be observed trapped in the deceased hermaphrodite's cuticle (**Fig 3.3**). Like the *lin-28* single mutants vulval development begins one stage early, VPCs dividing at the L2 molt. Unlike the *lin-28* null and *hbl-1* hypomorphic alleles, vulva development did not complete and appeared to arrest during morphogenesis. This explains the lack of protruding vulva that is seen in the single mutants where the vulva completes morphogenesis one stage

Table 3.1 Phenotypes of mutant strains

|	Genotype[a,b]	Number of seam cells at adulthood[c]	% fertile	Average brood size of fertile animals[c,d]
1	wild type	16.1 (n = 30)	100	262.7 (n = 10)[+]
2	*lin-28(ga54)*	10.8 (n = 28)	100	20.3 (n = 57)
3	*hbl-1(ve18)*	20.9 (n = 27)	95	48.4 (n = 44)
4	*hbl-1(ae23)*	16.1 (n = 31)	100	248.9 (n = 24)[+]
5	*lin-28(ga54); hbl-1(ve18)*	10.4 (n = 21)	10.6	3.4 (n = 66)
6	*lin-28(ga54); hbl-1(ae23)*	11.1(n = 22)	19	1.92 (n = 68)
7	*lin-28(ga54); hbl-1(ae26)*	11.1(n = 41)	100	24.4 (n = 70)
8	*lin-28(ga54); lin-46(ma174); hbl-1(ve18)*	12.6 (n = 46)	65	4.5 (n = 40)
9	*lin-28(ga54); lin-46(ma174); hbl-1(ae23)*	15.8 (n = 37)	92	10.7 (n = 62)
10	*lin-46(ae30)*	12.1 (n = 31)	100	56.5 (n = 36)
11	*lin-46(ae30); hbl-1(ae23)*	12.2 (n = 34)	100	48.4 (n = 35)
12	*lin-46(ae30); hbl-1(ve18)*	13.5 (n = 35)	82.8	9.6 (n = 35)
13	*hbl-1(aeIS8 AID) w/o auxin*	16.1 (n = 36)	100	323.5 (n = 10)[+]
14	*hbl-1(aeIS8 AID)*	14.1 (n = 36)	100	51.1 (n = 15)
15	*hbl-1(aeIS8 AID) on RNAi w/o auxin*	12.2 (n = 24)	91	12.1 (n = 43)
16	*hbl-1(aeIS8 AID + RNAi)*	11.8 (n = 26)	50	6.9 (n = 36)
17	*lin-28(ga54); hbl-1(aeIS8 AID + RNAi)*	10.9 (n = 39)	0	0 (n = 39)
18	*lin-28(ga54); lin-46(ma174); hbl-1(aeIS8 AID + RNAi)*	13.5 (n = 35)	27	2.9 (n = 30)

[a]All strains examined were homozygous for alleles indicated and carry an integrated transgene *wIs78(ajm-1::GFP +SCMp::GFP)* to mark seam cells.

[b] Strains were maintained at 20ºC.

[c] Number of animals (n) scored for each genotype is indicated in parenthesis.

[d]All strains examined were egg laying defective unless otherwise labeled with "+" superscript denoting wild type egg laying capability.

early (Euling and Ambros, 1996a; Abrahante et al., 2003) (**Fig 3.2**). However, this

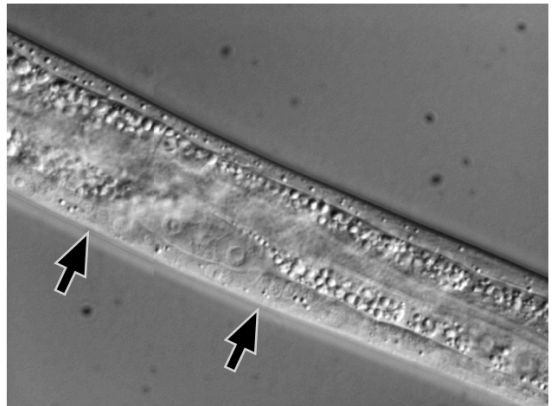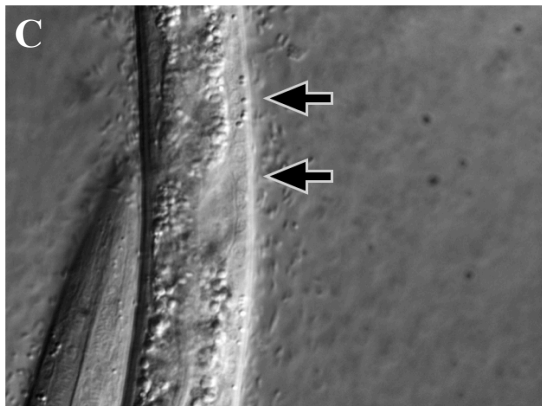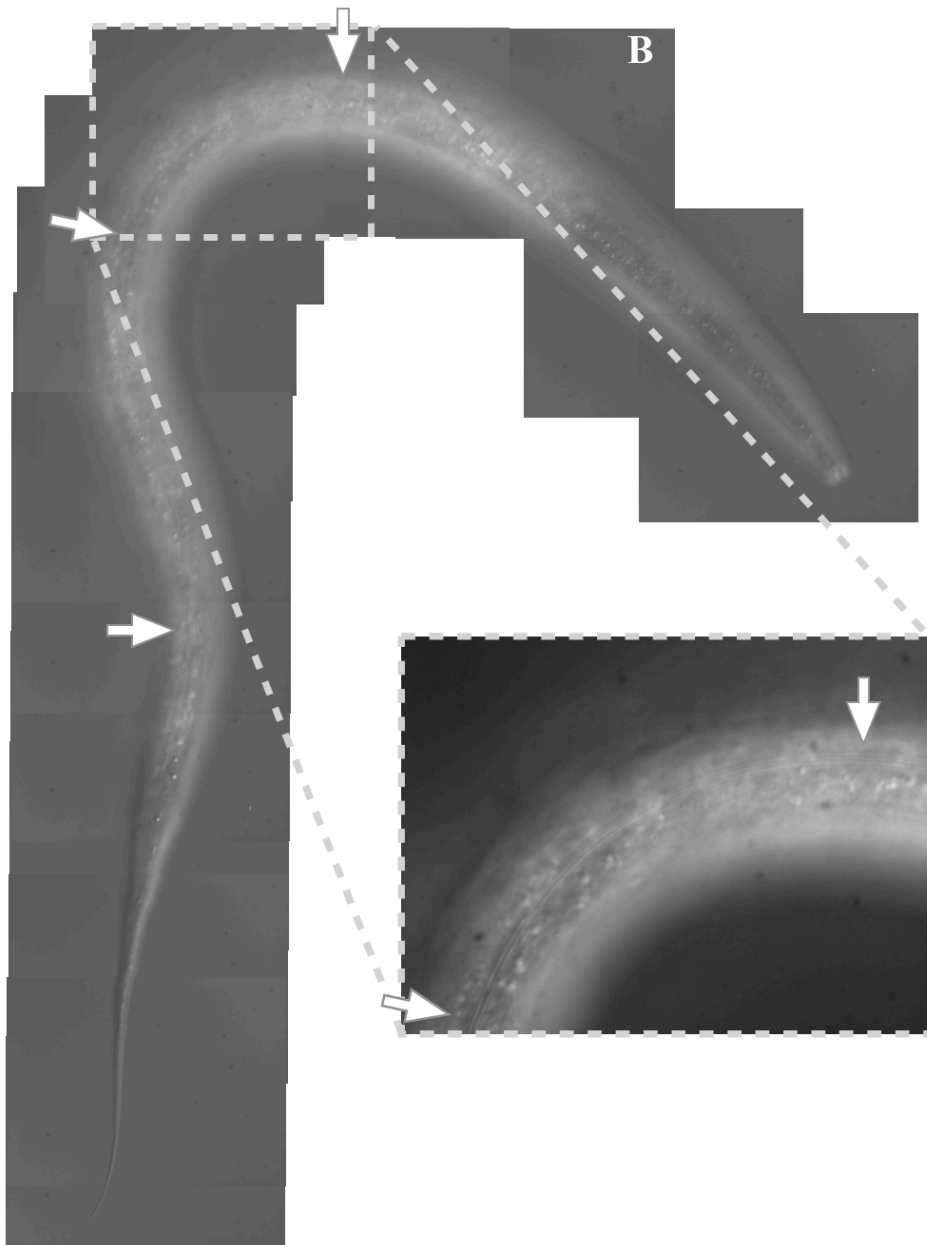arrested development of the vulva was displayed by wild type animals grown on
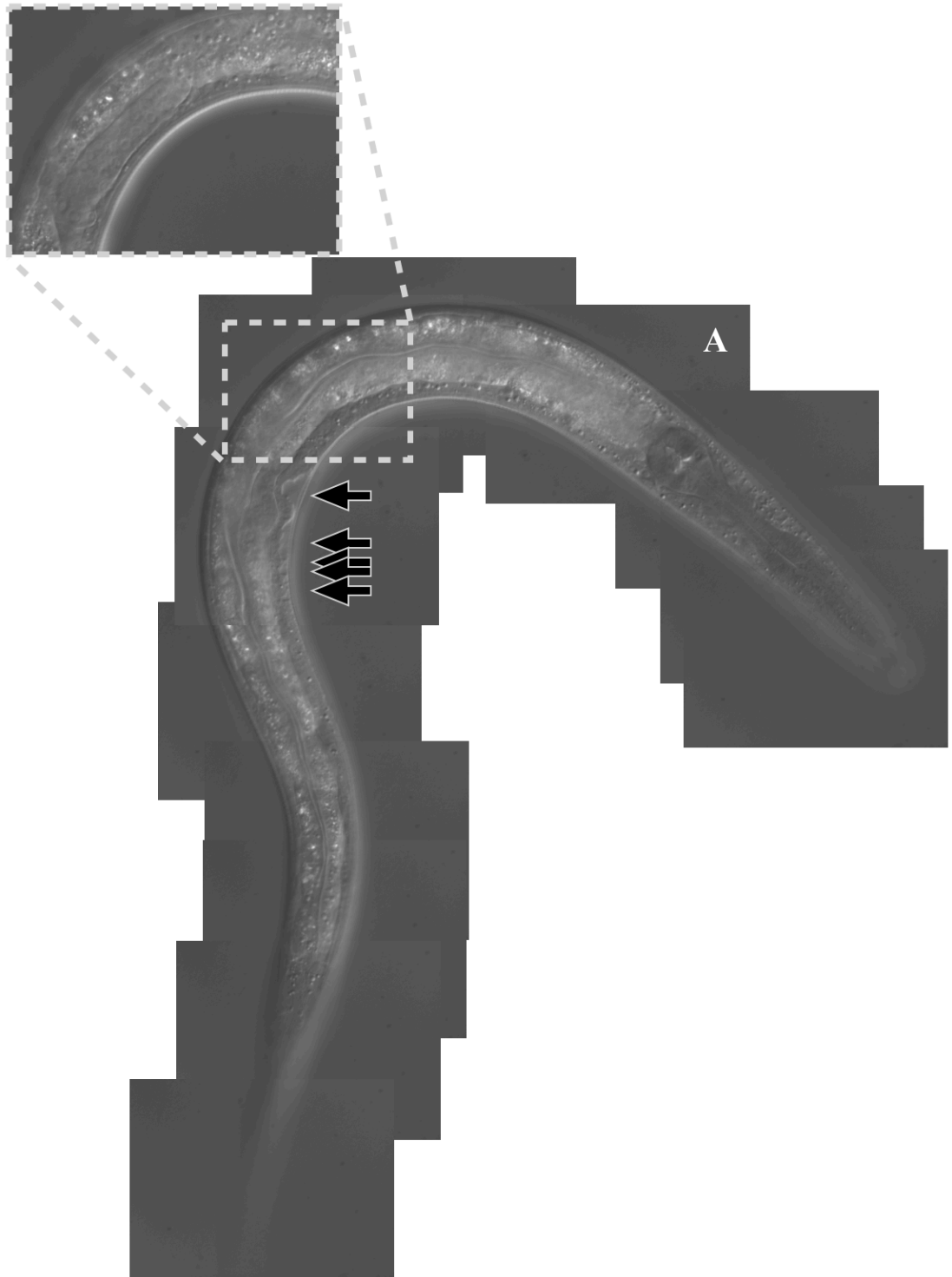
Fig. 3.2 Phenotypes displayed by *lin-28; hbl-1* L2/L3 larvae. A. *lin-28; hbl-1* L3 larvae displaying advanced vulva morphogenesis and abnormal multivulva (Muv) morphology (black arrows). Inset Box showing stage in larval development based on gonad arm position. **B.** *lin-28; hbl-1* L3 larvae displaying full adult-specific lateral alae (white arrows). Inset Box showing magnified view of alae. **C.** Individual *lin-28; hbl-1* larvae at L2 molt displaying precocious VPC divisions (black arrows).

hbl-1 RNAi (Fay et al., 1999). Like the *lin-28* single mutant, formation of one or more pseudovulva was typical, although they too arrested mid morphogenesis. Like the *lin-28* single mutants vulval development begins one stage early, VPCs dividing at the L2 molt. Unlike the *lin-28* null and *hbl-1* hypomorphic alleles, vulva development did not complete and appeared to arrest during morphogenesis. This explains the lack of protruding vulva seen in the single mutants where the vulva completes morphogenesis one stage early (Euling and Ambros, 1996a; Abrahante et al., 2003) (**Fig 3.2**). However, this arrested development of the vulva was displayed by wild type animals grown on *hbl-1* RNAi. Like the *lin-28* single mutant, formation of one or more pseudovulva was typical, although they too arrested mid morphogenesis.

To test whether a milder version of this phenotype might result from using a weaker allele of *hbl-1*, I characterized the phenotype of a double mutant containing *hbl-1(ae23)*. The *hbl-1(ae23)* allele has a small deletion at the 3' end of the open reading frame that disrupts the two C-terminal dimerization zinc fingers of the protein. The *hbl-1(ae23)* allele produces no apparent phenotype on its own. Surprisingly, the *lin-28(ga54); hbl-1(ae23)* double mutant displayed a strong sterility
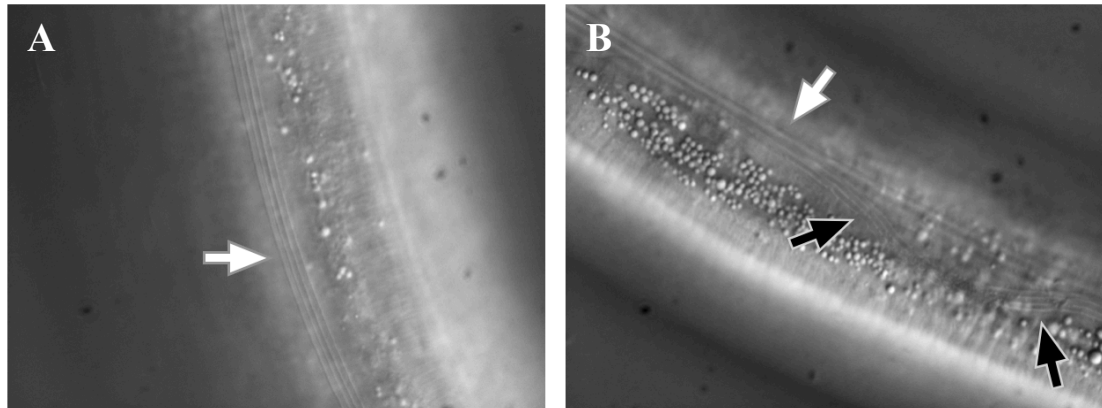
59

Fig. 3.3 Molting defects displayed by *lin-28; hbl-1* mutant. A. Wild type *C. elegans* adult displaying alae on single cuticle (white arrow). **B.** A similarly-aged *lin-28; hbl-1* double mutant displaying alae ridges from older, unshed cuticle (white arrow) as well as a newer cuticle that can be seen underneath (black arrows).

phenotype, albeit slightly less penetrant than that of *lin-28(ga54); hbl-1(ve18)* (**Table 3.1**). For all other traits, including heterochronic phenotype as well as gross body morphology, the *lin-28(ga54); hbl-1(ae23)* double mutant resembled the *lin-28(ga54); hbl-1(ve18)* double mutant.

LIN-28 and HBL-1 are necessary for normal gonad morphogenesis

To explore the basis of this sterility, I examined gonad development in *lin-28; hbl-1* double mutants. The gonad appears normal through the L2 when the first heterochronic defects of these genes appear. Beginning in the L3, defects in gonad development become apparent: the gonad arms do not follow their normal reflexive path to the center of the animal. By the late L4 the malformation of the uterus is observable. By adulthood, the gonads of these animals appeared disorganized with significant accumulation of yolk in the pseudocoelomic space. This pooling of yolk

within the pseudocoelom indicates additional defects in the *lin-28(0); hbl-1(lf)* double mutants. It is likely there are further abnormalities of the somatic gonad in which sheath cells surrounding the proximal gonad are unable to transport yolk from the intestine to the oocytes. It is also possible there is a reduction in oocyte production which would suggest heterochronic genes exert influence on the germ line.

To quantify gonad migration, I used a *lag-2::GFP* reporter which marks the distal tip cells (DTCs) as the gonad grows and migrates during the larval stages (Blelloch et al., 1999). Through the L2, DTC shape, position, and movement appeared normal in *lin-28(0); hbl-1(lf)* animals. Beginning in the L3, when the DTCs normally turn dorsally and reverse their direction along the anterior-posterior axis, the DTCs in *lin-28(0); hbl-1(lf)* animals migrated abnormally, often continuing toward the head or tail, and this wayward migration continues into the L4 (**Fig. 3.4**). This deviation in migration was the first visible defect identifiable in this double mutant's gonad development. Abnormal migration of the DTC of one arm was displayed by 24% of animals, abnormal migration of the DTCs in both arms was displayed by 54% of animals (**Table 3.2**).

The sterility phenotype observed in the double mutants could be due to defects in spermathecal development (Choi and Ambros, 2019). Specifically, I observed that although oocytes are formed in these double mutant animals, few gained entry into the spermatheca. The oocytes that were able to enter the spermatheca were unable to exit the spermatheca. To assess spermathecal development, I examined *l lin-28(0);*
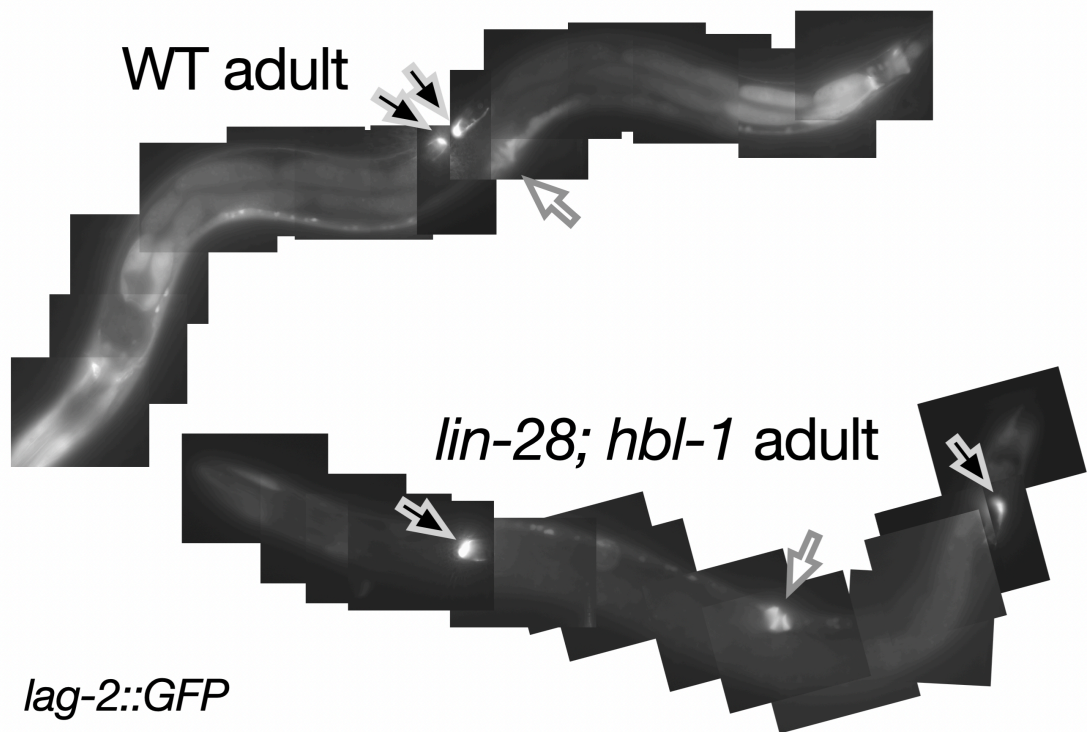
Fig. 3.4 Abnormal DTC migration in *lin-28; hbl-1*. Top, Wildtype *C. elegans* adult expressing *lag-2::GFP*. **Black arrows**, locations of fluorescing distal tip cells (DTCs). **White arrows**, vulva. The DTCs are located near one another on the dorsal side opposite the vulva. **Bottom,** a similarly-aged *lin-28; hbl-1* double mutant, also expressing *lag-2::GFP*. The DTCs are located far from each other near the head and tail.

hbl-1(lf) double mutants using a *fkh-6::GFP* reporter (Chang et al., 2004). This reporter is expressed in the spermatheca as well as spermathecal precursor cells. In wild type animals, the reporter is consistently expressed in both spermathecae and displays the typical tube shape assumed by the spermatheca at the end of development (Chang et al., 2004). The spermathecae were analyzed with both fluorescence and DIC microscopy. In the *lin-28(0); hbl-1(lf)* double mutant, the presentation of the reporter was markedly different in two ways. First, the fluorescent signal was variable, present in either both, one, or neither arms of the gonad (**Table**

Table 3.2 Abnormal DTC migration in single and double mutants

		A	B	C
	Genotype[a]	% normal DTC migration[b,c,d]	% abnormal DTC migration of 1 arm[b,c,d]	% abnormal DTC migration of both arms[b,c,d]
1	wild type	100 (n = 32)	0	0
2	*lin-28(ga54)*	94 (n = 51)	6	0
3	*hbl-1(ve18)*	91 (n = 51)	9	0
4	*lin-28(ga54); hbl-1(ae23)*	22 (n = 41)	24	54

[a] Strains were maintained at 20ºC.

[b] Somatic gonad defects were assessed using DIC and fluorescence microscopy.

[c] Strains examined contain *qIs56* an integrated *lag-2::GFP* reporter.

[d] Number of animals (n) scored for each genotype is indicated in parenthesis.

3.3). As best that could be determined, the variability in reporter expression was not due to reporter failure. Gonad arms that displayed little to no reporter expression were also observed to be abnormal in appearance. The area of the gonad arm at which the spermatheca should be present appeared to resemble that of an earlier stage of development, in which conspicuous features of the spermatheca were not observable. This suggested failure of spermathecal development at an early stage rather than reporter failure. The absence of spermathecae was not seen in either single mutant alone. There are 24 cells that make up the spermatheca, and when the spermatheca did not form, occasionally a few cells (3-12) expressing the reporter could be seen. Second, when a spermatheca did form and expressed the marker throughout, the reporter showed irregular morphology. The reporter frequently formed irregular

Table 3.3 fkh-6::GFP reporter expression in single and double mutants

	Genotype[a]	B % with 2 fluorescent spermathecae[b,c,d]	C % with 1 fluorescent spermatheca[b,c,d]	D % with 0 spermathecae[b,c,d]
1	wild type	100 (n = 63)	0	0
2	*lin-28(ga54)*	100 (n = 62)	0	0
3	*hbl-1(ve18)*	100 (n = 62)	0	0
4	*lin-28(ga54); hbl-1(ae23)*	44 (n = 61)	38	18

[a] Strains were maintained at 20ºC.

[b] Somatic gonad defects were assessed using DIC and fluorescence microscopy.

[c] Strains examined contain *ezIs2* an integrated *fkh-6::GFP* reporter.

[d] Number of animals (n) scored for each genotype is indicated in parenthesis.

shapes instead of the wild type tube shape (**Fig. 3.5**). It sometimes exhibited what looked to be a cross-section of the spermatheca rather than the left or right side, possibly indicating improper orientation of the spermatheca. When the spermatheca did form properly, sperm could be observed within it by adulthood. In most cases, the proximal gonad of adult mutant became clogged with a mass of oocytes and oocyte fragments (**Fig. 3.5**). This observation indicated the primary cause for the failure of fertilization of mature oocytes was defective spermathecal development. In addition to these spermathecal defects, *lin-28(0); hbl-1(lf)* double mutants also displayed malformed uteri. Analysis of vulva morphology by DIC microscopy revealed morphogenesis ceasing after vulval cell invagination but before anchor cell invasion. Consequently, the formation of the uterine seam cell (utse) does not occur.
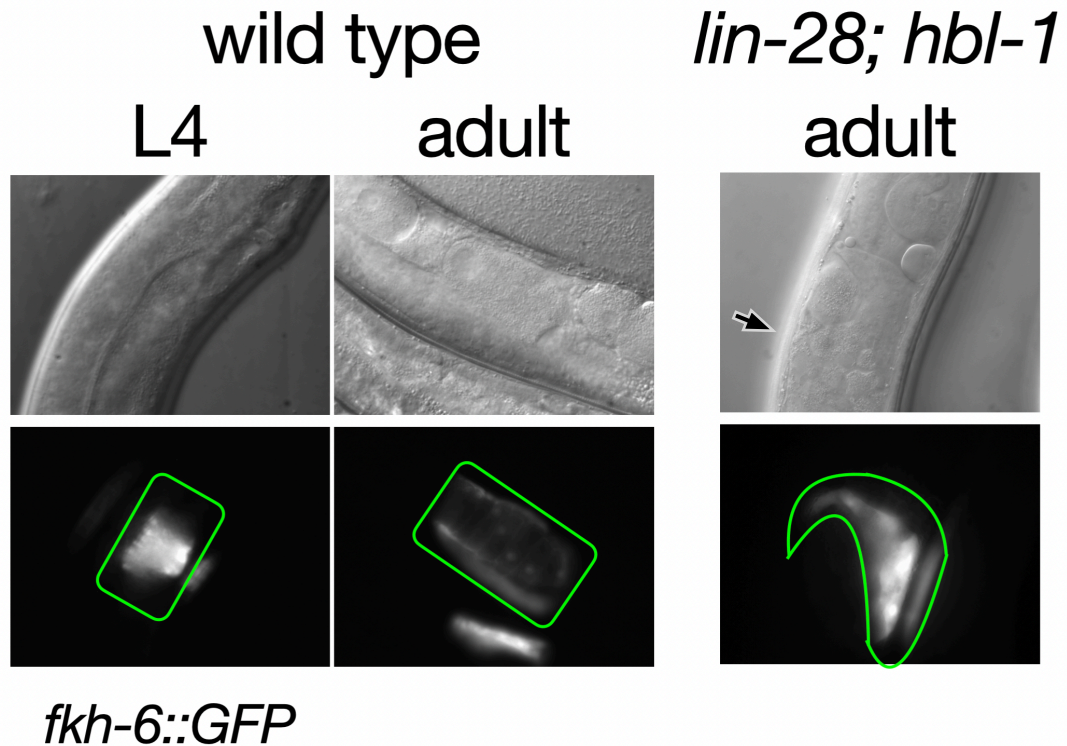
wild type

lin-28; hbl-1

L4   adult   adult

fkh-6::GFP

Fig. 3.5 Abnormal spermatheca formation in *lin-28; hbl-1*. Left, Wild type *C. elegans* L4 and adult expressing *fkh-6::GFP* in the developing and mature spermathecae, respectively. **Right,** adult *lin-28; hbl-1* double mutant showing an incompletely developed and abnormally shaped spermatheca. **Black arrow** indicating mass of oocytes in proximal gonad.

Because *lin-28* and *hbl-1* act during early stages of larval development, I wanted to examine the effect their loss would have on the early stages of somatic gonad development. I used a strain containing the *ckb-3p::mCherry::H2B* reporter, which is expressed in the daughter cells of Z1 and Z4 in the first and second larval stage (Tenen and Greenwald, 2019). I compared the number of mCherry labeled cells in the first and second larval stages of wild type animals to *lin-28(0); hbl-1(lf)* double mutant and found no change. While I was not able to visualize a cell lineage defect in these particular cells of the somatic gonad, it is possible the lineage defect occurs in

other cells formed in the early larval stages or the defect simply occurs at a later developmental stage. It is also possible the defect affects the inherent identity of certain cells. This would not effect cell number or location initially but morphological defects would result later in development.

Given that the loss of both *lin-28* and *hbl-1* dramatically impacts hermaphrodite somatic gonad development, I reasoned the male somatic gonad might also be affected. To address this question, I generated and examined males of the *lin-28(0); hbl-1(lf)* double mutant strain containing the *lag-2::GFP* reporter, which is expressed in the linker cell that leads gonad migration in males. While the loss of *lin-28* and *hbl-1* activity causes deformities in the male tail due to heterochronic defects, I observed no abnormal gonad migration.

LIN-28 binds lin-46's 5'UTR, and LIN-46 binds two of HBL-1's zinc fingers

To gain an understanding of the relationship between *lin-28* and *hbl-1*, I characterized the mechanistic relationships of *lin-46* to *lin-28* as well as *lin-46* to *hbl-1*. By certain genetic criteria, *lin-46* appears to act downstream of *lin-28* and upstream of *hbl-1* (Pepper et al., 2004; Abbott et al., 2005). *lin-46* null mutations display an incompletely penetrant reiterative phenotype, and *lin-28* and *lin-46* mutations suppress each other, suggesting either that *lin-28* acts partly through regulation of *lin-46* or that they act in parallel (Pepper et al., 2004). A recent study showed that mutations in *lin-46*'s 5'UTR cause a phenotype similar to *lin-28* loss-of-function, showing that the RNA-binding protein LIN-28 may act at least in part by

directly regulating *lin-46* expression (Ilbay et al., 2021). This 35-nucleotide 5' UTR is conserved among nematodes but shows no secondary structure motifs or sequence similarity to other LIN-28 targets.

First, I performed a yeast three hybrid assay to test whether LIN-28 directly interacts with *lin-46* mRNA. I found that LIN-28 bound the 35-nucleotide wild type *lin-46* 5'UTR sequence, but not to the mutant 5'UTR of allele *ae30*, which contains a 6-nucleotide deletion (**Table 3.4**). *lin-46(ae30)* is one of several lesions in the gene's 5'UTR that cause a *lin-28(0)*-like phenotype (**Table 3.1** and E. Moss, unpublished). The fact that mutations in the *lin-28* ORF and in the *lin-46* 5'UTR cause similar precocious phenotypes suggests that LIN-28 negatively regulates *lin-46* expression through direct binding (Ilbay et al., 2021).

The *lin-46* gene encodes an unusual protein whose distant relatives are involved in diverse biological activities, although its mechanism of action in *C. elegans* isn't known (Pepper et al., 2004). Ilbay and Ambros showed that *lin-46* mutations affect the nuclear localization of the transcription factor HBL-1 in lateral hypodermal seam cells (Ilbay and Ambros, 2019b). To understand LIN-46 protein function, I investigated its interaction with HBL-1. Previous work in the lab, using a yeast two-hybrid screen and GST pull-down, identified HBL-1 as a LIN-46-interacting protein (Kevin Kemper, unpublished). HBL-1 is a Hunchback-like Ikaros family member with nine zinc-fingers (Abrahante et al., 2003). Several of these are believed to be involved in DNA binding as they are in other family members (Iuchi,

Table 3.4 Interaction of LIN-28 protein with lin-46 5'UTR

Protein	RNA	Interaction	Sequence*
LIN-28	SL1+*lin-46* 5'UTR	+++	`GUAAAACCAAGAAUUGUAUCAGUGGGAGUCAAUCCAAUG`
IRP	SL1+*lin-46* 5'UTR	–	`GUAAAACCAAGAAUUGUAUCAGUGGGAGUCAAUCCAAUG`
LIN-28	SL1+*lin-46(ae30)* 5'UTR	–	`GUAAAACCAAGAAUU------GUGGGAGUCAAUCCAAUG`
LIN-28	SL1+*lin-46(ae31)* 5'UTR	+/–	`GUAAA--------------AGUGGGAGUCAAUCCAAUG`
LIN-28	SL1+*lin-46(ae40)* 5'UTR	+++	`GUAAAACCAAGAAUUGUGGGAGUGGGAGUCAAUCCAAUG`
LIN-28	*lin-46* 5'UTR only	+++	`GUAAAACCAAGAAUUGUAUCAGUGGGAGUCAAUCCAAUG`
IRP	*lin-46* 5'UTR only	–	`GUAAAACCAAGAAUUGUAUCAGUGGGAGUCAAUCCAAUG`
LIN-28	*lin-46* 3'UTR	–	`CGUGGUUGAUCUACGCUUUGCAUGA...153nt...AAA`

* SL1 (trans-spliced leader) sequence not shown

IRP, Iron Regulatory Protein (negative control)

+++, X-gal blue after ≤ 6 hr at 30°

+/–, X-gal pale blue after 24 hr at 30°

–, X-gal white after 24 hr at 30°

2001). The Ikaros family is characterized by two C-terminal zinc-fingers involved in

homo- and heterodimerization (McCarty et al., 2003). I mapped the LIN-46-

interacting portions of HBL-1 using a yeast two-hybrid assay and found that LIN-46

interacted only with segments of the protein containing either zinc-fingers 5 or 9 (**Fig.**

3.5). The 5th Zinc-finger of HBL-1 is highly conserved among its relatives, and is

likely involved in DNA binding, whereas the 9th zinc-finger is variable in sequence

among relatives. Interestingly, HBL-1 is unique in the Ikaros family in that it does not
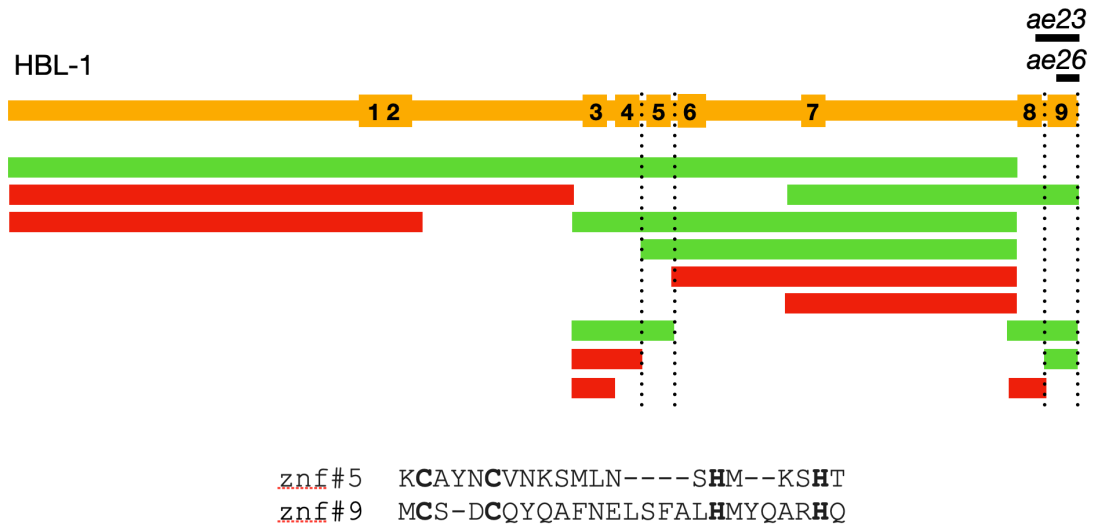
Fig. 3.5 Interaction of HBL-1 with LIN-46. **Top,** A schematic of the HBL-1 protein sequence showing the locations of 9 zinc fingers and indicating the portion missing in alleles *ae23* and *ae26*. **Green,** Sequence fragments that show a strong positive interaction with LIN-46 in a yeast two-hybrid assay. **Red,** Fragments that show no interaction with LIN-46. **Bottom,** The sequences of zinc fingers 5 and 9 aligned by their cystine and histidine residues showing their dissimilarity.

homodimerize via its C-terminal zinc-fingers in in vitro assays (McCarty et al., 2003; Giesecke et al., 2006; Kevin Kemper, unpublished). Notably, the allele *hbl-1(ae23)*, which lacks the C-terminal zinc-fingers, has no noticeable phenotype on its own (**Table 3.1**). Because *hbl-1(ae23)* does not have a phenotype resembling *lin-46* mutations, LIN-46 binding to zinc-finger 5 appears to be sufficient to inhibit HBL-1 activity.

lin-28 acts both through lin-46 and independently of lin-46 to affect gonad

development

Because the sterility of the *lin-28(0); hbl-1(lf)* double mutant was unlike either mutant alone, it is possible that loss of *lin-28* further reduced the activity of a *hbl-1* hypomorphic allele. Previous work from the lab suggested that *lin-28* might act via *hbl-1*'s 3'UTR (Vadla et al. 2012) (discussed further in Chapter 4). However, because the results presented above suggest *lin-28* regulates *hbl-1* via *lin-46*, I characterized *lin-46*'s role with respect to the gonad phenotype of *lin-28; hbl-1* double mutants by genetic analysis. Specifically, I sought to determine whether *lin-28* acts entirely or in part through *lin-46* to regulate *hbl-1*.

As mentioned above *lin-46*, like *lin-28*, acts in the L2 stage. Null mutations of these genes cause opposite heterochronic defects; *lin-28(0)* causing a precocious phenotype and *lin-46(0)* causing a reiterative phenotype. However there is a difference in the penetrance of their effects indicating the genes impose different levels of influence. Still, a null allele of *lin-46* fully suppresses the heterochronic phenotypes of a *lin-28* null allele. This mutual suppression indicates that *lin-28* and *lin-46* do not act in a simple linear pathway but instead act on a common factor. (Pepper et al., 2005). Additionally, alleles of *lin-46* are not able to suppress *hbl-1* hypomorphic allele phenotypes which resemble those of *lin-28* null mutants (Pepper et al., 2004; Abbott et al., 2005). To test whether *lin-46* null would suppress the sterility phenotype of the *lin-28(null); hbl-1(hypomorph)* double mutant, I generated a *lin-28(null); lin-46(null); hbl-1(hypomorph)* strain. The sterility phenotype was partially rescued in this strain (**Table 3.1**). Suppression occurred with both weak and

strong *hbl-1* alleles, although fertility was not fully restored for either. The brood sizes of fertile triple mutants were similar to those seen in *lin-28* and *hbl-1* single mutants.

The *lin-46(ae30)* allele, which has a 6-bp deletion in its 5'UTR preventing LIN-28 binding (**Table 3.4**) produces a precocious phenotype similar to a *lin-28* null allele (**Table 3.1**). If *lin-28* supports *hbl-1* expression or activity via *lin-46,* then *lin-46(ae30)* when combined with a *hbl-1* hypomorphic allele should cause sterility. Surprisingly, a slight increase in sterility was seen in the *lin-46(ae30); hbl-1(ve18)* double mutant in comparison to *hbl-1(ve18)* alone, with 83% of the *lin-46(ae30); hbl-1(ve18)* double mutants being fertile. However, no reduction in fertility was seen in the double mutant containing *lin-46(ae30)* and the weaker *hbl-1(ae23)* allele, unlike the *lin-28(0); hbl-1(ae23)* double mutant (**Table 3.1**). This suggests that a significant fraction of *lin-28*'s affect on *hbl-1* occurs independently of *lin-46*.

HBL-1 activity is necessary for gonad development

Because a null allele of *hbl-1* is embryonic lethal, it was difficult to directly address whether *lin-28* and *hbl-1* act redundantly or in a linear pathway to affect gonad development. To resolve this issue, I supplied HBL-1 during embryogenesis, removed HBL-1 activity during larval development and then analyzed gonad development. To control when HBL-1 is active, I utilized the auxin-inducible degron (AID) system where the addition of the plant hormone auxin to nematode growth

media causes *in vivo* degradation of proteins containing the auxin-binding degron (Zhang et al., 2015).

In collaboration with Maria Ivanova, I generated strains that contained endogenous *hbl-1* locus tagged at its C-terminus with the degron sequence (*hbl-1::AID*). On auxin-containing media, these animals displayed a phenotype like that of the *hbl-1(ve18)* allele where HBL-1 activity is reduced. To maximally reduce *hbl-1* activity, we grew *hbl-1::AID* animals on media containing auxin and *E. coli* expressing *hbl-1* dsRNA (*hbl-1* RNAi). Eggs from this strain were transferred onto auxin-RNAi plates at the threefold stage of development and grown to adulthood. Under these conditions, I observed that 50% of the *hbl-1::AID* animals displayed a sterility phenotype with fertile animals producing a reduced number of offspring (**Table 3.1**). Many sterile animals of adult age were found to display abnormal gonad morphology with no fertilized embryos present. Removal of *lin-28* from *hbl-1::AID* animals reared on *hbl-1 RNAi* and auxin increased the severity of the sterility defect (**Table 3.1**). Nevertheless, these observations support a model whereby *lin-28* regulates the expression and/or activity of *hbl-1*, and *hbl-1* is the proximal gene responsible for normal gonad development.

Conclusions

When combining alleles of *lin-28* and *hbl-1* that on their own cause heterochronic phenotypes that skip events of the second larval stage in the

hypodermis, I discovered an unexpected level of sterility due to abnormal gonad growth and development. In particular, the formation of the spermatheca and uterus of the hermaphrodite is severely abnormal. The migration pattern of *lin-28(0); hbl-1(lf)* double mutant DTCs in later stages of development is also affected. Single mutants of *lin-28* and *hbl-1* consistently display the FKH-6::GFP reporter indicative of normal spermathecal formation. However a small percentage of single mutants display abnormal migration of DTCs. This demonstrates the effects of *lin-28* and *hbl-1* are separable, providing strong evidence that the heterochronic pathway controls more than one aspect of somatic gonad development.

It is difficult to determine whether cell lineage transformations analogous to the seam cell fate transformations occurred. Rather, it seems that morphogenesis depends on these genes. Initial gonad growth and development of the double mutant through the L2 is normal. The first major defect occurs when the distal tip cells (DTCs) migrate aberrantly at the start of the L3. This indicates that *lin-28* and *hbl-1* act to control hypodermal development and somatic gonad development in the L2 larval stage. This leads me to speculate that the hypodermis may be compromised by the severity of effects of combining *lin-28* and *hbl-1* mutations. Loss of both LIN-28 and HBL-1 activity may cause malformation of the basement membrane resulting in abnormal DTC migration. Loss of LIN-28 and HBL-1 activity may also result in loss of an intracellular signal sent from the hypodermis to the somatic gonad to control spermathecal development.

The relative roles of *lin-28*, *hbl-1*, and *lin-46* in gonad development seem to reflect their roles in hypodermal developmental timing. Genetic analyses indicate that the multiple factors of the heterochronic pathway converge onto *hbl-1*, which is seemingly the most proximal acting gene involved in controlling seam cell fate events of the L2 stage. My data indicate that sterility is the true null phenotype of *hbl-1* during larval development. Additionally, it suggests *lin-46* is only able to suppress the somatic gonad defect in the *lin-28(0); hbl-1(lf)* mutants due to *hbl-1* hypomorphic alleles producing some functional protein. This designates *hbl-1* as the most proximal acting heterochronic gene for controlling somatic gonad development.

Null mutations of *lin-46* are able to suppress heterochronic defects seen in *lin-28* null mutants. The mutual suppression of their heterochronic phenotypes implies they affect a common target. While early and late heterochronic defects in *lin-28(0)* animals are equal in penetrance, the early and late defects in *lin-46(0)* animals are unequal in penetrance. This relative penetrance of *lin-46(0)* defects indicates that its influence on cell fate is uneven with *lin-28*'s influence on cell fate. Thus, even though there is mutual suppression between the genes, they do not act in a linear pathway. My data further support this forked pathway model by the rescue of sterility in *lin-28(0); hbl-1(lf)* mutants by a *lin-46* null mutation. While *lin-46* is able to restore fertility to a degree, it is not able to completely suppress the sterility phenotype. Taken together this evidence indicates that *lin-28* is partially acting

through *lin-46* to regulate *hbl-1* but also utilizing other mechanisms independent of *lin-46*.

Finally, it is important to note the complete loss of LIN-28 activity does not result in sterility. Null mutants of *lin-28* display only weak somatic gonad defects. This demonstrates that complete loss of LIN-28 is not sufficient to eliminate HBL-1 activity. Therefore, there must be another gene also acting to positively regulate *hbl-1* to control somatic gonad development.

Chapter 4: Removal of *hbl-1 3'UTR* is insufficient to abolish negative regulation of *hbl-1* expression in seam cells

Abstract

Two early acting heterochronic genes *lin-28* and *hbl-1*, function to control cell

fate events of the L2 developmental stage in *C.elegans*. The gene *hbl-1* is the most

proximal regulator, controlling the double division event of hypodermal seam cells in

the L2 stage. Evidence suggests *lin-28* positively regulates *hbl-1* expression in

opposition to the *3 let-7* negative regulatory miRNAs. I show that removal of the

hbl-1 3'UTR results in its constitutive expression and yields a mutant with a

reiterative phenotype. This further supports the idea that the *3 let-7* miRNAs act

within the *hbl-1* 3'UTR to repress its expression in the L2 stage. Surprisingly, the loss

of LIN-28 activity in mutants with lesions in the *hbl-1* 3'UTR results in a precocious

phenotype. I propose that *lin-28* negatively regulates an additional factor in the

heterochronic pathway; it is this factor that remains to repress *hbl-1* in *lin-28(0)*;

hbl-1(Δ 3'UTR) double mutants. Analyses of the genetic interactions of *lin-28* and

hbl-1 within the somatic gonad suggest *lin-46* is the remaining factor negatively

regulating *hbl-1*.

Introduction

Proper development requires the precise control of gene expression. Too much

or not enough, early or late, will result in defects that can change the morphology and

overall robustness of the animal. Permutations in the expression of heterochronic

genes, at the very least, cause changes in the physiology of the animal, and at most, cause fatality. Animals lacking LIN-28 activity skip cell fate events characteristic of the L2 developmental stage resulting in a precocious cell differentiation (Ambros and Horvitz, 1981; Ambros and Horvitz, 1987). In the heterochronic pathway multiple transcription factors play the role of gate-keeper for cells to transition from early to late stage fates. Specifically, the transcription factor *hbl-1* acts to control the transition of seam cell fates from the L2 to L3 stage (Abrahante et al., 2003; Lin et al., 2003). Vadla et al. (2012) previously found that the 3'UTR of *hbl-1* is a target of the *3 let-7* miRNAs. Further, they found that the RNA-binding protein LIN-28 positively regulated the expression of a transgene containing the 3'UTR of *hbl-1*. Analysis by our lab and others have shown that *hbl-1* has approximately ten to fifteen *let-7* complementary sites (LeCSs) in its 3'UTR. In light of this evidence, it is plausible that *lin-28* could be acting in opposition to the down regulation imposed on *hbl-1* by the *3 let-7*s through a post-translational mechanism (Ilbay and Ambros, 2019b).

Results

Partial deletion of the hbl-1 3'UTR is not sufficient for eliminating negative regulation of hbl-1

To determine if *lin-28* is acting through the 3'UTR of *hbl-1*, I created a lesion in endogenous *hbl-1* to remove the LeCSs from its 3'UTR. This modification should prevent the *3 let-7s* from binding the regulatory sites of the *hbl-1* 3'UTR and allow
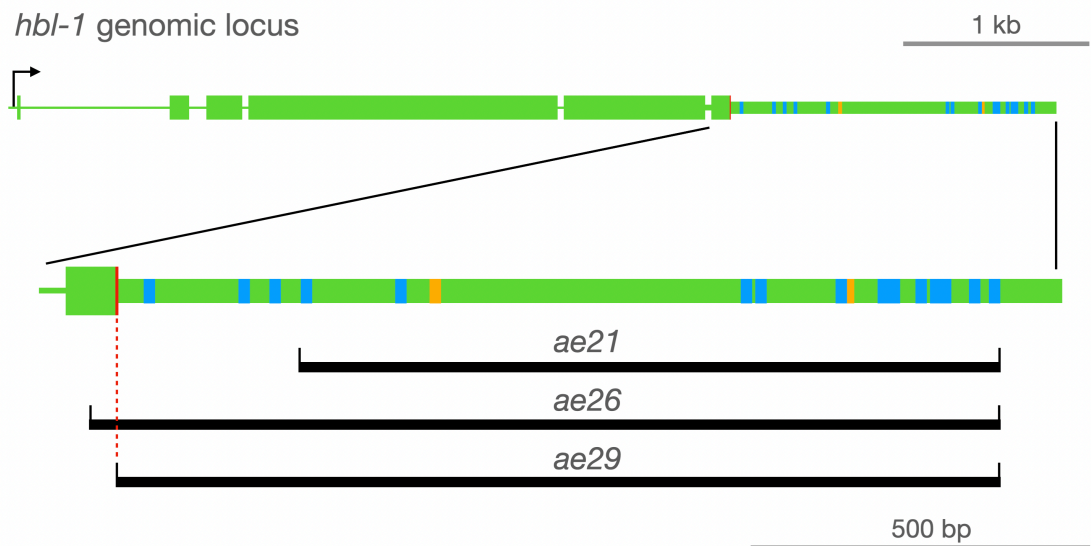
Fig. 4.1 3'UTR deletions of *hbl-1*. Top, A schematic of the *hbl-1* genomic locus showing the locations of the transcription start site, exons, and 3'UTR (narrower green box). Scale bar, upper right. **Bottom,** The *hbl-1* 3'UTR with the stop codon in red, let-7 sites in blue, and lin-4 sites in orange. The extent of the deletion alleles *ae21*, *ae26*, and *ae29* are shown. *ae26* deletes part of the open reading frame encoding HBL-1's non-essential C-terminal zinc fingers 8 and 9. *ae29* is a precise deletion after the stop codon. All deletions retain the normal poly(A) addition signal.

increased *hbl-1* expression. First, a lesion that deleted 12 of the 15 predicted LeCSs from the 3'UTR of *hbl-1* was made, *hbl-1(ae21)* (**Fig. 4.1**). Mutants with the *hbl-1(ae21)* allele display a reiterative phenotype of an increased number of seam cells at adulthood. This phenotype is indicative of L2 larval stage events being repeated (**Table 4.1**). In addition, these animals often burst at the vulva at the transition to adulthood or shortly after, indicative of delayed vulva morphogenesis.

To determine whether an activity of *lin-28* is to oppose the negative regulation of the *3 let-7s* on the 3'UTR of *hbl-1*, I performed epistasis analysis on a double mutant containing a null allele of *lin-28(ga54)*, and the 3'UTR deletion mutant

Table 4.1 Phenotypes of hbl-1 3'UTR deletion mutants and double mutants

	Genotype[a,b]	Number of seam cells at adulthood[c]
1	wild type	16.1 (n = 30)
2	*lin-28(ga54)*	10.8 (n = 28)
3	*hbl-1(ve18)*	20.9 (n = 27)
4	*hbl-1(ae23)*[d]	16.0 (n = 31)
5	*hbl-1(ae21)*	22.6 (n = 32)
6	*lin-28(ga54); hbl-1(ae21)*	11.0 (n = 37)
7	*hbl-1(ae26)*	25.5 (n = 38)
8	*lin-28(ga54); hbl-1(ae26)*	11.1 (n = 41)
9	*hbl-1(ae29)*	25.7 (n = 35)
10	*lin-28(ga54); hbl-1(ae29)*	11.0 (n = 40)

[a]All strains examined were homozygous for alleles indicated and carry an integrated transgene *wIs78(ajm-1::GFP +SCMp::GFP)* to mark seam cells.

[b] Strains were maintained at 20ºC.

[c] Number of animals (n) scored for each genotype is indicated in parenthesis.

[d] *hbl-1(ae23)* allele has lesion removing C-terminal dimerization fingers.

hbl-1(ae21). If both positive and negative regulation by *lin-28* and the *3 let-7s*, respectfully, is occurring solely through the 3'UTR of *hbl-1*, then the double mutant should also display a reiterative phenotype. This is due to the fact that while there is no LIN-28 to enact positive regulation on *hbl-1* expression, there is also no negative regulation by the *3 let-7s* due to their inability to bind the *hbl-1* mRNA lacking LeCSs in the 3'UTR. This combined loss of regulation would result in conditions allowing for the constitutive expression of *hbl-1*. Surprisingly, the *lin-28(ga54); hbl-1(ae21)* double mutant surprisingly displays a precocious phenotype of a reduced number of seam cells at adulthood mimicking the phenotype displayed by *lin-28(ga54)* single mutants (**Table 4.1**). A possible explanation of this result lies in nature of the *hbl-1* 3'UTR lesion. While the *hbl-1(ae21)* lesion removes the majority of LeCSs in the

3'UTR, there are still three LeCSs remaining (**Fig. 4.1**). It is possible that these remaining LeCSs were sufficient for the *3 let-7s* to bind and down regulate *hbl-1* expression. If this were the case, the incomplete deletion of LeCSs could mask the regulatory interactions between *lin-28*, *hbl-1* and the *3 let-7s*.

Full deletion of the hbl-1 3'UTR is not sufficient for eliminating negative regulation of hbl-1

Due to the uncertainty that the *hbl-1(ae21)* allele generated *hbl-1* mRNA invulnerable to negative regulation through the 3'UTR, a second *hbl-1* 3'UTR deletion allele was made, *hbl-1(ae26)*, in which all the LeCSs were removed. This second lesion also removed a small, C-terminal portion, of the HBL-1 protein (**Fig. 4.1**). The lab previously constructed a *hbl-1(ae23)* allele in which the C-terminal dimerization zinc-fingers of the HBL-1 protein were absent (**Fig. 4.1**). Animals containing the this allele are essentially wild type in continuous development. Because *hbl-1(ae23)* has a wild type hypodermal phenotype, the *hbl-1(ae26)* allele was regarded as valid lesion with which results could be interpreted (**Table 4.1**). Animals with the *hbl-1(ae26)* allele display a reiterative phenotype of an increased number of seam cells at adulthood (**Table 4.1**).

The *hbl-1(ae26)* allele was then combined with the *lin-28(ga54)* null allele to create a double mutant. The *lin-28(ga54); hbl-1(ae26)* double mutant displays a precocious phenotype of low seam cell number at adulthood (**Table 4.1**). This result supported the possibility that the current understanding of the relationship between

lin-28, *hbl-1* and the *3 let-7s* was either incorrect or incomplete. However, another possible explanation was that the loss of a small portion of the HBL-1 C-terminus reduced its activity. While this possibility is not supported by the observations made of the *hbl-1(ae23)* allele, if protein function was compromised, removal of negative regulation through the 3' UTR would have little to no affect. If this was the case for the *hbl-1(ae26)* allele, that would again mask the regulatory interactions between *lin-28*, *hbl-1* and the *3 let-7s*.

Because of the concern that removal of a small, C-terminal portion, of the HBL-1 protein reduced its activity, a final lesion in the 3'UTR of *hbl-1* was made. The *hbl-1(ae29)* lesion removed all 15 LeCSs while leaving the protein coding region unmodified (**Fig. 4.1**). Animals with the *hbl-1(ae29)* allele also display a reiterative phenotype of an increased number of seam cells at adulthood (**Table 4.1**). It is worth noting that while 12 of the 15 LeCSs were removed in the *hbl-1(ae21)* allele, the *hbl-1(ae29)* allele did show a slight increase in seam cell number. This suggests that the remaining LeCSs in the *hbl-1(ae21)* allele impacted the regulation of *hbl-1* expression.

The *lin-28(ga54); hbl-1(ae29)* double mutant was created and epistasis analysis performed. Remarkably, the *lin-28(ga54); hbl-1(ae29)* double mutants display a precocious phenotype of low seam cell number (**Table 4.1**). This final result suggests that our understanding of the relationship between *lin-28*, *hbl-1* and the *3 let-7* miRNAs is incomplete. It is still possible that *lin-28* does regulate *hbl-1* through

its 3'UTR but in a *lin-28* null background, the magnitude of this effect is not enough to cause an observable phenotypic change. These results support the idea that another factor carries out additional negative regulation of *hbl-1* expression in the L2 stage.

Conclusions

Deletion of the *hbl-1* 3'UTR creates a gain-of-function phenotype indicative of a reduction in negative regulation of expression. Surprisingly, removal of *lin-28* in a *hbl-1(gf)* mutant decreased HBL-1 activity. Further, in contrast to my analyses of *hbl-1* activity in the somatic gonad, it does not seem to require its C-terminal zinc-fingers to fulfill its activity in the seam cells. It is likely that the C-terminal zinc-fingers of *hbl-1* play a role in modulating HBL-1 protein activity. While I am unable to draw a definitive conclusion from the results of my epistasis analysis of seam cell phenotypes, the data do not refute my current model. Given the global impact of *hbl-1* disregulation, both post-transcriptional and post-translational control of *hbl-1* would be expedient for robust regulation of its expression. My results support a pathway in which *hbl-1* is regulated in opposite ways. In the first, the *3 let-7s* act at the *hbl-1* 3'UTR to inhibit HBL-1 protein synthesis. In the second, *lin-28* indirectly increases HBL-1 activity. Results from my analysis of somatic gonad development suggests *lin-46* plays a role in this post-translational regulation.

Chapter 5: Summary and Conclusions

The LIN-28 protein has both an early and late activity necessary for proper development in *C. elegans* (Vadla et al., 2012). The late activity, regulation of *let-7* expression, as well as the mechanism by which it carries out this activity is highly conserved in both worms and humans. The early activity in which *lin-28* controls seam cell fate of the L2 developmental stage does not require *let-7*. Precisely how *lin-28* controls the L2 cell fate event, until recently, was somewhat unclear. Analysis of *hbl-1* expression through the use of transgenes suggested positive regulation of *hbl-1* by *lin-28* through the *hbl-1* 3'UTR. However the ambiguous results of epistasis analysis, presented above, of seam cell phenotype call this conclusion into question. Thus *lin-28* is likely exerting positive regulation of *hbl-1* through some other mechanism. Ilbay and Ambros (2019b) recently demonstrated that *lin-28* negatively regulates *lin-46,* which in turn negatively regulates *hbl-1*. This result provides strong evidence that *lin-28* acts solely through *lin-46*. However, if *lin-28* acts solely through *lin-46*, then why are *lin-46(ae30); hbl-1(lf)* double mutants fertile when *lin-28(0); hbl-1(lf)* double mutants are sterile?

My analysis of the relationship between *lin-28*, *lin-46* and *hbl-1* in the somatic gonad reinforces the existence of a second mechanism for positive regulation of *hbl-1* by *lin-28*. As shown above, I was able to identify two distinct zinc-fingers of the HBL-1 protein that LIN-46 is able to bind. One of these zinc-fingers is zinc-finger number nine, which is one in a pair of dimerization zinc-fingers located at the very C-

terminus of the protein. The *hbl-1(ae23)* allele does not contain zinc-finger number nine and single mutants are fertile (**Fig. 3.5**). This *hbl-1(ae23)* allele in combination with a *lin-28* null allele results in a sterile animal. The *hbl-1(ae26)* gain-of-function allele does not contain any miRNA binding sites in the 3'UTR nor does it contain zinc-finger number nine (**Fig. 3.5**). Single *hbl-1(ae26)* mutants are fertile. This *hbl-1(ae26)* allele in combination with a *lin-28* null allele results in a fertile animal. The key difference between the two double mutants is the presence of the *hbl-1* 3'UTR in the sterile *lin-28(0); hbl-1(ae23)* double mutant and the absence of the *hbl-1* 3'UTR in the fertile *lin-28(0); hbl-1(ae26)* double mutant. Removing the negative regulation of *hbl-1* exerted by the *3 let-7s* allows for an increase in HBL-1 activity, rescuing the sterility phenotype.

The loss of *hbl-1* activity may produce a cumulative effect as development progresses due to pleiotropic nature of HBL-1. This would explain the disparity in the effect of *hbl-1* loss in the hypodermis versus in the gonad. Because of the relative simplicity of hypodermal development, it is possible that moderate disregulation of *hbl-1* is not enough to cause an observable phenotype if there is also disregulation of other heterochronic genes. The development of the gonad is considerably more complicated, requiring the coordination of multiple tissues and cell types, allowing for disregulation of *hbl-1* to have a much larger downstream effect. In this manner, it is important to recognize that while the constitutive expression of *hbl-1* causes defective development of the hypodermis, it does not affect somatic gonad

development. This suggests that for somatic gonad development, it is not so much the timing of *hbl-1* expression but the occurrence of *hbl-1* expression that is important. Further, the threshold for the level of HBL-1 activity required for proper somatic gonad development is ostensibly lower than the level of activity required by the hypodermis. Thus, even though the defects caused by disregulation of *hbl-1* in the hypodermis are less severe than those seen in the somatic gonad, the hypodermis is more sensitive to disregulation of *hbl-1* than the somatic gonad.

Future analyses should also attempt to determine if there is variation in the influences of the different regulatory mechanisms utilized by *lin-28*. It is possible *lin-28* may rely more heavily on a certain mechanism depending on the tissue type. This could also help elucidate if there are other developmental regulators involved in the developmental pathway. Future analyses should also attempt to determine where this *hbl-1* activity is needed in order for somatic gonad development to progress properly.

Attempting to define the genetic relationships among *lin-28* and *hbl-1* through the development of the hypodermis alone was unsuccessful. However, I found that gonad development could be used as a tool to analyze the heterochronic pathway. By studying the developing gonad and observing defects caused by *lin-28*, *lin-46* and *hbl-1*, I was able to further distinguish their relationships. This new means of analysis allows for further study of their genetic relationships and possible expansion of the pathway.

The spermathecal defects that result from *hbl-1* depletion indicate normal development of the somatic gonad relies on proper temporal control of development of the hypodermis. As discussed in the introduction, heterochronic genes *let-7* and *lin-29* are able to control processes in the intestine through their activity in the hypodermis. Also, LIN-28 activity in the hypodermis has been shown to regulate spermathecal-uterine valve formation in the somatic gonad (Choi, 2018). It is very possible that *hbl-1* may regulate somatic gonad development from the hypodermis in a non-autonomous manner. Previous studies provide strong support for this mechanism of regulation. The *sym-1* gene encodes a protein secreted from the hypodermis. This gene has been shown to be a downstream target of *hbl-1* in controlling temporal hypodermal differentiation (Niwa et al., 2009). Additionally, SYM-1 has also been shown to aid in anchoring muscle to the extracellular cuticle in embryonic development (Davies et al. 1999).

A simple model of this mechanism could be *hbl-1* as an upstream regulator of a metalloprotease which acts to remodel the basement membrane of the hypodermis for somatic gonad development. An appropriate example of somatic gonad regulation through gene expression in a different tissue is the expression of *gon-1* in muscle tissue. Mutations in the *gon-1* (abnormal Gonad development) gene, which encodes a metalloprotease, cause some shared phenotypic defects (Blelloch et. al., 1999). Null *gon-1* mutants have a similar phenotype to that seen in the *lin-28; hbl-1* double mutant. Animals containing *gon-1* mutations display DTC migration defects as well

as gonadal morphogenesis defects such as failing to properly form the uterus

(Blelloch and Kimble, 1999; Blelloch et. al., 1999). Additionally *gon-1* has been

shown to be expressed in both the DTCs and the body wall muscle. The main activity

of GON-1 in both tissues is to direct and permit the growth and expansion of the

somatic gonad. Expression of *gon-1* in the DTCs is responsible for DTC migration

creating the characteristic U shape of the gonad arms. Expression of *gon-1* in the

muscle is responsible for growth and expansion of the gonad within the basement

membrane. Importantly, *gon-1* is expressed in the muscle to then be secreted. This

allows GON-1 to act over a distance on the somatic gonad in a non-autonomous

manner. (Blelloch and Kimble, 1999). It is possible that the activity of a

metalloprotease regulated by *hbl-1* not only allows for DTC movement but also

creates the proper environment for intertissue signaling as well as space for new

tissue growth and morphogenesis (Blelloch et. al., 1999).

This mechanism of intercellular signaling utilized by *hbl-1* could give an

indication as to possible targets for the regulation of spermathecal development in the

somatic gonad. A strong candidate for a down stream target of *hbl-1* is *nhr-6* (<u>N</u>uclear

<u>h</u>ormone <u>r</u>eceptor family). The gene *nhr-6* encodes the sole *C. elegans* homolog of

the NR4A subgroup of nuclear receptor transcription factors (Gissendanner et al.,

2008). Nuclear receptors are able to regulate gene expression in response to a wide

variety of intercellular and intracellular signals (Mangelsdorf et al. 1995). Contrary to

most nuclear receptors, NR4A nuclear receptors are not ligand regulated, instead

controlling cellular processes though signal transduction pathways (Pekarsky et al., 2001; Maira et al., 2003; Wang et al., 2003; Wingate et al., 2006). In mammals, the NR4A subgroup has been shown to play important roles in developmental regulation such as tissue differentiation and organogenesis (Castro et al., 2001; Zeng et al., 2006). In *C.elegans nhr-6* has been shown to be required for proper morphogenesis of the spermatheca. Null *nhr-6* mutants display abnormal spermatheca morphology resulting in low progeny number. In addition to disorganization of the spermathecal cells, *nhr-6(0)* mutants display around half the number of spermathecal cells in comparison to wild type (Gissendanner et al., 2008). Further, recent studies have shown the reduced number of spermathecal cells in *nhr-6(0)* mutants is the result of a cell lineage defect in spermathecal precursor cells. In wild type animals during the L3 molt, four spermathecal precursor cells are produced; one large distal daughter cell and one smaller proximal daughter cell per gonad arm. In the early L4 stage the large and small spermathecal precursor cells undergo rapid division creating twelve cells. In *nhr-6* null mutants, frequently the smaller proximal daughter spermathecal precursor cells do not divide. Occasionally, the large distal daughter and the smaller proximal daughter will divide once then cease divisions (Praslicka and Gissendanner, 2015). The spermathecal phenotype observed in the *lin-28(0);hbl-1(lf)* double mutants seemingly phenocopy *nhr-6(0)* mutants. The stage in development at which these division defects are occurring in *nhr-6* mutants is also the same stage at which developmental defects are first observed in *lin-28(0);hbl-1(lf)* double mutants.

Further, this would also explain why no cell lineage defects were observed in the

lin-28(0);hbl-1(lf) double mutants at the L1 and L2 stages.

Because the consequences of *hbl-1* disregulation are so extensive, it stands to

reason that there are redundancies built into the heterochronic pathway to ensure strict

regulation of *hbl-1* expression. Several experiments suggest that *lin-28* implements

two mechanisms to promote *hbl-1* expression: opposing *3 let-7s* activity on the *hbl-1*

3'UTR and negative regulation of *lin-46* expression by binding its 5'UTR. The

possibility of a third mechanism is further compounded when considering the nature

of *lin-28*, *lin-46* and *hbl-1*. The gene *lin-46* is specific to *Caenorhabditis* genus of

nematodes while *lin-28* and *hbl-1* are conserved in many species. It is therefore not

unreasonable to speculate that a third mechanism of *lin-28* would target another

conserved developmental regulator. To address this possibility, multiple approaches

can be taken. The most straightforward of approaches is to carry out further epistasis

experiments. It is possible that other genes already associated with the heterochronic

pathway are involved in gonad development as well. There are a few strong gene

candidates that are already known be associated with *lin-28,* the *3 let-7s* and *hbl-1*,

these include *lin-14*, *nhl-1*, and *cgh-1*.

The heterochronic gene *lin-14* is a compelling candidate for regulation by

lin-28. As stated previously, *lin-28* and *lin-14* are known to positively regulate each

other via a feedback loop in the L1 developmental stage. It is possible that the

regulation of *lin-14* by *lin-28* continues into the early L2 stage. As mentioned above,

lin-14 has two genetically separable activities that are dosage dependent and by the late L1 stage the level of LIN-14 protein is significantly reduced. Though diminished, it was demonstrated that this this low protein level allows for the second of LIN-14's activities, which is the continuation of the positive feedback loop with *lin-28* (Ambros and Horvitz, 1987; Seggerson et al., 2002). Loss-of-function (*lf*) alleles of *lin-14b* have been observed to cause L2 cell fates to be skipped, supporting the hypothesis that *lin-14* does play some role in L2 cell fate regulation (Ambros and Horvitz, 1987). This premise is also supported by previous work demonstrating that a *lin-46(lf)* allele is able to suppress *lin-14* hypomorphic alleles but not *lin-14* null alleles. This suggests that *lin-46* is only able to achieve suppression when some level of LIN-14 activity is occurring (Pepper et al., 2004). Further, our lab has previously determined that *lin-14* acts to inhibit the transcription of *mir-48* and *mir-84* of the 3 *let-7* miRNA family as well as *mir-237* the only other known *lin-4* miRNA in *C.elegans* (Tsialikas et al., 2017). Given this evidence, it is then plausible that a further reduced, yet not depleted, LIN-14 protein level present at the beginning of the L2 stage is able to exercise a low level of activity exerting weak positive regulation of *hbl-1*. Because it seems the abundance of HBL-1 protein must reach an extremely low level in order for sterility to occur, it could very well be possible that a small, secondary boost from *lin-14* activity might just be enough, in combination with *lin-28* activity, to elevate HBL-1 over a threshold, allowing for fertility to be restored in an animal.

A second promising candidate for regulation by *lin-28* is *nhl-1* (<u>NHL</u> (ring finger b-box coiled coil) domain containing). The *nhl-1* gene encodes a *C. elegans* TRIM-NHL protein. Null alleles of *nhl-1* cause a weak penetrance of the reiterative heterochronic phenotype. Only around 3% of animals display an increased number of seam cells and failure to produce complete adult-specific hypodermal alae. However, null alleles of *nhl-1* enhance the reiterative defects caused by null alleles of the individual *3 let-7* miRNAs. The depletion of HBL-1 through *hbl-1* RNAi is able to suppress these reiterative defects seen in the *nhl-1; 3 let-7s* double mutant. Further, the null allele of *nhl-1* enhances the reiterative phenotype of a hypomorphic allele of *let-7* (Hammell et al., 2009).

The *cgh-1* (<u>C</u>onserved germline <u>h</u>elicase) gene is another candidate for regulation by *lin-28*. The aforementioned NHL-1 protein, along with interacting with core miRISC components, physically interacts with the dead box protein encoded by *cgh-1* (Hammell et al., 2009). A supposed null allele of *cgh-1* causes an animal to display sterility as well as abnormal oocytes and sperm at adulthood (Navarro et al., 2001; Boag et al., 2005). Similarly to *nhl-1*, *cgh-1* null alleles also cause a weak penetrance of a reiterative heterochronic phenotype with only 3% of animals producing complete adult-specific hypodermal alae. Likewise, *cgh-1* null alleles were shown to not only enhance the reiterative heterochronic defects caused by individual loss-of-function alleles of *mir-48* and *mir-84* (of the *3 let-7s*) but enhance the reiterative defects seen in the *mir-48(0); mir-84(0)* double mutant as well as. *cgh-1*

91

null alleles enhanced the increased seam cell number, the defects in adult-specific alae formation, as well as the defective expression of adult specific genes. It is important to note that inhibition of HBL-1 activity through *hbl-1* RNAi suppresses the reiterative defects displayed in the *cgh-1(0); mir-48(0); mir-84(0)* triple mutant (Hammell et al., 2009). This result places *cgh-1* upstream of *hbl-1*.

Taken together *nhl-1* and *cgh-1* are believed to promote miRNA activity by acting with or in parallel to the *3 let-7s* in the heterochronic pathway. In further support of their possible significance to the genes of interest is the observation that depletion of CGH-1 activity in a *nhl-1* null single mutant causes a stronger reiterative phenotype than that seen in the *nhl-1* null mutant alone. As a result, *nhl-1(0)* mutants on *cgh-1* RNAi exhibit HBL-1 expression inappropriately in the L3 developmental stage. It is also of significance that 40% of *cgh-1(0); nhl-1(0)* double mutants were found to expire during embryogenesis or early larval development (Hammell et al., 2009). Because *nhl-1* and *cgh-1* have been shown to help with the negative regulation of *hbl*-1 they should not be ignored as possible targets of *lin-28* regulation.

To test these gene candidates, double and triple mutants could be made by combining the candidate genes with *lin-28* and *hbl-1* single mutants as well as the *lin-28; hbl-1* double mutant. Epistasis analysis could then be performed to look for both enhancers of the sterility defect as well as possible suppressors of the sterility defect. If none of the apparent candidates prove fruitful, a genetic screen looking for

suppressors or enhancers of the double mutant may help discern new candidates that were previously overlooked.

Further analysis of what structures are defective in the double mutant may also help identify new factors in the pathway. A null mutation of *lin-28* was shown to affect brood size not only as a result of desynchronization of gonad and vulva development, but also due to a resulting malformation of the spermathecal uterine valve (Choi and Ambrose, 2019). Our analysis of what causes the sterility defect in the double mutant focused on a possible cell lineage defect of gonad precursor cells (GPC) in the second larval stage. It was hypothesized that a potential spermathecal precursor cell was not produced during the L2 stage and thus all of its decedents that go on to form parts of the spermatheca would not be properly formed. My analysis of GPCs in both the L1 and L2 stages did not reveal a cell lineage defect, all GPCs appeared to be present at the proper time. Although I did not detect a cell lineage defect in early stages it is possible a cell lineage defect could still occur at a later stage. There is also the possibility that a cell fate defect occurs instead of a cell lineage defect. A cell fate defect would also result in an abnormal morphogenesis of the somatic gonad. In the future a more comprehensive and thorough analysis of the cell lineage of the somatic gonad in the *lin-28; hbl-1* mutants could be done. This could establish not only if there is a cell lineage defect, but also what structures formed by the somatic gonad are most affected with the loss of HBL-1 activity. A

better understanding of the morphogenesis defects of the spermatheca could also help distinguish possible candidates for the regulation of gonadal development.

The role of *hbl-1* in vulval development should should not be overlooked. Multiple heterochronic genes have been found to affect the timing and efficacy of vulval development. *lin-12* encodes a Notch protein receptor and is fundamental for proper vulval development. LIN-12/Notch activity specifies cell fate of VPCs. *lin-12* is expressed in the L2 stage to specify the anchor cell/ventral uterine (AC/VU) cell fate decision. Though LIN-12 is constitutively active during this time, LIN-12 does not specify 2º VPC cell fate until the L3 stage (Greenwald et al., 1983; Levitan and Greenwald, 1998). Li and Greenwald (2010) found LIN-12/Notch activity to be inhibited by LIN-14, and only when *lin-14* is down regulated by *lin-4* can *lin-12/ Notch* signaling be initiated. Further, the genetic relationship between heterochronic genes have been studied previously through vulval phenotypes. As mentioned previously, *let-7* null mutants have a terminal bursting vulva phenotype. Ecsedi et al. (2015) found *let-7* directs vulval development through multiple targets, the most important of which is *lin-41*, which is both necessary and sufficient for the bursting vulva phenotype. Further they found that *let-7* activity is needed in both hypodermal and vulval tissues in order to prevent vulval bursting (Ecsedi et al., 2015). These examples validate a comprehensive study of vulval development in *hbl-1* mutants as a possible next step in clarifying its genetic relationships.

Presently, analysis of development in other tissues is the most powerful method to determine the genetic relationships of *hbl-1*. The expression of *hbl-1* is strictly regulated resulting in the HBL-1 protein have a high rate of turnover. I believe it is because of this rigorous regulation that detection of HBL-1 in larval development has been difficult. The development of more robust fluorescent reporters would allow for better understanding of what tissues express *hbl-1* and at what time this expression occurs. Further, more accurate measurements of HBL-1 protein formation could be taken with the production of new antibodies with higher specificity. These techniques could be utilized in animals of different mutant backgrounds to determine their association with *hbl-1*.

The objective of my project was to determine the genetic relationship among the heterochronic genes *lin-28* and *hbl-1*. I found that loss of HBL-1 activity results in sterility, a phenotype not before associated with heterochronic genes. Using somatic gonad development as a new means of analysis I found that *lin-28* regulation of *hbl-1* is not solely through *lin-46*. *lin-28* circumvents *lin-46* through the regulation of *hbl-1* through its 3'UTR. Whether, there is yet another branch involving another heterochronic gene or an unassociated factor has yet to be determined.

References

Abbott, A.L., Alvarez-Saavedra, E., Miska, E.A., Lau, N.C., Bartel, D.P., Horvitz, H.R., and Ambros, V. (2005). The let-7 MicroRNA Family Members mir-48, mir-84, and mir-241 Function Together to Regulate Developmental Timing in Caenorhabditis elegans. Dev. Cell *9*, 403–414. https://doi.org/10.1016/j.devcel.2005.07.009.

Abrahante, J.E., Daul, A.L., Li, M., Volk, M.L., Tennessen, J.M., Miller, E.A., and Rougvie, A.E. (2003). The Caenorhabditis elegans hunchback-like Gene lin-57/hbl-1 Controls Developmental Time and Is Regulated by MicroRNAs. Dev. Cell *4*, 625–637. https://doi.org/10.1016/S1534-5807(03)00127-8.

Albert, P.S., and Riddle, D.L. (1983). Developmental alterations in sensory neuroanatomy of theCaenorhabditis elegans dauer larva. J. Comp. Neurol. *219*, 461–481. https://doi.org/10.1002/cne.902190407.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in C. elegans. Cell *57*, 49–57. https://doi.org/10.1016/0092-8674(89)90171-2.

Ambros, V., and Horvitz, H.R. (1984). Heterochronic Mutants of the Nematode *Caenorhabditis elegans*. Science *226*, 409–416. https://doi.org/10.1126/science.6494891.

Ambros, V., and Horvitz, H.R. (1987). The lin-14 locus of Caenorhabditis elegans controls the time of expression of specific postembryonic developmental events. Genes Dev. *1*, 398–414. https://doi.org/10.1101/gad.1.4.398.

Ambros, V., and Moss, E.G. (1994). Heterochronic genes and the temporal control of C. elegans development. Trends Genet. *10*, 123–127. https://doi.org/10.1016/0168-9525(94)90213-5.

Angelo, G., and Van Gilst, M.R. (2009). Starvation Protects Germline Stem Cells and Extends Reproductive Longevity in *C. elegans*. Science *326*, 954–958. https://doi.org/10.1126/science.1178343.

Antebi, A., Culotti, J.G., and Hedgecock, E.M. (1998). daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans. Development *125*, 1191–1205. https://doi.org/10.1242/dev.125.7.1191.

Antebi, A., Yeh, W.-H., Tait, D., Hedgecock, E.M., and Riddle, D.L. (2000). *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes Dev. *14*, 1512–1527. https://doi.org/10.1101/gad.14.12.1512.

Arasu, P., Wightman, B., and Ruvkun, G. (1991). Temporal regulation of lin-14 by the antagonistic action of two other heterochronic genes, lin-4 and lin-28. Genes Dev. *5*, 1825–1833. https://doi.org/10.1101/gad.5.10.1825.

Austin, J., and Kimble, J. (1987). glp-1 Is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. Cell *51*, 589–599. https://doi.org/10.1016/0092-8674(87)90128-0.

Avery, L. (2014). A Model of the Effect of Uncertainty on the C elegans L2/L2d Decision. PLoS ONE *9*, e100580. https://doi.org/10.1371/journal.pone.0100580.

Azzi, C., Aeschimann, F., Neagu, A., and Großhans, H. (2020). A branched heterochronic pathway directs juvenile-to-adult transition through two LIN-29 isoforms. ELife *9*, e53387. https://doi.org/10.7554/eLife.53387.

Balzer, E., Heine, C., Jiang, Q., Lee, V.M., and Moss, E.G. (2010). LIN28 alters cell fate succession and acts independently of the *let-7* microRNA during neurogliogenesis in vitro. Development *137*, 891–900. https://doi.org/10.1242/dev.042895.

Baugh, L.R. (2013). To Grow or Not to Grow: Nutritional Control of Development During *Caenorhabditis elegans* L1 Arrest. Genetics *194*, 539–555. https://doi.org/10.1534/genetics.113.150847.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D.J., and Antebi, A. (2009). Nuclear Hormone Receptor Regulation of MicroRNAs Controls Developmental Progression. Science *324*, 95–98. https://doi.org/10.1126/science.1164899.

Bettinger, J.C., Euling, S., and Rougvie, A.E. (1997). The terminal differentiation factor LIN-29 is required for proper vulval morphogenesis and egg laying in Caenorhabditis elegans. Development *124*, 4333–4342. https://doi.org/10.1242/dev.124.21.4333.

Blelloch, R., and Kimble, J. (1999). Control of organ shape by a secreted metalloprotease in the nematode Caenorhabditis elegans. Nature *399*, 586–590. https://doi.org/10.1038/21196.

Blelloch, R., Anna-Arriola, S.S., Gao, D., Li, Y., Hodgkin, J., and Kimble, J. (1999). The gon-1 Gene Is Required for Gonadal Morphogenesis in Caenorhabditis elegans. Dev. Biol. *216*, 382–393. https://doi.org/10.1006/dbio.1999.9491.

Boag, P.R., Nakamura, A., and Blackwell, T.K. (2005). A conserved RNA-protein complex component involved in physiological germline apoptosis regulation in *C. elegans*. Development *132*, 4975–4986. https://doi.org/10.1242/dev.02060.

Brenner, S. (1974). THE GENETICS OF *CAENORHABDITIS ELEGANS*. Genetics *77*, 71–94. https://doi.org/10.1093/genetics/77.1.71.

Byrd, D.T., Kawasaki, M., Walcoff, M., Hisamoto, N., Matsumoto, K., and Jin, Y. (2001). UNC-16, a JNK-Signaling Scaffold Protein, Regulates Vesicle Transport in C. elegans. Neuron *32*, 787–800. https://doi.org/10.1016/S0896-6273(01)00532-3.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev. Biol. *46*, 326–342. https://doi.org/10.1016/0012-1606(75)90109-8.

Castro, D.S., Hermanson, E., Joseph, B., Wallén, Å., Aarnisalo, P., Heller, A., and Perlmann, T. (2001). Induction of Cell Cycle Arrest and Morphological Differentiation by Nurr1 and Retinoids in Dopamine MN9D Cells. J. Biol. Chem. *276*, 43277–43284. https://doi.org/10.1074/jbc.M107013200.

Chalfie, M., Horvitz, H.R., and Sulston, J. (1981). Mutations that lead to reiterations in the cell lineages of C. elegans. Cell *24*, 59–69. https://doi.org/10.1016/0092-8674(81)90501-8.

Chang, W., Tilmann, C., Thoemke, K., Markussen, F.H., Mathies, L., Kimble, J., and Zarkower, D. (2004). A forkhead protein controls sexual identity of the C. elegans male somatic gonad. Development *131*, 1425–1436. https://doi.org/10.1242/dev.01012.

Choi, S. (2018). Investigation of LIN-28 Function in Somatic Gonadal Development and Fertility, and Characterization of the LIN-28 Isoforms in C. elegans Hermaphrodites. University of Massachusetts Medical School.

Choi, S., and Ambros, V. (2019). The *C. elegans* heterochronic gene *lin-28* coordinates the timing of hypodermal and somatic gonadal programs for hermaphrodite reproductive system morphogenesis. Development dev.164293. https://doi.org/10.1242/dev.164293.

Davies, A.G., Spike, C.A., Shaw, J.E., and Herman, R.K. (1999). Functional Overlap Between the mec-8 Gene and Five sym Genes in Caenorhabditis elegans. Genetics *153*, 117–134. https://doi.org/10.1093/genetics/153.1.117.

Dickinson, D.J., and Goldstein, B. (2016). CRISPR-Based Methods for *Caenorhabditis elegans* Genome Engineering. Genetics *202*, 885–901. https://doi.org/10.1534/genetics.115.182162.

Dowen, R.H., Breen, P.C., Tullius, T., Conery, A.L., and Ruvkun, G. (2016). A microRNA program in the *C. elegans* hypodermis couples to intestinal mTORC2/PQM-1 signaling to modulate fat transport. Genes Dev. *30*, 1515–1528. https://doi.org/10.1101/gad.283895.116.

Ecsedi, M., Rausch, M., and Großhans, H. (2015). The let-7 microRNA Directs Vulval Development through a Single Target. Dev. Cell *32*, 335–344. https://doi.org/10.1016/j.devcel.2014.12.018.

El Mouridi, S., Lecroisey, C., Tardy, P., Mercier, M., Leclercq-Blondel, A., Zariohi, N., and Boulin, T. (2017). Reliable CRISPR/Cas9 Genome Engineering in *Caenorhabditis elegans* Using a Single Efficient sgRNA and an Easily Recognizable Phenotype. G3 GenesGenomesGenetics *7*, 1429–1437. https://doi.org/10.1534/g3.117.040824.

Esquela-Kerscher, A., Johnson, S.M., Bai, L., Saito, K., Partridge, J., Reinert, K.L., and Slack, F.J. (2005). Post-embryonic expression of *C. elegans* microRNAs belonging to the *lin-4* and *let-7* families in the hypodermis and the reproductive system. Dev. Dyn. *234*, 868–877. https://doi.org/10.1002/dvdy.20572.

Euling, S., and Ambros, V. (1996a). Heterochronic Genes Control Cell Cycle Progress and Developmental Competence of C. elegans Vulva Precursor Cells. Cell *84*, 667–676. https://doi.org/10.1016/S0092-8674(00)81045-4.

Euling, S., and Ambros, V. (1996b). Reversal of cell fate determination in Caenorhabditis elegans vulval development. Development *122*, 2507–2515. https://doi.org/10.1242/dev.122.8.2507.

Faas, L., Warrander, F.C., Maguire, R., Ramsbottom, S.A., Quinn, D., Genever, P., and Isaacs, H.V. (2013). Lin28 proteins are required for germ layer specification in *Xenopus*. Development *140*, 976–986. https://doi.org/10.1242/dev.089797.

Fay, D.S., Stanley, H.M., Han, M., and Wood, W.B. (1999). ACaenorhabditis elegansHomologue ofhunchbackIs Required for Late Stages of Development but Not

Early Embryonic Patterning. Dev. Biol. *205*, 240–253. https://doi.org/10.1006/dbio.1998.9096.

Ferguson, E.L., and Horvitz, H.R. (1985). IDENTIFICATION AND CHARACTERIZATION OF 22 GENES THAT AFFECT THE VULVAL CELL LINEAGES OF THE NEMATODE *CAENORHABDITIS ELEGANS*. Genetics *110*, 17–72. https://doi.org/10.1093/genetics/110.1.17.

Fielenbach, N., and Antebi, A. (2008). *C. elegans* dauer formation and the molecular basis of plasticity. Genes Dev. *22*, 2149–2165. https://doi.org/10.1101/gad.1701508.

Gerisch, B., and Antebi, A. (2004). Hormonal signals produced by DAF-9/cytochrome P450 regulate *C. elegans* dauer diapause in response to environmental cues. Development *131*, 1765–1776. https://doi.org/10.1242/dev.01068.

Giesecke AV, Fang R, Joung JK. (2006) Synthetic protein-protein interaction domains created by shuffling Cys2His2 zinc-fingers. Mol Syst Biol. 2:2006.2011.

Gissendanner, C.R., Kelley, K., Nguyen, T.Q., Hoener, M.C., Sluder, A.E., and Maina, C.V. (2008). The Caenorhabditis elegans NR4A nuclear receptor is required for spermatheca morphogenesis. Dev. Biol. *313*, 767–786. https://doi.org/10.1016/j.ydbio.2007.11.014.

Golemis EA, Serebriiskii I, Finley RL Jr, Kolonin MG, Gyuris J, Brent R. (2001) Interaction trap/two-hybrid system to identify interacting proteins. Curr Protoc Cell Biol. Chapter 17:Unit 17.3.

Golden, J.W., and Riddle, D.L. (1984). The Caenorhabditis elegans dauer larva: Developmental effects of pheromone, food, and temperature. Dev. Biol. *102*, 368–378. https://doi.org/10.1016/0012-1606(84)90201-X.

Goodwin, P.R., and Juo, P. (2013). The Scaffolding Protein SYD-2/Liprin-α Regulates the Mobility and Polarized Distribution of Dense-Core Vesicles in C. elegans Motor Neurons. PLoS ONE *8*, e54763. https://doi.org/10.1371/journal.pone.0054763.

Grant, B., and Hirsh, D. (1999). Receptor-mediated Endocytosis in the *Caenorhabditis elegans* Oocyte. Mol. Biol. Cell *10*, 4311–4326. https://doi.org/10.1091/mbc.10.12.4311.

Greenwald, I.S., Sternberg, P.W., and Robert Horvitz, H. (1983). The lin-12 locus specifies cell fates in caenorhabditis elegans. Cell *34*, 435–444. https://doi.org/10.1016/0092-8674(83)90377-X.

Hall, D.H., Winfrey, V.P., Blaeuer, G., Hoffman, L.H., Furuta, T., Rose, K.L., Hobert, O., and Greenstein, D. (1999). Ultrastructural Features of the Adult Hermaphrodite Gonad of Caenorhabditis elegans: Relations between the Germ Line and Soma. Dev. Biol. *212*, 101–123. https://doi.org/10.1006/dbio.1999.9356.

Hallam, S.J., and Jin, Y. (1998). lin-14 regulates the timing of synaptic remodelling in Caenorhabditis elegans. Nature *395*, 78–82. https://doi.org/10.1038/25757.

Hammell, C.M., Lubin, I., Boag, P.R., Blackwell, T.K., and Ambros, V. (2009). nhl-2 Modulates MicroRNA Activity in Caenorhabditis elegans. Cell *136*, 926–938. https://doi.org/10.1016/j.cell.2009.01.053.

Hanna, J., Saha, K., Pando, B., van Zon, J., Lengner, C.J., Creyghton, M.P., van Oudenaarden, A., and Jaenisch, R. (2009). Direct cell reprogramming is a stochastic process amenable to acceleration. Nature *462*, 595–601. https://doi.org/10.1038/nature08592.

Harandi, O.F., and Ambros, V.R. (2015). Control of stem cell self-renewal and differentiation by the heterochronic genes and the cellular asymmetry machinery in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. *112*. https://doi.org/10.1073/pnas.1422852112.

Hedgecock, E.M., and White, J.G. (1985). Polyploid tissues in the nematode Caenorhabditis elegans. Dev. Biol. *107*, 128–133. https://doi.org/10.1016/0012-1606(85)90381-1.

Henderson, S.T., Gao, D., Lambie, E.J., and Kimble, J. (1994). lag-2 may encode a signaling ligand for the GLP-1 and LIN-12 receptors of C. elegans. Development *120*, 2913–2924. https://doi.org/10.1242/dev.120.10.2913.

Hills-Muckey, K., Martinez, M.A.Q., Stec, N., Hebbar, S., Saldanha, J., Medwig-Kinney, T.N., Moore, F.E.Q., Ivanova, M., Morao, A., Ward, J.D., et al. (2022). An engineered, orthogonal auxin analog/ *At* TIR1(F79G) pairing improves both specificity and efficacy of the auxin degradation system in *Caenorhabditis elegans*. Genetics *220*, iyab174. https://doi.org/10.1093/genetics/iyab174.

Hirsh, D., Oppenheim, D., and Klass, M. (1976). Development of the reproductive system of Caenorhabditis elegans. Dev. Biol. *49*, 200–219. https://doi.org/ 10.1016/0012-1606(76)90267-0.

Hong, Y., Roy, R., and Ambros, V. (1998). Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in Caenorhabditis elegans. Development *125*, 3585–3597. https://doi.org/10.1242/ dev.125.18.3585.

Hook B, Bernstein D, Zhang B, Wickens M (2005) RNA-protein interactions in the yeast three-hybrid system: affinity, sensitivity, and enhanced library screening. RNA 11: 227–233.

Horvitz, H.R., and Sulston, J.E. (1980). ISOLATION AND GENETIC CHARACTERIZATION OF CELL-LINEAGE MUTANTS OF THE NEMATODE *CAENORHABDITIS ELEGANS*. Genetics *96*, 435–454. https://doi.org/10.1093/ genetics/96.2.435.

Hubbard, E.J.A., and Greenstein, D. (2005) Introduction to the germ line. WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/ wormbook.1.18.1, http://www.wormbook.org.

Ilbay, O., and Ambros, V. (2019a). Pheromones and Nutritional Signals Regulate the Developmental Reliance on let-7 Family MicroRNAs in C. elegans. Curr. Biol. *29*, 1735-1745.e4. https://doi.org/10.1016/j.cub.2019.04.034.

Ilbay, O., and Ambros, V. (2019b). Regulation of nuclear-cytoplasmic partitioning by the *lin-28 - lin-46* pathway reinforces microRNA repression of HBL-1 to confer robust cell-fate progression in *C. elegans*. Development dev.183111. https://doi.org/ 10.1242/dev.183111.

Ilbay, O., Nelson, C., and Ambros, V. (2019). *C. elegans* LIN-28 controls temporal cell-fate progression by regulating LIN-46 expression via the 5'UTR of *lin-46* mRNA (Developmental Biology).

Iuchi, S. (2001). Three classes of C2H2 zinc finger proteins: Cell. Mol. Life Sci. *58*, 625–635. https://doi.org/10.1007/PL00000885.

Jeon, M., Gardner, H.F., Miller, E.A., Deshler, J., and Rougvie, A.E. (1999). Similarity of the *C. elegans* Developmental Timing Protein LIN-42 to Circadian Rhythm Proteins. Science *286*, 1141–1146. https://doi.org/10.1126/ science.286.5442.1141.

Johnson, S.M., Lin, S.-Y., and Slack, F.J. (2003). The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. Dev. Biol. *259*, 364–379. https://doi.org/10.1016/S0012-1606(03)00202-1.

Kaech, S.M., Whitfield, C.W., and Kim, S.K. (1998). The LIN-2/LIN-7/LIN-10 Complex Mediates Basolateral Membrane Localization of the C. elegans EGF Receptor LET-23 in Vulval Epithelial Cells. Cell *94*, 761–771. https://doi.org/10.1016/S0092-8674(00)81735-3.

Karp, X., and Ambros, V. (2012). Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in *C. elegans*. Development *139*, 2177–2186. https://doi.org/10.1242/dev.075986.

Katz, W.S., Hill, R.J., Clandinin, T.R., and Sternberg, P.W. (1995). Different Levels of the C. elegans growth factor LIN-3 promote distinct vulval precursor fates. Cell *82*, 297–307. https://doi.org/10.1016/0092-8674(95)90317-8.

Kimble, J., and Crittenden, S.L. (2007). Controls of Germline Stem Cells, Entry into Meiosis, and the Sperm/Oocyte Decision in *Caenorhabditis elegans*. Annu. Rev. Cell Dev. Biol. *23*, 405–433. https://doi.org/10.1146/annurev.cellbio.23.090506.123326.

Kimble, J., and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Dev. Biol. *70*, 396–417. https://doi.org/10.1016/0012-1606(79)90035-6.

Kimble, J., and Sharrock, W.J. (1983). Tissue-specific synthesis of yolk proteins in Caenorhabditis elegans. Dev. Biol. *96*, 189–196. https://doi.org/10.1016/0012-1606(83)90322-6.

Kimble, J.E., and White, J.G. (1981). On the control of germ cell development in Caenorhabditis elegans. Dev. Biol. *81*, 208–219. https://doi.org/10.1016/0012-1606(81)90284-0.

Kloosterman, W.P., and Plasterk, R.H.A. (2006). The Diverse Functions of MicroRNAs in Animal Development and Disease. Dev. Cell *11*, 441–450. https://doi.org/10.1016/j.devcel.2006.09.009.

Koštál, V. (2006). Eco-physiological phases of insect diapause. J. Insect Physiol. *52*, 113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008.

Kovacevic, I., and Cram, E.J. (2010). FLN-1/Filamin is required for maintenance of actin and exit of fertilized oocytes from the spermatheca in C. elegans. Dev. Biol. *347*, 247–257. https://doi.org/10.1016/j.ydbio.2010.08.005.

Large, E.E., and Mathies, L.D. (2010). hunchback and Ikaros-like zinc finger genes control reproductive system development in Caenorhabditis elegans. Dev. Biol. *339*, 51–64. https://doi.org/10.1016/j.ydbio.2009.12.013.

Lehrbach, N.J., Armisen, J., Lightfoot, H.L., Murfitt, K.J., Bugaut, A., Balasubramanian, S., and Miska, E.A. (2009). LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans. Nat. Struct. Mol. Biol. *16*, 1016–1020. https://doi.org/10.1038/nsmb.1675.

Levitan, D., and Greenwald, I. (1998). LIN-12 protein expression and localization during vulval development in C. elegans. Development *125*, 3101–3109. https://doi.org/10.1242/dev.125.16.3101.

Li, C., and Chalfie, M. (1990). Organogenesis in C. elegans: Positioning of neurons and muscles in the egg-laying system. Neuron *4*, 681–695. https://doi.org/10.1016/0896-6273(90)90195-L.

Li, J., and Greenwald, I. (2010). LIN-14 Inhibition of LIN-12 Contributes to Precision and Timing of C. elegans Vulval Fate Patterning. Curr. Biol. *20*, 1875–1879. https://doi.org/10.1016/j.cub.2010.09.055.

Lin, R., Hill, R.J., and Priess, J.R. (1998). POP-1 and Anterior–Posterior Fate Decisions in C. elegans Embryos. Cell *92*, 229–239. https://doi.org/10.1016/S0092-8674(00)80917-4.

Lin, S.-Y., Johnson, S.M., Abraham, M., Vella, M.C., Pasquinelli, A., Gamberi, C., Gottlieb, E., and Slack, F.J. (2003). The C. elegans hunchback Homolog, hbl-1, Controls Temporal Patterning and Is a Probable MicroRNA Target. Dev. Cell *4*, 639–650. https://doi.org/10.1016/S1534-5807(03)00124-2.

Liu, Z., and Ambros, V. (1991). Alternative temporal control systems for hypodermal cell differentiation in Caenorhabditis elegans. Nature *350*, 162–165. https://doi.org/10.1038/350162a0.

Liu, Z.C., and Ambros, V. (1989). Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in Caenorhabditis elegans. Genes Dev. *3*, 2039–2049. https://doi.org/10.1101/gad.3.12b.2039.

Ludewig, A.H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., Gerisch, B., Hutter, H., and Antebi, A. (2004). A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging. Genes Dev. *18*, 2120–2133. https://doi.org/10.1101/gad.312604.

Maira, M., Martens, C., Batsché, É., Gauthier, Y., and Drouin, J. (2003). Dimer-Specific Potentiation of NGFI-B (Nur77) Transcriptional Activity by the Protein Kinase A Pathway and AF-1-Dependent Coactivator Recruitment. Mol. Cell. Biol. *23*, 763–776. https://doi.org/10.1128/MCB.23.3.763-776.2003.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995). The nuclear receptor superfamily: The second decade. Cell *83*, 835–839. https://doi.org/10.1016/0092-8674(95)90199-X.

Marin, V.A., and Evans, T.C. (2003). Translational repression of a *C. elegans* Notch mRNA by the STAR/KH domain protein GLD-1. Development *130*, 2623–2632. https://doi.org/10.1242/dev.00486.

McCarter, J., Bartlett, B., Dang, T., and Schedl, T. (1997). Soma–Germ Cell Interactions inCaenorhabditis elegans:Multiple Events of Hermaphrodite Germline Development Require the Somatic Sheath and Spermathecal Lineages. Dev. Biol. *181*, 121–143. https://doi.org/10.1006/dbio.1996.8429.

McCarty, A.S., Kleiger, G., Eisenberg, D., and Smale, S.T. (2003). Selective Dimerization of a C2H2 Zinc Finger Subfamily. Mol. Cell *11*, 459–470. https://doi.org/10.1016/S1097-2765(03)00043-1.

Mihailovich, M., Militti, C., Gabaldón, T., and Gebauer, F. (2010). Eukaryotic cold shock domain proteins: highly versatile regulators of gene expression. BioEssays *32*, 109–118. https://doi.org/10.1002/bies.200900122.

Molnár, A., and Georgopoulos, K. (1994). The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. Mol. Cell. Biol. *14*, 8292–8303. https://doi.org/10.1128/mcb.14.12.8292-8303.1994.

Morita, K., and Han, M. (2006). Multiple mechanisms are involved in regulating the expression of the developmental timing regulator lin-28 in Caenorhabditis elegans. EMBO J. *25*, 5794–5804. https://doi.org/10.1038/sj.emboj.7601451.

Moss, E.G. (2007). Heterochronic Genes and the Nature of Developmental Time. Curr. Biol. *17*, R425–R434. https://doi.org/10.1016/j.cub.2007.03.043.

Moss, E.G., and Tang, L. (2003). Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. Dev. Biol. *258*, 432–442. https://doi.org/10.1016/S0012-1606(03)00126-X.

Moss, E.G., Lee, R.C., and Ambros, V. (1997). The Cold Shock Domain Protein LIN-28 Controls Developmental Timing in C. elegans and Is Regulated by the lin-4 RNA. Cell *88*, 637–646. https://doi.org/10.1016/S0092-8674(00)81906-6.

Motola, D.L., Cummins, C.L., Rottiers, V., Sharma, K.K., Li, T., Li, Y., Suino-Powell, K., Xu, H.E., Auchus, R.J., Antebi, A., et al. (2006). Identification of Ligands for DAF-12 that Govern Dauer Formation and Reproduction in C. elegans. Cell *124*, 1209–1223. https://doi.org/10.1016/j.cell.2006.01.037.

Navarro, R.E., Shim, E.Y., Kohara, Y., Singson, A., and Blackwell, T.K. (2001). *cgh-1*, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. Development *128*, 3221–3232. https://doi.org/10.1242/dev.128.17.3221.

Newman, M.A., Thomson, J.M., and Hammond, S.M. (2008). Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. RNA *14*, 1539–1549. https://doi.org/10.1261/rna.1155108.

Niwa, R., Hada, K., Moliyama, K., Ohniwa, R.L., Tan, Y.-M., Carter, K.O., Chi, W., Reinke, V., and Slack, F.J. (2009). *C. elegans sym-1* is a downstream target of the Hunchback-like-1 developmental timing transcription factor. Cell Cycle *8*, 4147–4154. https://doi.org/10.4161/cc.8.24.10292.

Olsson-Carter, K., and Slack, F.J. (2010). A Developmental Timing Switch Promotes Axon Outgrowth Independent of Known Guidance Receptors. PLoS Genet. *6*, e1001054. https://doi.org/10.1371/journal.pgen.1001054.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86–89. https://doi.org/10.1038/35040556.

Pekarsky, Y., Hallas, C., Palamarchuk, A., Koval, A., Bullrich, F., Hirata, Y., Bichi, R., Letofsky, J., and Croce, C.M. (2001). Akt phosphorylates and regulates the orphan nuclear receptor Nur77. Proc. Natl. Acad. Sci. *98*, 3690–3694. https://doi.org/10.1073/pnas.051003198.

Pepper, A.S.-R., McCane, J.E., Kemper, K., Yeung, D.A., Lee, R.C., Ambros, V., and Moss, E.G. (2004). The C. elegans heterochronic gene lin-46 affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin. Development *131*, 2049–2059. https://doi.org/10.1242/dev.01098.

Perez, M.F., and Lehner, B. (2019). Vitellogenins - Yolk Gene Function and Regulation in Caenorhabditis elegans. Front. Physiol. *10*, 1067. https://doi.org/10.3389/fphys.2019.01067.

Perez, M.F., Francesconi, M., Hidalgo-Carcedo, C., and Lehner, B. (2017). Maternal age generates phenotypic variation in Caenorhabditis elegans. Nature *552*, 106–109. https://doi.org/10.1038/nature25012.

Praslicka, B., and Gissendanner, C.R. (2015). The *C. elegans* NR4A nuclear receptor gene *nhr-6* promotes cell cycle progression in the spermatheca lineage: Spermatheca Cell Cycle Regulation by NHR-6. Dev. Dyn. *244*, 417–430. https://doi.org/10.1002/dvdy.24244.

Procko, C., Lu, Y., and Shaham, S. (2012). Sensory Organ Remodeling in *Caenorhabditis elegans* Requires the Zinc-Finger Protein ZTF-16. Genetics *190*, 1405–1415. https://doi.org/10.1534/genetics.111.137786.

Ramboarina, S., Druillennec, S., Morellet, N., Bouaziz, S., and Roques, B.P. (2004). Target Specificity of Human Immunodeficiency Virus Type 1 NCp7 Requires an Intact Conformation of Its CCHC N-Terminal Zinc Finger. J. Virol. *78*, 6682–6687. https://doi.org/10.1128/JVI.78.12.6682-6687.2004.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901–906. https://doi.org/10.1038/35002607.

Resnick, T.D., McCulloch, K.A., and Rougvie, A.E. (2010). miRNAs give worms the time of their lives: Small RNAs and temporal control in *Caenorhabditis elegans*. Dev. Dyn. NA-NA. https://doi.org/10.1002/dvdy.22260.

Romano, M., Rosanova, P., Anteo, C., and Limatola, E. (2004). Vertebrate yolk proteins: A review. Mol. Reprod. Dev. *69*, 109–116. https://doi.org/10.1002/mrd.20146.

Rougvie, A.E., and Moss, E.G. (2013). Developmental Transitions in C. elegans Larval Stages. In Current Topics in Developmental Biology, (Elsevier), pp. 153–180.

Roush, S., and Slack, F.J. (2008). The let-7 family of microRNAs. Trends Cell Biol. *18*, 505–516. https://doi.org/10.1016/j.tcb.2008.07.007.

Roush, S.F., and Slack, F.J. (2009). Transcription of the C. elegans let-7 microRNA is temporally regulated by one of its targets, hbl-1. Dev. Biol. *334*, 523–534. https://doi.org/10.1016/j.ydbio.2009.07.012.

Ruvkun, G., and Giusto, J. (1989). The Caenorhabditis elegans heterochronic gene lin-14 encodes a nuclear protein that forms a temporal developmental switch. Nature *338*, 313–319. https://doi.org/10.1038/338313a0.

Ruvkun, G., Ambros, V., Coulson, A., Waterston, R., Sulston, J., and Horvitz, H.R. (1989). Molecular genetics of the Caenorhabditis elegans heterochronic gene lin-14. Genetics *121*, 501–516. https://doi.org/10.1093/genetics/121.3.501.

Ruvkun, G., Wightman, B., Bürglin, T., and Arasu, P. (1991). Dominant gain-of-function mutations that lead to misregulation of the *C. elegans* heterochronic gene *lin-14* , and the evolutionary implications of dominant mutations in pattern-formation genes. Development *113*, 47–54. https://doi.org/10.1242/dev.113.Supplement_1.47.

Sapir, A., Choi, J., Leikina, E., Avinoam, O., Valansi, C., Chernomordik, L.V., Newman, A.P., and Podbilewicz, B. (2007). AFF-1, a FOS-1-Regulated Fusogen, Mediates Fusion of the Anchor Cell in C. elegans. Dev. Cell *12*, 683–698. https://doi.org/10.1016/j.devcel.2007.03.003.

Schindler, A.J., and Sherwood, D.R. (2013). Morphogenesis of the *Caenorhabditis elegans* vulva: Morphogenesis of the *C. elegans* vulva. Wiley Interdiscip. Rev. Dev. Biol. *2*, 75–95. https://doi.org/10.1002/wdev.87.

Seggerson, K., Tang, L., and Moss, E.G. (2002). Two Genetic Circuits Repress the Caenorhabditis elegans Heterochronic Gene lin-28 after Translation Initiation. Dev. Biol. *243*, 215–225. https://doi.org/10.1006/dbio.2001.0563.

Seydoux, G., Salvage, C., and Greenwald, I. (1993). Isolation and Characterization of Mutations Causing Abnormal Eversion of the Vulva in Caenorhabditis elegans. Dev. Biol. *157*, 423–436. https://doi.org/10.1006/dbio.1993.1146.

Sharma-Kishore, R., White, J.G., Southgate, E., and Podbilewicz, B. (1999). Formation of the vulva in Caenorhabditis elegans: a paradigm for organogenesis. Development *126*, 691–699. https://doi.org/10.1242/dev.126.4.691.

Sharrock, W.J. (1983). Yolk proteins of Caenorhabditis elegans. Dev. Biol. *96*, 182–188. https://doi.org/10.1016/0012-1606(83)90321-4.

Sherwood, D.R., and Sternberg, P.W. (2003). Anchor Cell Invasion into the Vulval Epithelium in C. elegans. Dev. Cell *5*, 21–31. https://doi.org/10.1016/S1534-5807(03)00168-0.

Shi, X.-B., Tepper, C.G., and deVere White, R.W. (2008). Cancerous miRNAs and their regulation. Cell Cycle *7*, 1529–1538. https://doi.org/10.4161/cc.7.11.5977.

Shostak, Y., Van Gilst, M.R., Antebi, A., and Yamamoto, K.R. (2004). Identification of *C. elegans* DAF-12-binding sites, response elements, and target genes. Genes Dev. *18*, 2529–2544. https://doi.org/10.1101/gad.1218504.

Singson, A. (2001). Every Sperm Is Sacred: Fertilization in Caenorhabditis elegans. Dev. Biol. *230*, 101–109. https://doi.org/10.1006/dbio.2000.0118.

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., and Ruvkun, G. (2000). The lin-41 RBCC Gene Acts in the C. elegans Heterochronic Pathway between the let-7 Regulatory RNA and the LIN-29 Transcription Factor. Mol. Cell *5*, 659–669. https://doi.org/10.1016/S1097-2765(00)80245-2.

Smith, K.K. (2002). Sequence Heterochrony and the Evolution of Development. J. Morphol. *252*, 82–97. https://doi.org/10.1002/jmor.10014.

Sornda, T., Ezcurra, M., Kern, C., Galimov, E.R., Au, C., de la Guardia, Y., and Gems, D. (2019). Production of YP170 Vitellogenins Promotes Intestinal Senescence in Caenorhabditis elegans. J. Gerontol. Ser. A *74*, 1180–1188. https://doi.org/10.1093/gerona/glz067.

Sternberg, P.W. (2005) Vulval development. WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.6.1, http://www.wormbook.org.

Sturm, Á., Saskői, É., Tibor, K., Weinhardt, N., and Vellai, T. (2018). Highly efficient RNAi and Cas9-based auto-cloning systems for C. elegans research. Nucleic Acids Res. *46*, e105–e105. https://doi.org/10.1093/nar/gky516.

Sulston, J.E., and Horvitz, H.R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev. Biol. *56*, 110–156. https://doi.org/10.1016/0012-1606(77)90158-0.

Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The Caenorhabditis elegans male: Postembryonic development of nongonadal structures. Dev. Biol. *78*, 542–576. https://doi.org/10.1016/0012-1606(80)90352-8.

Sulston, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Dev. Biol. *100*, 64–119. https://doi.org/10.1016/0012-1606(83)90201-4.

Sun, L., Liu, A., and Georgopoulos, K. (1996). Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. EMBO J. *15*, 5358–5369. https://doi.org/10.1002/j.1460-2075.1996.tb00920.x.

Tax, F.E., Yeargers, J.J., and Thomas, J.H. (1994). Sequence of C. elegans lag-2 reveals a cell-signalling domain shared with Delta and Serrate of Drosophila. Nature *368*, 150–154. https://doi.org/10.1038/368150a0.

Tenen CC, and Greenwald I (2019). Cell non-autonomous function of daf-18/PTEN in the somatic gonad coordinates somatic gonad and germ-line development in C. elegans Dauer larvae. Curr. Biol 29, 1064–1072.e8.

Tennessen, J.M., Gardner, H.F., Volk, M.L., and Rougvie, A.E. (2006). Novel heterochronic functions of the Caenorhabditis elegans period-related protein LIN-42. Dev. Biol. *289*, 30–43. https://doi.org/10.1016/j.ydbio.2005.09.044.

Terns, R.M., Kroll-Conner, P., Zhu, J., Chung, S., and Rothman, J.H. (1997). A Deficiency Screen for Zygotic Loci Required for Establishment and Patterning of the Epidermis in *Caenorhabditis elegans*. Genetics *146*, 185–206. https://doi.org/10.1093/genetics/146.1.185.

Thomas, J.H., Stern, M.J., and Horvitz, H.R. (1990). Cell interactions coordinate the development of the C. elegans egg-laying system. Cell *62*, 1041–1052. https://doi.org/10.1016/0092-8674(90)90382-O.

Timmons, L., Luna, H., Martinez, J., Moore, Z., Nagarajan, V., Kemege, J.M., and Asad, N. (2014). Systematic comparison of bacterial feeding strains for increased yield of *Caenorhabditis elegans* males by RNA interference-induced non-disjunction. FEBS Lett. *588*, 3347–3351. https://doi.org/10.1016/j.febslet.2014.07.023.

Tsialikas, J., and Romer-Seibert, J. (2015). LIN28: roles and regulation in development and beyond. Development *142*, 2397–2404. https://doi.org/10.1242/dev.117580.

Tsialikas, J., Romens, M.A., Abbott, A., and Moss, E.G. (2017). Stage-Specific Timing of the microRNA Regulation of *lin-28* by the Heterochronic Gene *lin-14* in *Caenorhabditis elegans*. Genetics *205*, 251–262. https://doi.org/10.1534/genetics.116.195040.

Vadla, B., Kemper, K., Alaimo, J., Heine, C., and Moss, E.G. (2012). lin-28 Controls the Succession of Cell Fate Choices via Two Distinct Activities. PLoS Genet. *8*, e1002588. https://doi.org/10.1371/journal.pgen.1002588.

Van Nostrand, E.L., Sánchez-Blanco, A., Wu, B., Nguyen, A., and Kim, S.K. (2013). Roles of the Developmental Regulator unc-62/Homothorax in Limiting Longevity in Caenorhabditis elegans. PLoS Genet. *9*, e1003325. https://doi.org/10.1371/journal.pgen.1003325.

Van Wynsberghe, P.M., Kai, Z.S., Massirer, K.B., Burton, V.H., Yeo, G.W., and Pasquinelli, A.E. (2011). LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in Caenorhabditis elegans. Nat. Struct. Mol. Biol. *18*, 302–308. https://doi.org/10.1038/nsmb.1986.

Van Wynsberghe, P.M., Finnegan, E.F., Stark, T., Angelus, E.P., Homan, K.E., Yeo, G.W., and Pasquinelli, A.E. (2014). The Period protein homolog LIN-42 negatively regulates microRNA biogenesis in C. elegans. Dev. Biol. *390*, 126–135. https://doi.org/10.1016/j.ydbio.2014.03.017.

Viswanathan, S.R., Daley, G.Q., and Gregory, R.I. (2008). Selective Blockade of MicroRNA Processing by Lin28. Science *320*, 97–100. https://doi.org/10.1126/science.1154040.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P.C., and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature *423*, 555–560. https://doi.org/10.1038/nature01645.

Wightman, B., Burglin, T.R., Gatto, J., Arasu, P., and Ruvkun, G. (1991). Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development. Genes Dev. *5*, 1813–1824. https://doi.org/10.1101/gad.5.10.1813.

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855–862. https://doi.org/10.1016/0092-8674(93)90530-4.

Wingate, A.D., Campbell, D.G., Peggie, M., and Arthur, J.S.C. (2006). Nur77 is phosphorylated in cells by RSK in response to mitogenic stimulation. Biochem. J. *393*, 715–724. https://doi.org/10.1042/BJ20050967.

Yu, J., Hecht, N.B., and Schultz, R.M. (2003). Requirement for RNA-binding activity of MSY2 for cytoplasmic localization and retention in mouse oocytes. Dev. Biol. *255*, 249–262. https://doi.org/10.1016/S0012-1606(02)00094-5.

Zeng, H., Qin, L., Zhao, D., Tan, X., Manseau, E.J., Van Hoang, M., Senger, D.R., Brown, L.F., Nagy, J.A., and Dvorak, H.F. (2006). Orphan nuclear receptor TR3/Nur77 regulates VEGF-A–induced angiogenesis through its transcriptional activity. J. Exp. Med. *203*, 719–729. https://doi.org/10.1084/jem.20051523.

Zhang L, Ward JD, Cheng Z, Dernburg AF. (2015) The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in C. elegans. Development (24):4374-4384.

Attributes of the Thesis

Madeleine Minutillo wrote and performed the experiments, took the micrographs, compiled the data, made the figures and tables, and wrote the text, except:

Chapter 3 was coauthored by Madeleine Minutillo and Eric Moss (manuscript in preparation).

Yeast Two Hybrid experiments were performed by Madeleine Minutillo and Kevin Kemper.

Yeast Three Hybrid experiments were performed by Madeleine Minutillo and Eric Moss.

Strain ME502 was made by Maria Ivanova.

Fig. 1.2 - Image modified from WormBook, wormbook.org. Copyright 2005 E. Jane Albert Hubbard and David Greenstein. Creative Commons License.

Fig. 1.3 - Image modified from WormBook, wormbook.org. Copyright 2005 Paul W. Sternberg. Creative Commons License.

Fig. 1.4 - Made by Eric Moss based in part on work in this dissertation.

Creative Commons Attribution License permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.