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ROBUSTNESS MECHANISMS OF TEMPORAL CELL-FATE PROGRESSION IN *C. ELEGANS*

A Dissertation Presented

By

Orkan Ilbay

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

16 December, 2019

ROBUSTNESS MECHANISMS OF TEMPORAL CELL-FATE PROGRESSION IN *C. ELEGANS*

A Dissertation Presented

By

Orkan Ilbay

This work was undertaken in the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

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ABSTRACT

Robustness is a ubiquitous property of biological systems, however, underlying mechanisms that help reinforce the optimal phenotypes despite environmental or physiological perturbations are poorly understood.

C. elegans development consists of four larval stages (L1-L4) and well-characterized invariant cell lineages, within which the heterochronic pathway controls the order and timing of cell-fates. Environmental or physiological stress signals can slow or temporarily halt larval stage progression; remarkably, however, temporal cell-fate progression remains unaffected.

We show that two widely conserved signaling pathways, insulin and TGF-β, that regulate *C. elegans* larval stage progression in response to starvation and crowding, respectively, also regulate a rewiring of the heterochronic pathway so that cell-fates remain temporally anchored to appropriate larval stages. This rewiring is mediated by the nuclear hormone receptor DAF-12, and it involves a shift from the reliance on *let*-7-family microRNAs to the reliance on LIN-46 for proper downregulation of the transcription factor, Hunchback-like-1 (HBL-1), which promotes L2 cell-fates and opposes L3 cell-fates. LIN-46 (which is a homolog of bacterial molybdopterin molybdenum transferase (moeA) and human gephyrin) post-translationally inhibits HBL-1 activity. LIN-46 expression is repressed by the RNA-binding protein LIN-28 at the early stages to permit HBL-1 activity and hence the proper execution of L2 cell-fates.

Our results indicate that robustness mechanisms of temporal cell-fate progression in *C. elegans* involves 1) coordinated regulation of temporal cell-fates and larval stage progression and 2) collaboration between translational regulation exerted by microRNAs and post-translational regulation exerted by LIN-46 to coordinate HBL-1 downregulation with stage progression.

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PREFACE

Chapter II of this dissertation was previously published: Ilbay, O. and Ambros, V. (2019) 'Pheromones and Nutritional Signals Regulate the Developmental Reliance on let-7 Family MicroRNAs in C. elegans.', *Current biology : CB*. England, 29(11), pp. 1735-1745.e4. doi: 10.1016/j.cub.2019.04.034.

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Chapter IV of this dissertation was previously published as a preprint: 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', *bioRxiv*, p. 697490. doi: 10.1101/697490. Experiments to determine *lin-46* mRNA levels as presented in Figure 4.3 was conducted by Charles Nelson.

Victor Ambros aided in the experimental design and data interpretation of the results presented from Chapter II to Chapter V.

CHAPTER I – General Introduction

Robustness in biological systems

Despite lacking perfect biochemical mechanisms and inevitably living in constantly changing environments, biological systems reproduce stereotyped phenotypes. DNA replication and repair, which are responsible to generate and maintain the copies of DNA that are inherited to daughter cells and next generations, are error-prone processes, resulting in the accumulation of mutations and hence genetic variation within populations. Complex signaling molecules, mRNAs, and proteins that specify the phenotypes -- often in a dose-dependent manner -- are produced by biochemical reactions that are intrinsically noisy, leading to unavoidable stochastic fluctuations in the quantities of these critical molecules in cells. The environments where the cells and organisms live are variable, changing within a range of favorable -- but not necessarily optimal -- conditions as well as between favorable and unfavorable conditions. Remarkably, however, regardless of the inherent genetic and biochemical noise and despite environmental perturbations, phenotypic outcomes are remarkably robust -- in some cases essentially invariant.

The ability of a biological system to generate and maintain form and function in the face of internal or external perturbations is defined as biological robustness (Kitano, 2004). Biological robustness, phenotypic stability in the face of perturbations, is an abstract concept, a ubiquitous but hidden property that is recognized only when it is lost and sensitivity to conditions or phenotypic variability emerges. Importantly, robustness mechanisms integrated into our

physiology is in part what allows us to remain healthy despite accumulating mutations throughout our lives and inevitably living under suboptimal and sometimes hazardous conditions. However, underlying genetic mechanisms of biological robustness, which appear to be diverse and complex (de Visser *et al.*, 2003; Kitano, 2004), have not been studied well and are only poorly understood.

The core conserved processes of living organisms are thought to confer robustness to biological systems, especially via deconstraining evolutionarily younger biological processes, allowing (by minimizing fitness costs) the accumulation of genotypic variation and hence increasing their evolvability (Kirschner and Gerhart, 1998). Deconstraining properties of core processes include versatile proteins (e.g. calmodulin), weak regulatory linkage (e.g. cascaded organization of signal transduction pathways), and flexibility conferred by exploratory mechanisms (e.g. vertebrate adaptive immunity or pathfinding behavior of microtubules) (Kirschner and Gerhart, 1998).

The best experimental evidence of a core biological process conferring robustness comes from the heat-shock protein Hsp90 -- a versatile chaperone capable of recognizing a set of diverse proteins that are intrinsically unstable -- which was shown to provide fidelity to wildtype phenotypes by buffering against otherwise cryptic mutations and environmental variations both in animals and plants (Rutherford and Lindquist, 1998; Queitsch, Sangster and Lindquist, 2002). In yeast, a systematic search for phenotypic capacitors, like Hsp90, revealed 300

gene products, most of which belong to core biological processes that resulted in increased morphological variation when perturbed (Levy and Siegal, 2008).

Robustness is evident and pervasive especially in the context of animal development -- exhibited by the constancy of morphological features within each species. Living organisms under natural selection have evolved genetic mechanisms to increase the robustness of optimal phenotypic outcomes, through a (hypothetical) process called “canalization” (Waddington, 1942). Mechanisms of canalization are thought to involve the integration of “buffering strategies” that confer robustness to gene regulatory networks controlling phenotypic outcomes (Rutherford, 2000).

Developmental gene regulatory networks have complex, scale-free network topologies (characterized by having widely different levels of connectivity among its nodes), which are shown to be robust against random errors (Albert, Jeong and Barabási, 2000; Jeong *et al.*, 2001). Aside from robustness perhaps being an inherent feature of gene regulatory networks (Siegal and Bergman, 2002; Bergman and Siegal, 2003), genetic redundancy (paralog genes), fail-safe (alternative or parallel) pathways, and system control (feedback and feedforward loops) are some of the prevalent features of gene regulatory networks that confer robustness to network circuits and contribute to overall robustness of gene regulatory networks (Wilkins, 1997; Freeman, 2000; Hartman, Garvik and Hartwell, 2001; Kitano, 2004; Stelling *et al.*, 2004).

Noise in gene expression, which is inevitable because it stems from the inherent stochasticity of the biochemical reactions of gene expression (Raser and O'Shea, 2005; Raj *et al.*, 2006; Raj and van Oudenaarden, 2008), can -- if not buffered -- lead to meaningful deviations in gene expression and consequently phenotypic variation (Raj *et al.*, 2010). Moreover, noise can propagate in gene regulatory networks (transmitted noise), which can also be stochastically amplified (Thattai and van Oudenaarden, 2001; Hooshangi, Thiberge and Weiss, 2005; Pedraza and van Oudenaarden, 2005), destabilizing genetic programs even when the individual components have low intrinsic noise (Pedraza and van Oudenaarden, 2005). Positive feedback loops can effectively amplify noise, which is beneficial in the context of generating bi-stable switches; but, more importantly, negative feedback loops -- and, in some cases, cooperativity -- reduce noise and/or prevent its propagation by distinguishing signal from noise (Thattai and van Oudenaarden, 2001; Raj and van Oudenaarden, 2008). When the rates of mRNA production (transcription) and degradation as well as the rates of protein production (translation) and degradation are taken into account, increased translation efficiency is found to be the predominant source of noise in gene expression in prokaryotes (Ozbudak *et al.*, 2002). In eukaryotes, both transcription and translation contribute to noise; and importantly, reduced translation efficiency (e.g. low codon optimality) is capable of absorbing the transcriptional noise in gene expression (Blake *et al.*, 2003; Ebert and Sharp, 2012).

In recent years, MicroRNAs, post-transcriptional regulators of gene expression (Lee, Feinbaum and Ambros, 1993; Reinhart *et al.*, 2000), have been increasingly associated with canalization of development and developmental robustness (Hornstein and Shomron, 2006; Posadas and Carthew, 2014). MicroRNAs are commonly found in feedback and feedforward control circuits (Tsang, Zhu and van Oudenaarden, 2007; Martinez *et al.*, 2008), regulate thousands of genes in animal cells (Enright *et al.*, 2004; Lewis, Burge and Bartel, 2005; Xie *et al.*, 2005; Friedman *et al.*, 2009), reduce noise in gene expression (Stark *et al.*, 2005; Schmiedel *et al.*, 2015) and confer robustness to developmental programs (Ebert and Sharp, 2012; Pelaez and Carthew, 2012).

MicroRNAs and their roles in developmental robustness

MicroRNAs are a class of small (22-nucleotides in length) non-coding RNAs that serve as specificity factors for certain Argonaute proteins (Lee, Feinbaum and Ambros, 1993; Hammond *et al.*, 2001; Schirle and MacRae, 2012). Arganaute-MicroRNA containing complexes [microRNA-induced silencing complexes (miRISCs)] interact with target messenger RNAs (mRNAs) through RNA-RNA base-pairing and interfere with their translation, reducing protein production and mRNA stability (Bartel, 2018).

Base-pairing between the microRNA seed region (nucleotides at positions 2-8 from the 5-prime end) and target mRNAs confers specificity and is sufficient for target regulation (Brennecke *et al.*, 2005) -- although base-pairing between the rest of the microRNA and target mRNA seems to support specificity and function

(Broughton *et al.*, 2016). Metazoan genomes encode tens to hundreds of microRNAs (e.g. 147 in *Caenorhabditis elegans* (Jan *et al.*, 2011), 556 in humans (Fromm *et al.*, 2015)), some of which share the same seed and are called microRNA seed families. Members of a microRNA seed family -- which are usually, but not necessarily, paralogs -- can have unique as well as redundant biological functions. For example, *Caenorhabditis elegans (C. elegans) let-7* controls cell-fates during L4 to adult larval transition (Reinhart *et al.*, 2000) whereas its paralogs *mir-48, mir-84* and *mir-241* function redundantly to control cell-fates during L2 to L3 larval transition (Abbott *et al.*, 2005).

MiRISC targeting of mRNAs can lead to substantial (10-20-fold) reductions in protein production (Stadler *et al.*, 2012), but more modest reductions (within 2-fold) seem to be more common (Baek *et al.*, 2008; Selbach *et al.*, 2008). These two classes of targets, which are substantially and modestly affected by microRNA-mediated regulation, are also called “switch” and “tuning” targets, respectively (Bartel and Chen, 2004). The impact of microRNAs on their targets depends on: 1) the relative abundances of the microRNA and the target (Mukherji *et al.*, 2011), 2) the strength of the microRNA-target interaction (e.g. number of microRNA complementary sites) (Mukherji *et al.*, 2011) 3) the organization of the control circuit containing the microRNA and the target, including feedback, and coherent or incoherent feedforward loops (Herranz and Cohen, 2010; Pelaez and Carthew, 2012). Accordingly, a particular microRNA can function to denoise gene expression, which includes dampening of

fluctuations in protein production as well as generating thresholds in the abundances of target mRNAs for effective protein production -- which can prevent gene expression from mRNAs that are inappropriately produced as a result of leaky transcription (Figure 1.1). Alternatively, a microRNA can function to fine-tune gene expression, which includes adjusting the steady-state protein levels, and facilitating target protein down-regulation (Figure 1.1).

Unlike the first examples of microRNA encoding genes, *lin-4* and *let-7*, whose inactivation resulted in evident phenotypes, most microRNA mutants in *C. elegans* did not produce any observable defects (Miska *et al.*, 2007). This was in part due to functional redundancy amongst microRNA family members (Alvarez-Saavedra and Horvitz, 2010) and with other microRNA families (Brenner *et al.*, 2010). A potential predominance of fine-tuning roles and the scarcity of switch-like roles of microRNAs in *C. elegans* would also explain the lack of evident phenotypes in many cases. Namely, whereas *lin-4* and *let-7* act as OFF switches and in their absence their targets remain ON, causing fully penetrant gain-of-function phenotypes, most other microRNAs might act as denoisers or fine-tuners rather than switches. The absence of such microRNAs may result in only partially penetrant phenotypes due to 1) lack of denoising: occasional protein bursts (especially in the cases where a positive feedback loop amplifies the initial leak in protein production) or fluctuations that can reach the phenotype-altering threshold (Raj *et al.*, 2010), 2) lack of fine-tuning: elevated protein production from target mRNAs or delayed and/or slowed

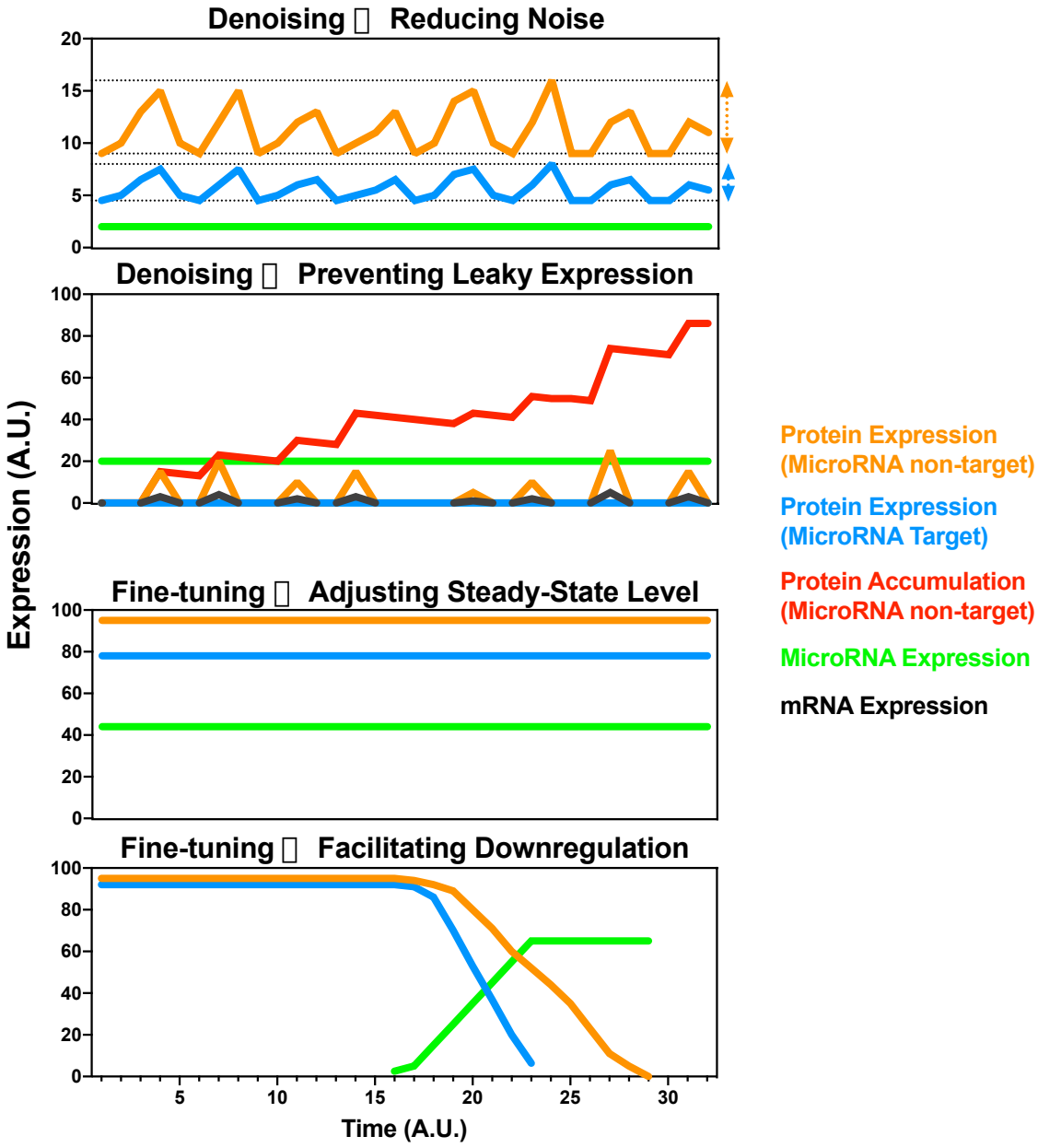
Denoising Reducing Noise
Denoising Preventing Leaky Expression
Fine-tuning Adjusting Steady-State Level
Fine-tuning Facilitating Downregulation
Expression (A.U.)
Time (A.U.)
Protein Expression (MicroRNA non-target)
Protein Expression (MicroRNA Target)
Protein Accumulation (MicroRNA non-target)
MicroRNA Expression
mRNA Expression

Figure 2.1. Denoising and fine-tuning of gene expression by microRNAs. Hypothetical profiles of microRNA target, non-target, and microRNA expression over time are plotted to illustrate denoising (upper two plots) and fine-tuning (lower to plots) functions of microRNAs.

downregulation, which may again lead to occasional threshold violations and hence a change in the phenotype. Lastly, in some cases, the overexpression of the target -- regardless of being a switch or tuning target -- may not be consequential; therefore, the loss of microRNAs regulating such targets may not produce any mutant phenotypes.

In many cases, MicroRNA loss-of-function phenotypes are revealed or enhanced under stress conditions, indicating that the biological functions of at least certain microRNAs include conferring robustness to phenotypic outcomes (Herranz and Cohen, 2010; Ebert and Sharp, 2012; Posadas and Carthew, 2014; Ambros and Ruvkun, 2018). In *Drosophila*, *mir-7* functions in two different gene regulatory circuits to regulate photoreceptor and sensory organ precursor (SOP) determination. Lack of *mir-7* results in mutant phenotypes in both circuits due to altered target gene expression under conditions of temperature fluctuations but not under normal conditions, demonstrating that *mir-7* imparts robustness to these developmental programs against variations in temperature (Li *et al.*, 2009). Similarly, in *C. elegans*, pathfinding defects of the distal tip cells observed in animals doubly mutant for *mir-34* and *mir-83* are enhanced when the animals are subjected to temperature oscillations, indicating that these microRNAs protect the distal tip cell migration program from the effects of unstable temperature (Burke, Hammell and Ambros, 2015).

The heterochronic microRNAs, in particular, *let-7*-family microRNAs, of *C. elegans* confer robustness to cell-fate progression (Ambros and Ruvkun, 2018).

Temporal cell-fate defects observed in the mutants of *let-7*-family microRNAs (*mir-48, mir-84, and mir-241*) are enhanced when the worms are fed with pathogenic bacteria instead of *E. coli* OP50 (Ren and Ambros, 2015). The nuclear hormone receptor DAF-12 is required to activate the transcription of *let-7*-family microRNAs (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009). Insufficient expression of *let-7*-family microRNAs in *daf-12*-null animals results in extra seam cell phenotype (Antebi *et al.*, 2000; Hammell, Karp and Ambros, 2009); and this phenotype is enhanced by temperature oscillations (Hochbaum *et al.*, 2011). These studies demonstrate that *let-7*-family microRNAs confer robustness to cell-fate progression programs in *C. elegans* against both pathogen stress and temperature variations.

C. elegans development and the heterochronic pathway

The nematode *C. elegans* is a relatively simple multicellular organism comprised of diverse tissues built from a defined number of somatic cells. *C. elegans* develops from a single cell through embryonic development followed by four larval stages (L1-L4), each of which consists of an invariant set of cell-division and cell-fate specification events (Sulston and Horvitz, 1977).

The order and timing of cell-fates within individual *C. elegans* cell lineages are controlled by genes in the heterochronic pathway (Ambros and Horvitz, 1984; Ambros, 2011). In this context, the seam cell lineage has been studied most extensively. Larval seam cells are hypodermal stem cells that are positioned side-by-side in a row along the lateral body axis from head to tail on each side of

the worms. L1 larvae have ten seam cells on each side, nine of which divide asymmetrically at the beginning of each larval stage and give rise to another seam cell and a differentiated (hyp7) hypodermal cell. Importantly, six of the ten seam cells divide symmetrically at the L2 stage, giving rise to six new (post-embryonic) seam cells. Thus, L3, L4 and adults worms have sixteen seam cells on each side of their bodies. In addition, at the end of the L4 stage, seam cells fuse with each other, forming a seam syncytium along the lateral body axis, and secrete special collagens to form a special cuticle structure called alae. These two events, the increase in the seam cell number at the L2 stage and the formation of alae structure at adult stage, are convenient markers of L2 and young adult stage cell-fates, respectively, and have been heavily used to study developmental timing in *C. elegans* (Figure 1.2 and 1.3).

Three transcription factors (TFs), LIN-14, HBL-1, and LIN-29, in the heterochronic pathway specify L1, L2, and adult cell-fates, respectively (Ambros and Horvitz, 1987; Rougvie *et al.*, 1995; Abrahante *et al.*, 2003; Lin *et al.*, 2003) (Figure 1.3). LIN-14 is regulated by *lin-4* (Lee, Feinbaum and Ambros, 1993; Wightman, Ha and Ruvkun, 1993) and HBL-1 is regulated by *let-7*-family (*mir-48, mir-241, mir-84*) microRNAs (Abbott *et al.*, 2005). LIN-29 is negatively regulated by the TRIM-NHL protein LIN-41 (Slack *et al.*, 2000), which is in turn regulated by

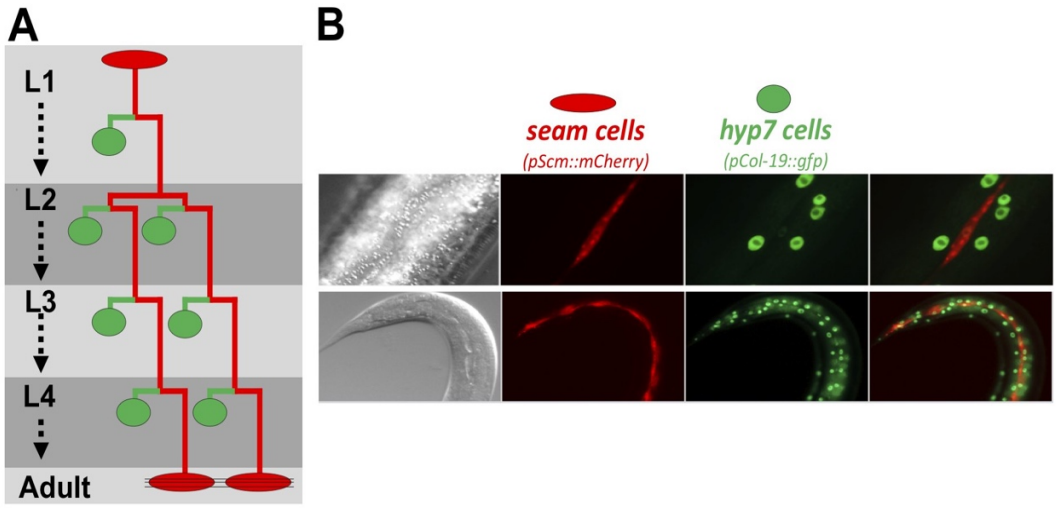
A
L1
L2
L3
L4
Adult
B
seam cells
(pScm::mCherry)
hyp7 cells
(pCol-19::gfp)

Figure 1.2. Hypodermal seam cell linage of *C. elegans.*

A) Lineage diagram of seam cells (called V1-V4 and V6) showing cell division and differentiation events during larval development. Seam cells (red) asymmetrically divide at all stages, giving rise to hyp7 cells (green). One round of symmetric cell division at the L2 stage results in an increase in the total seam cell number. At the adult stage, seam cells secrete special collagens, forming a cuticle structure called alae, which is represented by three horizontal lines over the adult stage seam cells. B) Microscopy images showing seam cells, which are marked with mCherry driven by pScm promoter and hyp7 cells, which are marked with GFP driven by pCol-19 promoter.

let-7 microRNA (Reinhart *et al.*, 2000). There are also other regulatory factors that act upstream of HBL-1; most notably, the RNA-binding protein LIN-28 (Moss, Lee and Ambros, 1997), the downstream effector gene *lin-46* (Pepper *et al.*, 2004), and a nuclear hormone receptor DAF-12 (Antebi *et al.*, 2000), which regulates the transcription of *let-7*-family microRNAs (Bethke *et al.*, 2009) (Figure 1.3).

The heterochronic TFs are necessary and sufficient to drive cell-fates of specific larval stages and the heterochronic microRNAs are required to facilitate their temporal regulation, ensuring cell-fate progression concomitant with larval stage progression. The heterochronic microRNAs *lin-4* and *let-7* act as off switches; in mutants lacking *lin-4* or *let-7*, the expression of the corresponding target, LIN-14 or LIN-41, persists into abnormally late stages, preventing appropriate cell-fate progression. On the other hand, *let-7*-family (*mir-48, mir-84, mir-241*) microRNAs, seem to serve as fine-tuners of gene expression acting in parallel with other factors in regulating HBL-1 expression. Accordingly, in their absence L2 to L3 cell-fate progression does not stop but is delayed, indicating a delay in HBL-1 down regulation.

Importantly, in *let-7*-family mutants, the normal synchrony among seam cells in their expression of stage-specific cell-fates is lost, presumably due to the variability in HBL-1 downregulation among the seam cells. In the same animal, some seam cells may reiterate L2 fates at the L3 stage, while other cells do not, and still others may reiterate L2 fates not only at the L3 stage but also at the L4

stage. This variability in the number of L2 fate reiterations results in variably number extra seam cells in young adults in *let-7*-family mutants, and the extra seam cell phenotype that is modified by certain conditions, for example, it is enhanced when the worms are fed with pathogenic bacteria instead of *E.coli* (Ren and Ambros, 2015). Therefore, *let-7*-family microRNAs, by ensuring uniform and robust HBL-1 downregulation in all seam cells and under different environmental conditions, prevent the variance in temporal cell-fate progression among the seam cell of an animal. Thus, in wildtype animals all seam cells execute L2 fates at the L2 stage and progress to L3 fates concomitant with the stage progression to L3 stage, maintaining the total number of seam cells in young adults—which is 16--regardless of various environmental or physiological perturbations.

The *C. elegans* genome encodes three more *let-7* seed family microRNAs, *mir-793, mir-794, and mir-795* (Ruby *et al.*, 2006). The seed sequence of two these microRNAs, *mir-793* and *mir-794*, differ from *let-7* at position 8, but are considered to be *let-7* family members because they could, in principle, regulate the same target mRNAs at the other family members. The functions of these newer members of *let-7*-family are not known. In particular, it is not known if these understudied *let-7*-family microRNAs might also be contributing to temporal downregulation of HBL-1, and hence to proper L2 to L3 cell-fate transitions, in parallel to *mir-48, mir-84*, and *mir-241*. And, if this were the case, it is not known

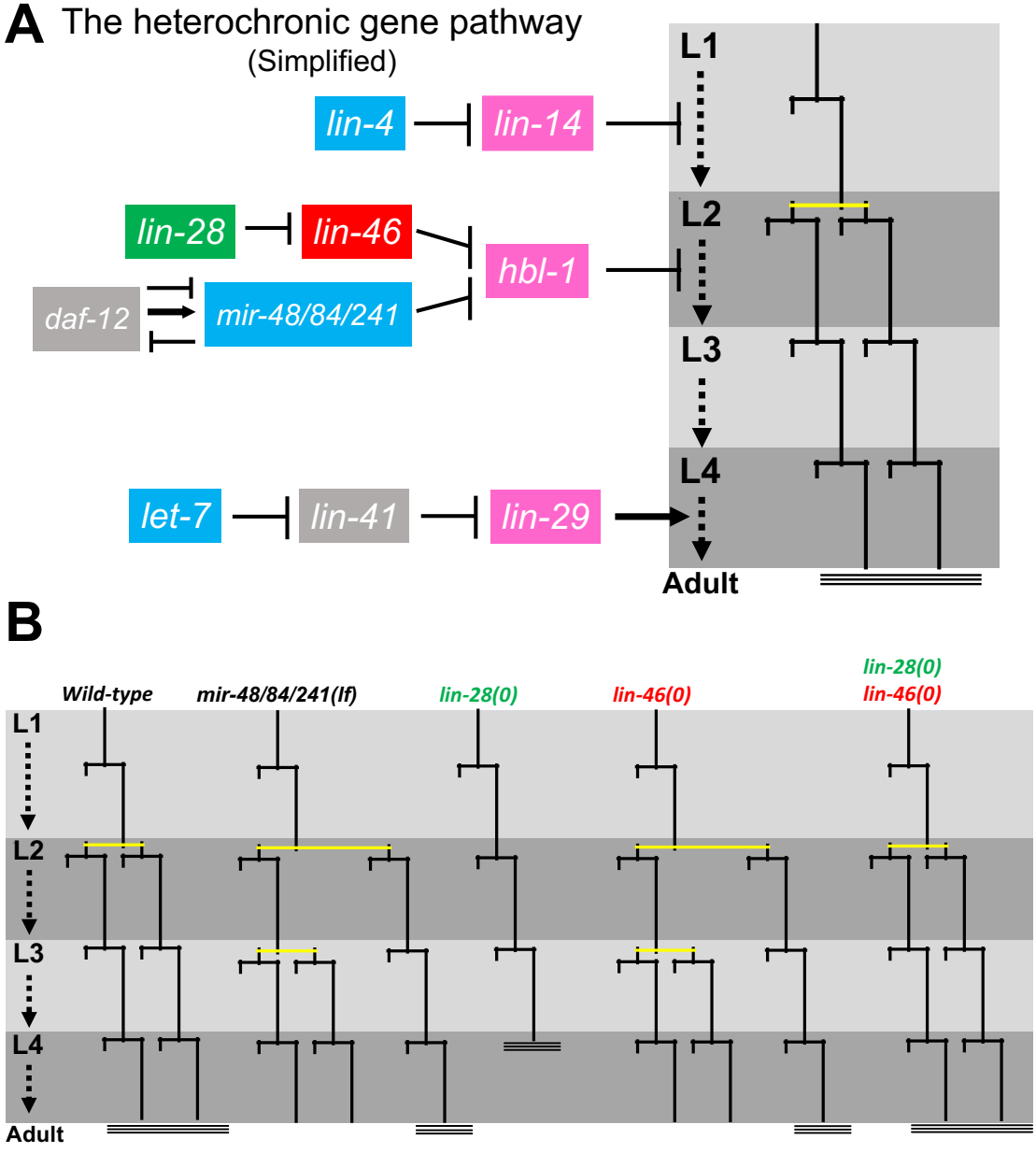
A
The heterochronic gene pathway
(Simplified)
lin-4
lin-14
lin-28
lin-46
hbl-1
daf-12
mir-48/84/241
let-7
lin-41
lin-29
L1
L2
L3
L4
Adult
B
Wild-type
mir-48/84/241(lf)
lin-28(0)
lin-46(0)
lin-28(0)
lin-46(0)
L1
L2
L3
L4
Adult

Figure 1.3. Heterochronic pathway and cell-fate progression defects in common heterochronic mutants.

(A) A simplified view of the *C. elegans* heterochronic pathway. Transcription factors are shown in pink boxes and microRNAs are in blue boxes. L2 stage-specific symmetric cell division is indicated by a yellow color in the lineage diagram (B) Developmental timing defects observed in the mutants of certain heterochronic genes are shown. The genotypes of the mutants are indicated at the top of the lineage diagrams.

if these new *let-7*-family members, similar to *mir-48, mir-84, and mir-241,* could also have roles in conferring robustness to temporal cell-fate progression.

The *C. elegans lin-28-lin-46* pathway acts in parallel to *let-7*-family microRNAs and upstream of HBL-1 (Abbott *et al.*, 2005; Vadla *et al.*, 2012): *lin-28* loss-of-function suppresses and *lin-46* loss-of-function substantially enhances the *let-7*-family mutant phenotypes (Abbott *et al.*, 2005). Therefore, the *lin-28-lin-46* pathway may be involved in modifying *let-7*-family mutant phenotypes by environmental or physiological stresses. For example, *lin-28* and/or *lin-46* activity might be affected by environmental stresses such that this parallel arm of the pathway could compensate for reduced *let-7*-family levels under such conditions. However, how LIN-28 regulates LIN-46 and how in turn LIN-46 regulates HBL-1, which might shed light into potential microRNA-compensatory roles of the *lin-28-lin-46* pathway*,* are not known. Likewise, the underlying mechanism by which LIN-46 could regulate HBL-1 activity and temporal cell-fates is unknown. *Lin-46* encodes a protein with sequence similarity to a conserved molybdenum co-factor biosynthesis enzyme and also to proteins shown to regulate the intracellular localization of other proteins in mammal—called gephyrins (Kneussel *et al.*, 1999; Fuhrmann *et al.*, 2002; Fritschy, Harvey and Schwarz, 2008). It is not known whether or how such hypothetical activities of *C. elegans* LIN-46 could be involved in its heterochronic pathway functions.

Alternative life-history trajectories and the regulation of developmental plasticity

Major natural habitats of *C. elegans* include ephemeral food sources such as bacterial populations growing on rotting fruits (Frézal and Félix, 2015). *C. elegans* is thought to colonize and rapidly populate these food sources owing to its rapid reproductive larval development and the ability to produce hundreds of eggs. However, as the population density increases and accordingly the resources for the worm population decline, the worms employ a different life-cycle strategy, which prioritizes survival and dispersal rather than reproduction: during larval growth, instead of the L3 stage, worms can elect to arrest development and enter a non-feeding and long-lived larval stage called dauer stage (Cassada and Russell, 1975; Hu, 2007). Dauer formation involves remodeling of larval tissues to render the larvae resistant to stress, and capable of dispersal to reach and colonize distant food sources.

Signaling pathways controlling the choice between the two major alternative life-history options have been studied very well (Fielenbach and Antebi, 2008). Population density and food availability are the two major factors that affect the decision between rapid reproductive larval development versus developmental arrest as dauer larva. Concentrations of certain pheromones secreted by the worms, called ascarosides (Butcher *et al.*, 2007; Srinivasan *et al.*, 2008), are sensed by the worms and translated into TGF-β/DAF-7 signals that regulate the dauer decision (Ren *et al.*, 1996). Similarly, nutritional status is sensed by an

insulin-like/DAF-2 signaling pathway that regulates the dauer decision (Kimura *et al.*, 1997) in parallel to the TGF-β/DAF-7 signals (Gottlieb and Ruvkun, 1994; Fielenbach and Antebi, 2008). The TGF-β/DAF-7 and Insulin/DAF-2 pathways converge on regulating the biosynthesis of the dafachronic acid (DA) hormone by the cytochrome P540 enzyme DAF-9 (Fielenbach and Antebi, 2008). DA is a ligand for the nuclear hormone receptor DAF-12 (Motola *et al.*, 2006), which mediates the dauer decision (Riddle, Swanson and Albert, 1981; Antebi *et al.*, 2000). Namely, ligand-bound DAF-12 allows reproductive development whereas unliganded DAF-12 promotes dauer formation.

Coordinate regulation of temporal cell-fates and developmental trajectory

Temporal cell-fate defects of many heterochronic mutants are suppressed or enhanced in larvae that develop through a temporary dauer arrest (Liu and Ambros, 1991; S Euling and Ambros, 1996; Karp and Ambros, 2012), indicating that proper temporal cell-fate regulation during different developmental trajectories (e.g. rapid reproductive vs. dauer-interrupted) require non-identical sets of heterochronic genes. These findings suggested that the heterochronic pathway is altered by dauer diapause, and perhaps specifically by certain signaling events or tissue remodeling programs occurring before, during, or after dauer commitment.

Dauer entry is preceded by a lengthened L2 stage, called the L2d stage (Golden and Riddle, 1984). Unlike the deterministic L2 stage, wherein progression to L3 stage is the only option, the L2d stage represents a bi-potential

developmental status. L2d animals are sensitive to the levels of dauer-inducing pheromones and are capable of either initiating the dauer program or advancing directly to the L3 reproductive trajectory, depending on the status of pheromone and nutritional signals at a decision point in the late L2d (Schaedel *et al.*, 2012).

L2d is initiated at the end of the L1 stage in response to dauer-inducing conditions (Golden and Riddle, 1984). L2d larvae continue to monitor the severity of the dauer-inducing conditions, and dauer-inductive signals -- TGF-β signaling effector DAF-3 (Patterson *et al.*, 1997) and/or insulin signaling effector DAF-16 (Ogg *et al.*, 1997) -- reach the threshold for dauer commitment, L2d animals enter dauer arrest. But, if these signals do not reach the threshold, L2d larvae develop continuously through L3 and L4 larval stages. It is, however, not known whether developing continuously through L2d or the presence of DAF-3 or DAF-16 signals, similar to the dauer-interrupted development, could have an impact on the heterochronic pathway; namely, could L2d modify heterochronic phenotypes? or could L2d alter the reliance to certain heterochronic genes for proper cell-fate progression?

The nuclear hormone receptor, DAF-12, is the main link between the regulation of diapause and developmental timing (Antebi *et al.*, 2000). Dafachronic acid (DA), which is a ligand for DAF-12, is produced by the worms when the conditions are favorable; and ligand-bound DAF-12 favors reproductive development (Motola *et al.*, 2006; Fielenbach and Antebi, 2008). Under unfavorable conditions, DA production is inhibited by upstream signaling

pathways, TGF- β or insulin; and, unliganded DAF-12 promotes dauer formation. Importantly, while mediating the decision between reproductive development and dauer arrest, DAF-12 also regulates *let-7*-family microRNA levels (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009). Liganded DAF-12 activates the transcription of *let-7*-family microRNAs and unliganded DAF-12 represses their transcription (Bethke *et al.*, 2009). Thus, *let-7*-family levels are high during the L2-to-L3 transition and low during L2d-to-dauer or L2d-to-L3 transition (Hammell, Karp and Ambros, 2009). It is, however, not clear how the *let-7*-family target HBL-1 is temporally downregulated during the L2d despite substantially reduced levels of *let-7*-family microRNAs.

On the other hand, while developmental trajectory (such as L2-L3 versus L2d-dauer-L3) can affect the expression of heterochronic phenotypes, genes in the heterochronic pathway can exert control on the choice of developmental trajectory. First, the heterochronic genes *lin-14* and *lin-4,* which control progression from L1 to L2 fates, also control at which larval stage dauer entry is permitted (Liu and Ambros, 1989)**.** Whilst wild-type animals enter dauer diapause after the L2 stage, *lin-14(lf)* larvae enter diapause one stage earlier, after the L1 stage, and some gain-of-function (gf) alleles of *lin-14* can cause larvae to enter diapause one stage later than normal, after the L3 stage. Moreover, high LIN-14 expression caused by *lin-4(lf)* or strong *lin-14(gf)* mutations prevents dauer commitment altogether. Second, the RNA-binding protein LIN-28, which is required for expressing L2 stage-specific cell-fates, is

also required for uniform remodeling of the tissues during dauer larva formation (Liu and Ambros, 1989). Third, *hbl-1*, which is expressed in the L1 and L2 and promotes proliferative seam cell divisions at the early L2 stage, opposes dauer formation induced by TGF-β or insulin signals (Karp and Ambros, 2011), which suggests that *hbl-1* downregulation that occur at the end of the L2 stage is required for dauer formation as well as proper progression to L3 stage cell-fates. Lastly, *let-7*-family microRNAs downregulate DAF-12; and in combination with the modulation of DA hormone levels, *let-7*-family mediated regulation of DAF-12 significantly affects the dauer decision (Hammell, Karp and Ambros, 2009).

CHAPTER II -- Pheromones and Nutritional Signals Regulate the Developmental Reliance on *let-7* Family MicroRNAs in *C. elegans*

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Abstract

Adverse environmental conditions can affect rates of animal developmental progression and lead to temporary developmental quiescence (diapause), exemplified by the dauer larva stage of the nematode *Caenorhabditis elegans* (*C. elegans*). Remarkably, patterns of cell division and temporal cell-fate progression in *C. elegans* larvae are not affected by changes in developmental trajectory. However, the underlying physiological and gene regulatory mechanisms that ensure robust developmental patterning despite substantial plasticity in developmental progression are largely unknown. Here, we report that diapause-inducing pheromones correct heterochronic developmental cell lineage defects caused by insufficient expression of *let-7* family microRNAs in *C. elegans*. Moreover, two conserved endocrine signaling pathways, DAF-7/TGF- β and DAF-2/Insulin, that confer on the larva diapause and non-diapause alternative developmental trajectories interact with the nuclear hormone receptor, DAF-12, to initiate and regulate a rewiring of the genetic circuitry controlling temporal cell-fates. This rewiring includes engagement of certain heterochronic genes, *lin-46*, *lin-4*, and *nhl-2*, that are previously associated with an altered genetic program in post-diapause animals, in combination with a novel ligand-independent DAF- 12 activity, to downregulate the critical *let-7* family target Hunchback-like-1 (HBL-1). Our results show how pheromone or endocrine signaling pathways can coordinately regulate both developmental progression and cell-fate transitions in

C. elegans larvae under stress so that the developmental schedule of cell-fates remains unaffected by changes in developmental trajectory.

Introduction

Despite the vast complexity of animal development, developmental processes are remarkably robust in the face of environment and physiological stresses. Multicellular animals develop from a single cell through a temporal and spatial elaboration of events that include cell division, differentiation, migration, and apoptosis. Early developmental cell lineages rapidly diverge functionally and spatially, and continue to follow distinct paths towards building diverse parts of the animal body. Marvelously, the sequence and synchrony of these increasingly complex programs of cell fate progression are precisely coordinated, regardless of various environmental and physiological stresses that the animal may encounter in its natural environment.

The nematode *C. elegans* develops through four larval stages, each of which consists of an invariant set of characteristic developmental events (Sulston and Horvitz, 1977). During larval development, stem cells and blast cells divide and progressively produce progeny cells with defined stage-specific fates. The timing of cell fate transitions within individual postembryonic cell lineages is regulated by genes of the heterochronic pathway, whose products include cell fate determinant transcription factors, as well as microRNAs (miRNAs) and other regulators of these transcription factors (Ambros and Horvitz, 1984; Ambros, 2011). In mutants defective in the activity of one or more heterochronic genes, the

synchrony between cell fates and developmental stages is lost in certain cell lineages, which results in dissonance in the relative timing of developmental events across the animal and consequently morphological abnormalities.

During *C. elegans* larval development, lateral hypodermal stem cells ('seam cells') express stage-specific proliferative or self-renewal behavior (Figure 2.1A). Particularly, whilst seam cells divide asymmetrically at each larval stage (L1-L4), giving rise to a new seam cell and a differentiating hypodermal (hyp7) cell, at the L2 stage, certain seam cells also undergo a single round of symmetric cell division, resulting in an increase in the number of seam cells on each side of the animal from ten to sixteen. This L2-specific proliferative cell fate is driven by a transcription factor, HBL-1, which specifies expression of the L2 cell fate, and also prevents the expression of the L3 cell fates (Abrahante *et al.*, 2003; Lin *et al.*, 2003). Therefore, in order to allow progression to L3 cell fates, HBL-1 must be downregulated by the end of the L2 stage. If HBL-1 is not properly downregulated, for example in mutants defective in upstream regulatory genes, seam cells inappropriately execute L2 cell fates at later stages, resulting in an enlarged and developmentally retarded population of seam cells in adult worms. Three *let-7* family miRNAs *(mir-48/84/241)* are redundantly required for proper temporal downregulation of HBL-1 (Abbott *et al.*, 2005). Larvae lacking all three *let-7* family miRNAs reiterate L2 cell fates in later stages of development. The degree of reiteration, hence the severity of the phenotype, varies depending on genetic

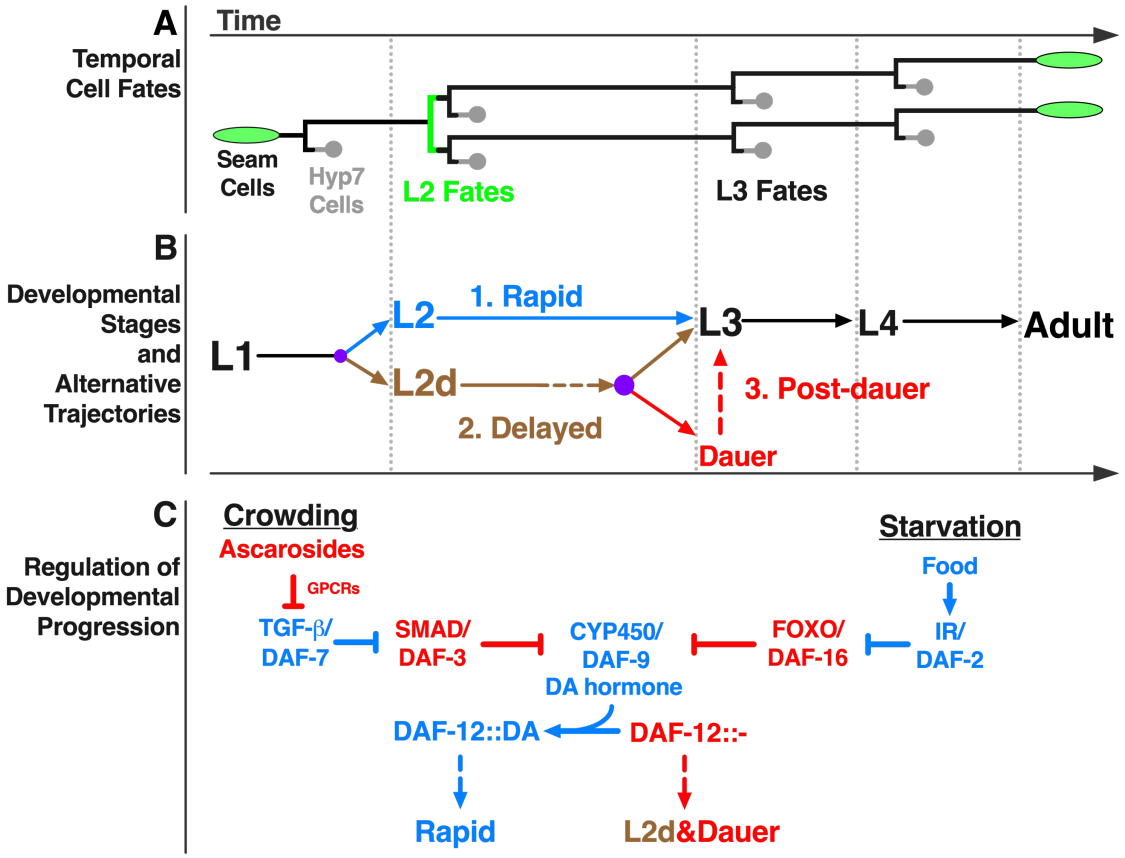

A
Temporal Cell Fates
Time
Seam Cells
Hyp7 Cells
L2 Fates
L3 Fates
B
Developmental Stages and Alternative Trajectories
L1
L2
1. Rapid
L2d
2. Delayed
L3
L4
Adult
3. Post-dauer
Dauer
C
Regulation of Developmental Progression
Crowding
Ascarosides
GPCRs
TGF-β/ DAF-7
SMAD/ DAF-3
CYP450/ DAF-9
DA hormone
FOXO/ DAF-16
Starvation
Food
IR/ DAF-2
DAF-12::DA
DAF-12::-
Rapid
L2d&Dauer

Figure 2.1. Temporal Fates of Hypodermal Seam Cells Are Robust against Changes in Developmental Trajectory Induced by Crowding or Starvation

(A) Lineage diagram showing temporal (stage-specific) hypodermal seam cell fates. Seam cells (green) divide asymmetrically at each larval stage, renewing themselves while giving rise to hyp7 cells (gray). At the L2 stage, seam cells undergo a single round of symmetric cell division, resulting in an increase in their number. Note that only six out of ten seam cells undergo symmetric cell division, which increases the total number of seam cells on each side of the worm from ten to sixteen.

(B) Developmental stages and three distinct developmental trajectories: (1) continuous, unipotent, and rapid progression define the L2 trajectory (blue); (2) continuous but bipotent and delayed progression define the L2d trajectory (brown); and (3) developmental progression is interrupted by a diapause in the dauer-interrupted trajectory (brown followed by red). The time axis indicates the order of events in time (not proportional to absolute time). Vertical dotted lines indicate the molts between stages. Purple dots represent decision points between different trajectory options.

(C) Regulation of developmental progression. Under favorable conditions, dafachronic acid (DA) hormone is abundant, and DA-bound DAF-12 promotes rapid development. Crowding or starvation induces L2d and dauer formation by repressing TGF-β/DAF-7 signals or insulin signaling (IR/DAF-2: insulin receptor), respectively. Activated effectors of these signaling pathways (DAF-3 or DAF-16) inhibit the biosynthesis of DA, and the unliganded DAF-12 interacts with DIN-1S, which together promote L2d and dauer formation.

and environmental factors (Ren and Ambros, 2015), and can be quantified by counting the number of seam cells in young adult worms.

C. elegans is a free-living nematode whose environment is prone to fluctuations between conditions that are favorable and unfavorable for completion of development (Frézal and Félix, 2015). Under favorable conditions (such as abundant food) *C. elegans* larvae develop rapidly and continuously progress through the four larval stages to the adult (Figure 2.1B, rapid). However, when the conditions are not favorable (for example, in the face of declining resources owing to high population density), the larva at the end of the L2 stage can elect to enter a developmentally arrested diapause, called the dauer larva, which is non-feeding, stress-resistant, and long-lived (Hu, 2007). When conditions improve, the dauer larva resumes development to the reproductive adult (Figure 2.1B, post-dauer). The DAF-7/TGF-β and DAF-2/insulin endocrine signaling pathways are the two major signaling pathways that regulate C. elegans dauer larva diapause. These two pathways act in parallel to integrate information about population density and nutritional status by co-modulating the biosynthesis of the dafachronic acid (DA) hormone. DA is the ligand of a nuclear hormone receptor, DAF-12, which opposes dauer formation when it is DA-bound and forms a repressor complex with DIN-1S and promotes dauer formation when it is unliganded (Figure 2.1C) (Fielenbach and Antebi, 2008).

The order and sequence of temporal cell fates in the various *C. elegans* larval cell lineages are robustly maintained regardless of developmental trajectory:

for example, blast cells properly transition from L2 to L3 fates whether the larva develops rapidly and continuously, or instead traverses dauer diapause, which imposes a lengthy (even months-long) interruption of the L2 to L3 transition (Figure 2.1A&B). Interestingly, cell fate transition defects of many heterochronic mutants are modified (either suppressed or enhanced) when larval development is interrupted by dauer diapause, suggesting that the genetic regulatory pathways regulating temporal cell fate progression are modified depending on whether the animal develops continuously vs undergoes dauer-interrupted development (Liu and Ambros, 1991; S Euling and Ambros, 1996; Karp and Ambros, 2012). The mechanisms by which temporal cell fate specification pathways are modified in association with the dauer larva trajectory are poorly understood, especially with regards to how modifications to the regulatory networks controlling temporal cell fate transitions may be coupled to particular steps in the specification and/or execution of the dauer larva diapause trajectory. Of particular interest is the question of whether and how the dauer-promoting signals that are monitored by L1 and L2d larvae might act prior to dauer commitment to directly modify gene regulatory mechanisms controlling temporal cell fate transitions.

To investigate the impact of dauer-inducing environmental and endocrine signals on the regulatory network controlling temporal cell fate transitions, we employed experimental conditions that induce the dauer formation program, but also efficiently prevent dauer commitment. We call these conditions “L2d-inducing” because the presence of both dauer-inducing and commitment-preventing

conditions results in worm populations growing continuously (without dauer arrest) but where all animals traverse the lengthened bi-potential L2d stage (Figure 2.1B, L2d/delayed) (Golden and Riddle, 1984; Avery, 2014).

We found that L2d-inducing pheromones suppress heterochronic defects caused by insufficient expression of *let-7* family microRNAs, suggesting that these pheromones that enable the dauer life history option also activate a program alternative to *let-7* family microRNAs in controlling stage-specific temporal cell fate progression. We found that the two major endocrine signaling pathways that regulate dauer formation in response to pheromones and food signals, the DAF-7/TGF-β and DAF-2/Insulin respectively, also mediate the effect of these same signals on temporal cell fates under L2d-inducing conditions. Moreover, we identified a previously undescribed ligand-independent activity of the nuclear hormone receptor DAF-12 that is responsible for activating the alternative program of cell fate specification in the L2d. This alternative program is responsible for correcting *let-7* family insufficiency phenotypes and it requires the activities of certain heterochronic genes, *lin-46, lin-4* and *nhl-2,* that are previously associated with an altered genetic program in post-diapause animals. This alternative program associated with L2d is coupled to a previously described reduction in the DAF-12-regulated expression of *let-7* family microRNAs (Hammell, Karp and Ambros, 2009). Hence, the overall L2d response is a “rewiring” program consisting two major operations: 1) repression of *let-7* family microRNA expression, and 2)

activation of an alternative program to downregulate the *let-7* family target (Hunchback-like-1) HBL-1.

Our results show that environmental signals and downstream endocrine signaling pathways are capable of coordinately regulating developmental progression and cell fate transitions in *C. elegans*. We propose that this capability confers elasticity to *C. elegans* development, whereby the proper developmental schedule of cell fates remains unaffected by changes or uncertainties in developmental trajectory.

Results

We developed three approaches to efficiently uncouple L2d from dauer commitment and thereby produce worm populations developing continuously through the bi-potential pre-dauer L2d phase directly to the L3, without dauer arrest. In the first approach, we employed the pheromone cocktail formula described by Butcher *et al.* (Butcher *et al.*, 2008), which contains three ascaroside molecules (ascr#2, ascr#3, and ascr#5) that synergistically induce L2d and dauer arrest (Butcher *et al.*, 2008) (Figure 2.2). At sufficiently high doses, the ascaroside cocktail can induce 100% dauer formation (Figure 2.2C). Previous findings showed that the presence of food can antagonize pheromones and prevent dauer formation (Golden and Riddle, 1984). However, it was not clear if the food signals also prevented the L2d. We observed that the presence of live bacteria food, or the presence of dafachronic acid (DA) hormone, could efficiently prevent dauer formation while not preventing the L2d, evidenced by dramatically

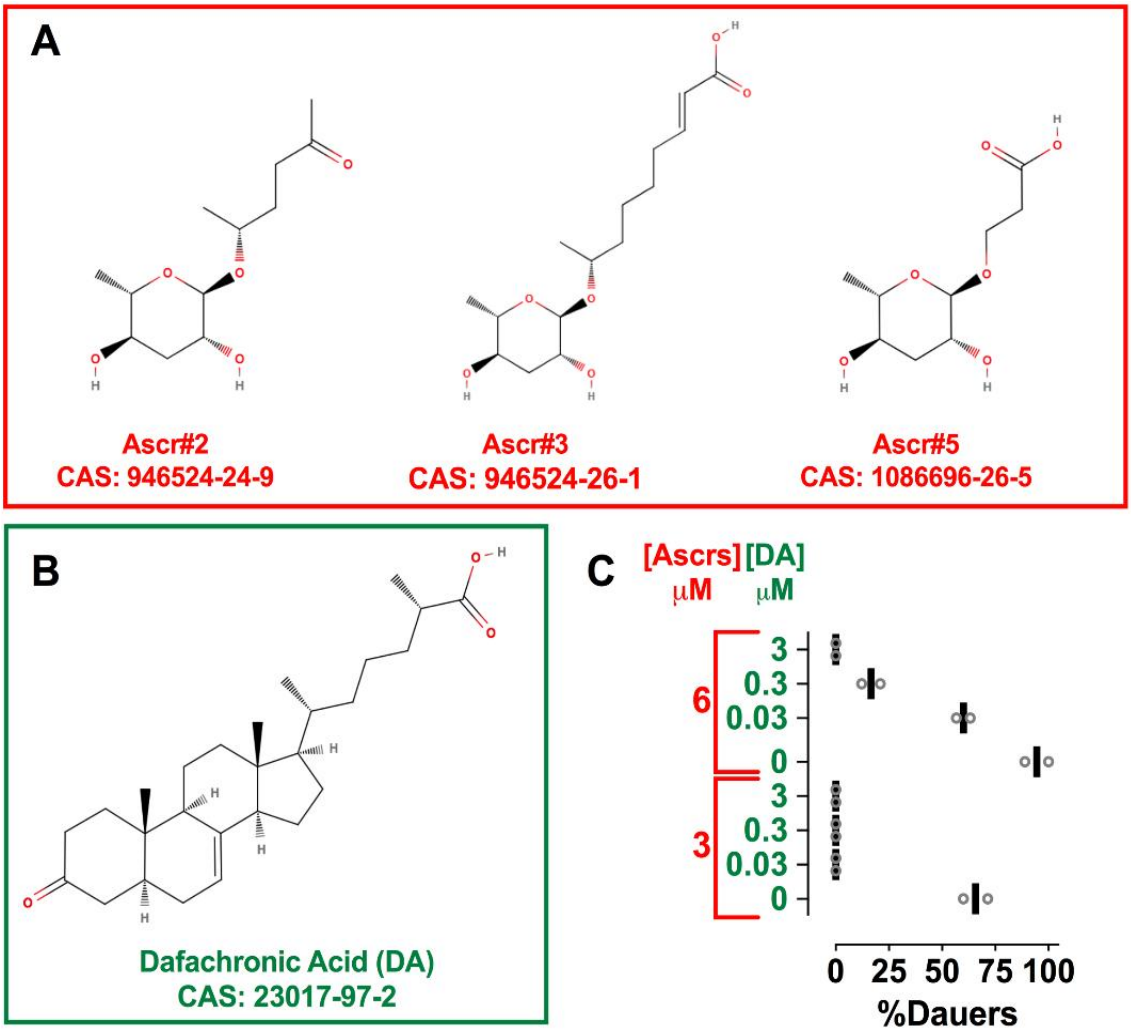

A
Ascr#2
CAS: 946524-24-9
Ascr#3
CAS: 946524-26-1
Ascr#5
CAS: 1086696-26-5
B
Dafachronic Acid (DA)
CAS: 23017-97-2
C
[Ascrs] μM
[DA] μM
6
3
0.3
0.03
0
3
3
0.3
0.03
0
0 25 50 75 100
%Dauers

Figure 2.2. Chemicals and conditions used to obtain worm populations developing continuously through L2d trajectory.

(A-B) Molecular structures (using www.molview.org), names, and CAS numbers of the chemicals used in the ascaroside (dauer-inducing), and ascaroside plus dafachronic acid (both dauer-inducing and dauer commitment inhibiting) plates.

(C) Two different concentrations of Ascrs assayed in combination with four different concentrations of dafachronic acid (DA) to determine conditions that prevent dauer formation in the presence of ascarosides. Percent dauer formation in the presence of different combinations of ascarosides (Ascrs: equimolar mixture of ascr#2, ascr#3, and ascr#5) and DA are plotted. DA inhibits dauer commitment but not L2d, which is evident by slowing of larval development. The combination of 3 μM of Ascrs and 0.03 μM DA was used as the L2d- inducing (Ascrs+DA) condition to test the effect of the L2d trajectory on the number of seam cells in wild-type and *mir-48/84/241* animals (Figure 2.3).

slowed second stage larval development. Therefore, to obtain animal populations traversing the L2d without committing to dauer arrest, we allowed larvae to develop in the presence of a combination of the ascaroside cocktail along with DA hormone (Motola *et al.*, 2006) (Figure 2.2B and 2.2C). Our second approach also uses the ascaroside cocktail, but to eliminate the need for DA hormone, dauer-commitment defective *daf-12(rh61)* mutant worms are employed. In the third approach, to genetically induce L2d, we combined *daf-12(rh61)* with a temperature-sensitive *daf-7* mutant that mimics the L2d-inducing pheromone conditions, or with a temperature-sensitive *daf-2* mutant that mimics the L2d-inducing starvation conditions.

L2d-inducing ascarosides reduce the reliance on the *let-7*-family microRNAs for proper L2-to-L3 cell fate transition

Wild-type larvae robustly execute L2 stage cell fates and transition to L3 stage cell fates (thus # of seam cell=16 in young adult worms) regardless of developmental trajectory (Figure 2.3, rows 1-3). *mir-48/84/241(0)* mutant larvae reiterate L2 stage cell fates at later stages due to prolonged HBL-1 expression, resulting in extra (>16) seam cells in young adult animals (Figure 2.3, row 4). We found that when *mir-48/84/241(0)* mutant larvae developed through L2d -- induced by a combination of the ascaroside cocktail and the DA hormone -- the extra seam cell phenotype was substantially (albeit partially) suppressed (Figure 2.3, row 4 vs 6). To compare the strength of this L2d suppression with the previously described post-dauer suppression of the *let-7* family phenotypes (Karp and Ambros, 2012),

we used the ascaroside cocktail but this time without the DA hormone. Under these conditions *mir-48/84/241(0)* larvae arrested as dauers; and as described previously (Karp and Ambros, 2012) this resulted in complete suppression of the extra seam cell phenotype in post-dauer adults (Figure 2.3, row 4 vs 5). Therefore, the L2d suppression is weaker than the post-dauer suppression (Figure 2.3, row 6 vs 5), and unlike dauer arrest, L2d-inducing ascarosides do not completely eliminate the need for *let-7* family microRNAs for proper L2-to-L3 cell fate transition. Nonetheless, the partial suppression of the extra seam cell phenotype of *let-7* family microRNAs suggests that the L2d-inducing ascarosides rewire the genetic regulatory pathway controlling temporal cell fate progression in a way to reduce the reliance on the *let-7* family microRNAs for proper L2-to-L3 cell fate transition.

L2d-inducing ascarosides or L2d-inducing mutations of *daf-7* and *daf-2* suppress heterochronic phenotypes caused by insufficient expression of *let-7* family microRNAs in *daf-12(rh61)* mutants

The *daf-12(rh61)* mutation combines three important properties which makes this mutation uniquely useful for studying the effects of L2d-inducing conditions on the regulation of temporal cell fates. These properties are: 1) *daf-12(rh61)* animals reiterate expression of L2 cell fates owing to reduced (insufficient) *let-7* family levels, 2) *daf-12(rh61)* larvae are unable to execute dauer larvae commitment or arrest (Antebi *et al.*, 2000), enabling the use of dauer-promoting conditions to obtain populations of *daf-12(rh61)* animals undergoing an

L2d-direct-to-L3 continuous development trajectory; 3) *daf-12(rh61)* animals are insensitive to the DA hormone (due to lack of the DAF-12 ligand binding domain) (Antebi *et al.*, 2000; Motola *et al.*, 2006), and so the levels of *let-7* family microRNAs are expected to be unresponsive to experimentally administered ascarosides, which are understood to regulate wild type DAF-12 activity by affecting the level of DA (Fielenbach and Antebi, 2008).

We observed that the presence of exogenous ascaroside cocktail during larval development almost completely suppressed the extra seam cell phenotype of *daf-12(rh61)* mutants (Figure 2.3, row 7 vs 8). To test the possibility that an unexpected elevation in the *let-7* family levels could be responsible for the suppression of the heterochronic phenotypes of *daf-12(rh61)* animals in the presence of the ascaroside cocktail, we quantified the levels of *let-7* family microRNAs in the absence and presence of the ascarosides (Figure 2.4). No elevation in the levels of these microRNAs in response to the ascaroside cocktail was evident (Figure 2.4). Therefore, the suppression of the heterochronic phenotypes of *daf-12(rh61)* mutants in the presence of the ascaroside cocktail is unlikely to result from restoration of normal levels of *mir-48/84/241* or an elevation of the other members of the *let-7* family microRNAs (Figure 2.4).

Similar to the ascaroside cocktail, conditional dauer-constitutive mutants of *daf-7* (mimicking high ascarosides) or *daf-2* (mimicking starvation) that allow continuous (L2d-to-L3 without dauer arrest) development at permissive temperatures (Swanson and Riddle, 1981), almost completely suppressed the

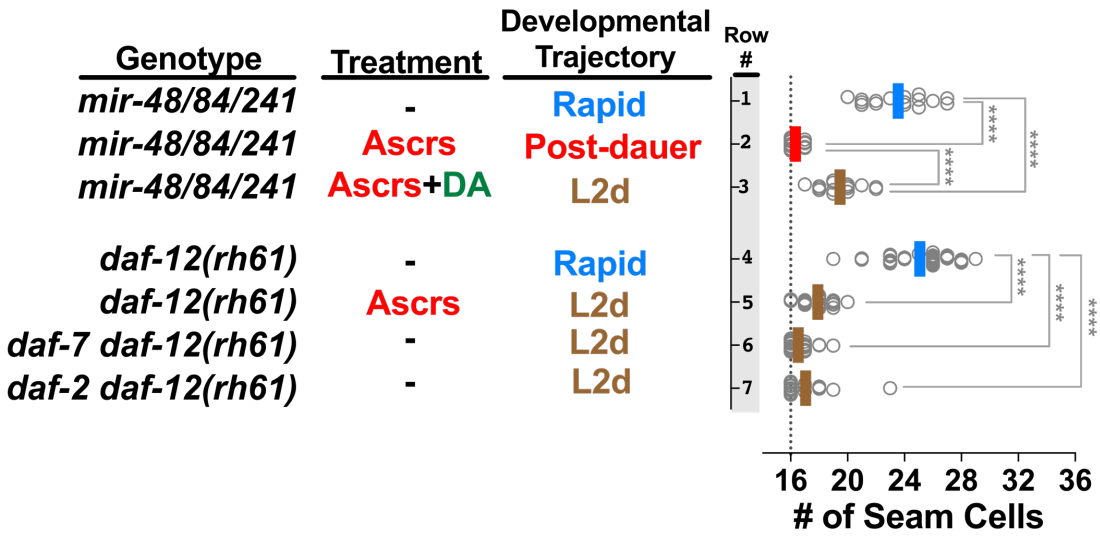

Genotype
Treatment
Developmental Trajectory
Row #
mir-48/84/241
-
Rapid
mir-48/84/241
Ascrs
Post-dauer
mir-48/84/241
Ascrs+DA
L2d
daf-12(rh61)
-
Rapid
daf-12(rh61)
Ascrs
L2d
daf-7 daf-12(rh61)
-
L2d
daf-2 daf-12(rh61)
-
L2d
1
2
3
4
5
6
7
****
16
20
24
28
32
36
# of Seam Cells

Figure 2.3. Sensitized Genetic Backgrounds Reveal that L2d-Inducing Environmental and Endocrine Signals Impact the Regulation of Temporal Cell Fates

Genotypes are indicated in the first column; treatments and corresponding developmental trajectories are indicated in the second and third columns, respectively. Each dot in the plots to the right shows the number of seam cells on one side (left side or right side, observed interchangeably) of a single young adult animal, and solid lines (color code matching the developmental trajectory) indicate the average seam cell number of the animals scored for each condition. Wild-type animals have sixteen seam cells per side (vertical dotted line), regardless of developmental trajectory (lines 1–3). Experiments involving temperature-sensitive alleles of *daf-2* and *daf-7* (lines 9 and 10) are performed at a permissive temperature (20° C) that allows continuous (L2d-to-L3 without dauer arrest) development. The Student's t test is used to calculate statistical significance (p): n.s. (not significant) $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

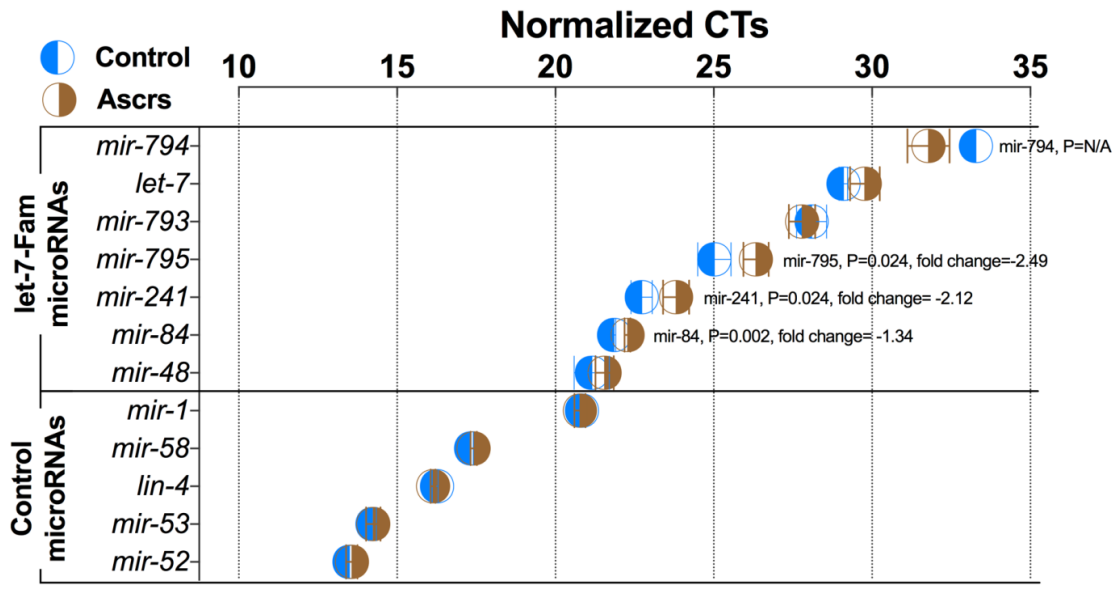

Normalized CTs
Control
Ascrs
10
15
20
25
30
35
let-7-Fam microRNAs
mir-794
let-7
mir-793
mir-795
mir-241
mir-84
mir-48
Control microRNAs
mir-1
mir-58
lin-4
mir-53
mir-52
mir-794, P=N/A
mir-795, P=0.024, fold change=-2.49
mir-241, P=0.024, fold change= -2.12
mir-84, P=0.002, fold change= -1.34

Figure 2.4. Ascarosides do not result in an increase in *let-7* family levels in *daf-12(rh61)* background.

let-7 family microRNAs in L2-to-L3 (control) vs. L2d-to-L3 (Ascrs) molting larvae of the *daf-12(rh61)* mutant were quantified using Taqman assays as described in the STAR methods. MicroRNAs that are highly expressed and not environmentally regulated were used as the normalization set (Control MicroRNAs). The expression levels of three *let-7* family microRNAs (*mir-84, mir-241, mir-795*) were slightly but statistically significantly reduced in the presence ascarosides (L2d-to-L3 molt). This reduction of *let-7* family levels is in contrast with the observed suppression of retarded heterochronic phenotypes of *daf-12(rh61)* in the presence of ascarosides. The lack of an upregulation of *let-7* family microRNAs in the presence of ascarosides is in line with the idea that an alternative, *let-7*-independent, mechanism is responsible for the ascaroside-mediated suppression of the heterochronic phenotypes. *mir-794* was not detected in two biological control samples (presumably due to low expression level); therefore, we do not know if there is a statistically significant up-regulation of *mir-794* in the presence of ascarosides.

extra seam cell phenotype of *daf-12(rh61)* mutants (Figure 2.3, row 7 vs 9 or 10). These results indicate that genetically induced L2d, whether by activation of the ascaroside response pathway (*daf-7(lf)*), or by activating the starvation response pathway (*daf-2(lf)*), results in an L2d-associated rewiring of the regulatory networks controlling temporal cell fate progression.

Ascarosides suppress the heterochronic phenotypes of *daf-12(rh61)* via *srg-36/37*-encoded GPCR signaling upstream of DAF-7/TGF-β-DAF-3 signaling

Each of the individual ascarosides in the cocktail (Ascrs#2,3,5) has been shown previously to be alone sufficient to induce dauer formation, although with reduced potency compared to the combined cocktail (Butcher *et al.*, 2007, 2008). Consistent with their individual capacities to induce L2d and dauer formation, we observed that each ascaroside ascr#2, ascr#3, and ascr#5 alone could suppress the extra seam cell phenotype of *daf-12(rh61)* mutants (Figure 2.5A). In the case of ascr#2 or ascr#3 alone, the suppression was partial, while for ascr#5 alone, the suppression was similar to the full cocktail (Figure 2.5A, rows 6 to 10). ascr#5 was the most potent of the three ascarosides in terms of both percent dauer formation of wild type larvae (Figure 2.5B) and suppression of the extra seam cell phenotype of *daf-12(rh61)* (Figure 2.5A, row 10 vs 8 and 9).

It has been shown previously that induction of dauer formation by ascarosides involves sensing of environmental ascaroside levels by specific G-protein coupled receptors (GPCRs) expressed in chemosensory neurons, wherein

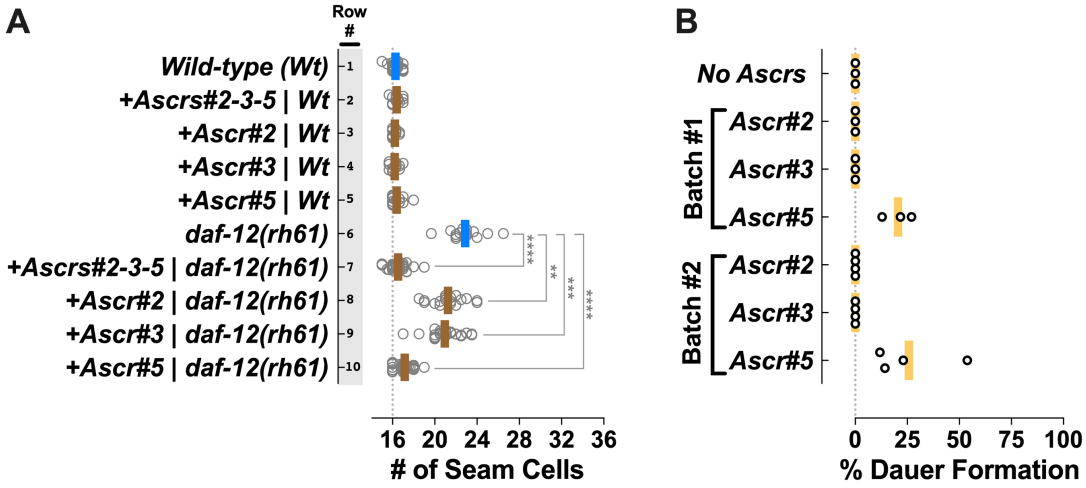

A
Row #
Wild-type (Wt)
+Ascrs#2-3-5 | Wt
+Ascr#2 | Wt
+Ascr#3 | Wt
+Ascr#5 | Wt
daf-12(rh61)
+Ascrs#2-3-5 | daf-12(rh61)
+Ascr#2 | daf-12(rh61)
+Ascr#3 | daf-12(rh61)
+Ascr#5 | daf-12(rh61)
1
2
3
4
5
6
7
8
9
10
****
**
***
****
16 20 24 28 32 36
# of Seam Cells
B
No Ascrs
Batch #1
Ascr#2
Ascr#3
Ascr#5
Batch #2
Ascr#2
Ascr#3
Ascr#5
0 25 50 75 100
% Dauer Formation

Figure 2.5. Testing of individual components of the pheromone cocktail for their potencies to suppress the extra seam cell phenotypes of *daf-12(rh61)* and to induce dauer formation

(A) Individual components of the pheromone cocktail can suppress the extra seam cell phenotypes of *daf- 12(rh61)* and ascr#5 is the most potent suppressor. Number of seam cells in young adult animals cultured under different ascaroside conditions are plotted. Each dot in the plot shows the number of seam cells of a single young adult animal, and solid lines (blue: rapid trajectory; brown: delayed [l2d] trajectory) indicate the average seam cell number of the animals scored for each condition. Ascrs#2-3-5 plates contained all three ascarosides at 3 μM final concentration of each ascaroside mixed in NGM-agarose media. Ascrs#2, Ascr#3, and Ascr#5 plates contained 3 μM final concentration of each ascaroside mixed in NGM-agarose media. The student's t-test is used to calculate statistical significance (p): n.s. (not significant) $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (B) Ascr#5 alone can induce dauer formation. At 3.3 μM concentration, using agarose NGM plates with no peptone, seeded with washed and concentrated *E. coli* OP50 culture as described in the materials and methods, Ascr#5 alone was sufficient to induce dauer formation but not Ascr#2 or Ascr#3. We tested single ascarosides in two different batches of plates and using three or four replicates. Both experiments were performed at 20°C. Each dot on the plots shows percent dauer formation on a single plate. We maintained population sizes small (<55 worms per plate) and comparable across different Ascr plates to minimize the potential effect of the accumulation of ascarosides secreted by the worms on the plates.

they repress DAF-7/TGF-β signals (Ren *et al.*, 1996; Kim *et al.*, 2009; McGrath *et al.*, 2011; Park *et al.*, 2012). To test whether these GPCRs were also required for the suppression of the heterochronic phenotypes of *daf-12(rh61)* mutants, we employed mutations of *srg-36* and *srg-37*, which encode GPCRs that are expressed in the ASI neurons and that are redundantly required for perceiving ascr#5 signal in the context of dauer induction (McGrath *et al.*, 2011). We observed that for *srg-36(0) srg-37(0); daf-12(rh61)* compound mutants, ascaroside (in this case ascr#5) failed to suppress the extra seam cell phenotype *daf-12(rh61)* (Figure 2.6A, compare row 1 vs 2 with row 3 vs 4). Moreover, we found that the TGF-β signaling effector *daf-3*, which is thought to function downstream of SRG-36/37, is required for the suppression of *daf-12(rh61)* by asaroside (Figure 2.6B). These results indicate that the same GPCRs that mediate dauer formation in response to asaroside are also required for mediating the effects of asaroside on temporal cell fates, and supports a common pathway for suppression of *daf-12(rh61)* by ascaroside and dauer induction, involving activation of SRG-36/37 GPCRs and the potential downstream TGF-β effector DAF-3.

DAF-7/TGF-β and DAF-2/Insulin signaling pathways act in parallel to mediate the suppression of the heterochronic phenotypes of *daf-12(rh61)*

As shown above, genetic activation of dauer-inductive signaling by *daf-7(lf)* or *daf-2(lf)* mutations is sufficient for suppression of *daf-12(rh61)* (Figure 2.3). In the context of dauer formation, DAF-7/TGF-β primarily mediates ascaroside signaling, and DAF-2/Insulin primarily mediates assessment of nutritional status

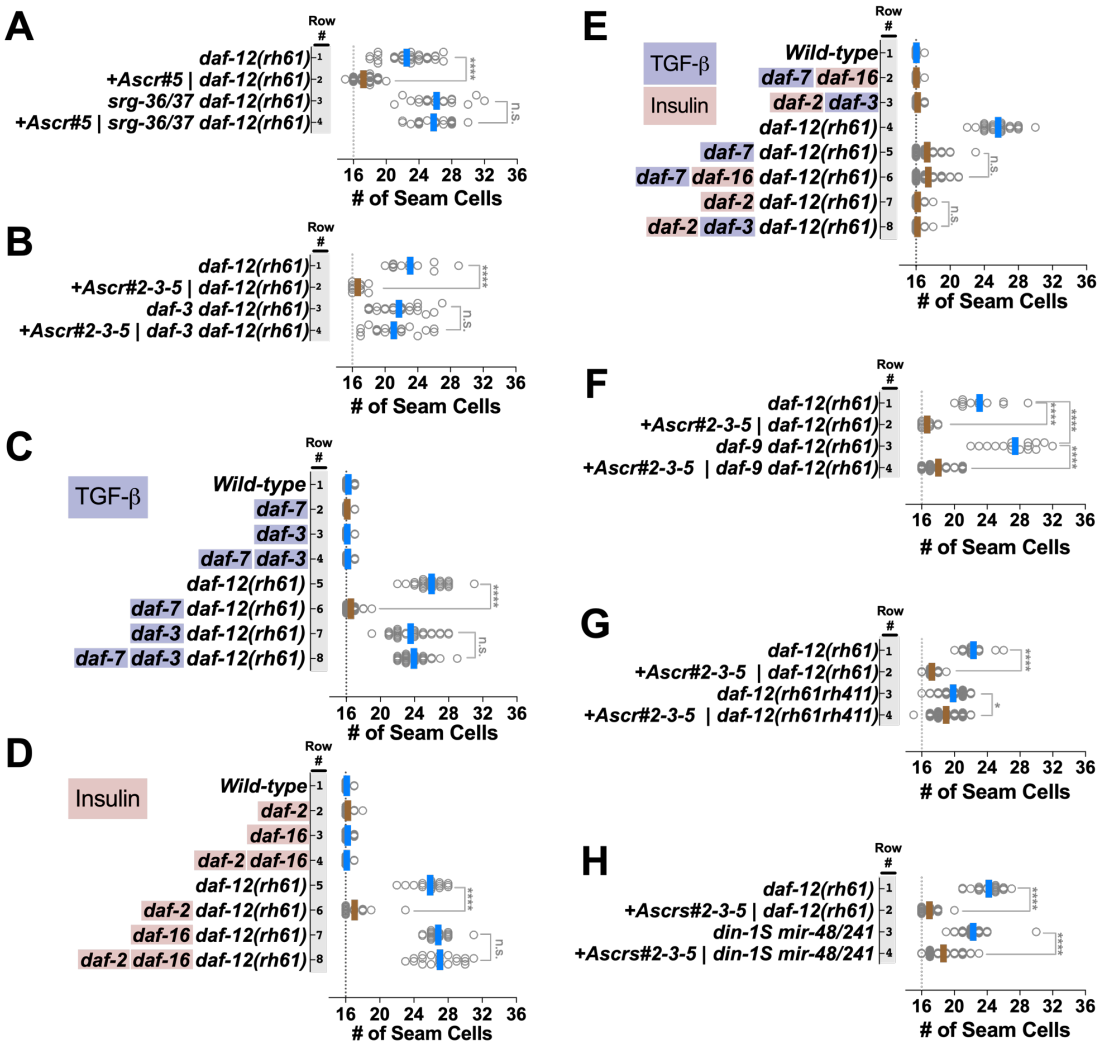

A
daf-12(rh61)
+Ascr#5 | daf-12(rh61)
srg-36/37 daf-12(rh61)
+Ascr#5 | srg-36/37 daf-12(rh61)
B
daf-12(rh61)
+Ascr#2-3-5 | daf-12(rh61)
daf-3 daf-12(rh61)
+Ascr#2-3-5 | daf-3 daf-12(rh61)
C
TGF-β
Wild-type
daf-7
daf-3
daf-7 daf-3
daf-12(rh61)
daf-7 daf-12(rh61)
daf-3 daf-12(rh61)
daf-7 daf-3 daf-12(rh61)
D
Insulin
Wild-type
daf-2
daf-16
daf-2 daf-16
daf-12(rh61)
daf-2 daf-12(rh61)
daf-16 daf-12(rh61)
daf-2 daf-16 daf-12(rh61)
E
TGF-β
Insulin
Wild-type
daf-7 daf-16
daf-2 daf-3
daf-12(rh61)
daf-7 daf-12(rh61)
daf-7 daf-16 daf-12(rh61)
daf-2 daf-12(rh61)
daf-2 daf-3 daf-12(rh61)
F
daf-12(rh61)
+Ascr#2-3-5 | daf-12(rh61)
daf-9 daf-12(rh61)
+Ascr#2-3-5 | daf-9 daf-12(rh61)
G
daf-12(rh61)
+Ascr#2-3-5 | daf-12(rh61)
daf-12(rh61rh411)
+Ascr#2-3-5 | daf-12(rh61rh411)
H
daf-12(rh61)
+Ascrs#2-3-5 | daf-12(rh61)
din-1S mir-48/241
+Ascrs#2-3-5 | din-1S mir-48/241
Row #
16 20 24 28 32 36
# of Seam Cells
****
n.s.
*

Figure 2.6. DAF-7/TGF-β and DAF-2/Insulin Signaling Pathways Act in Parallel to Modulate a Ligand-Independent Activity of DAF-12 That Is Responsible for Correcting Heterochronic Phenotypes Caused by Insufficient Expression of let-7 Family MicroRNAs

Number of seam cells in young adult animals of various mutants cultured on ascaroside or control plates (A, B and F, G), or on standard NGM plates (C–E): each dot in the plots shows the number of seam cells of a single young adult animal, and solid lines indicate the average seam cell number of the animals scored for each condition (blue lines, rapid trajectory; brown lines, L2d trajectory).

(A) Ascarosides suppress *daf-12(rh61)* via *srg-36/37*-encoded GPCR signaling upstream of DAF-7/TGF-β-DAF-3 signaling.

(B) *daf-3* activity is required for suppression of *daf-12(rh61)* by ascarosides.

(C–E) DAF-7/TGF-β and DAF-2/Insulin signaling pathway act in parallel to mediate the suppression of *daf-12(rh61).*

(F and G) Ligand-independent activity of *daf-12* is required for the ascaroside-mediated L2d rewiring of the pathways regulating temporal cell fates.

(H) The DAF-12 corepressor DIN-1S is not required for the ascaroside-mediated suppression of heterochronic phenotypes caused by insufficient expression of *let-7*-family microRNAs. Suppression of extra seam cell phenotype of *daf-12(rh61)* is shown as a measure of the strength of the ascaroside conditions tested for *din-1S(lf); mir-48/241(lf) animals*.

The Student's t test is used to calculate statistical significance (p): n.s. (not significant) $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

(Ren *et al.*, 1996; Kimura *et al.*, 1997; Fielenbach and Antebi, 2008). To determine if the known downstream effectors of DAF-7/TGF-β and DAF-2/Insulin signaling that mediate dauer formation are also required for mediating the L2d rewiring caused by *daf-7(lf)* or *daf-2(lf)* (Ogg *et al.*, 1997; Patterson *et al.*, 1997), we generated compound mutants carrying *daf-12(rh61)* in combination with mutations that impair these effectors of the TGF-β or insulin signaling pathways, and determined the number of seam cells in young adults. We found that the downstream effector of the TGF-β signaling pathway, *daf-3*, and the downstream effector of the insulin signaling pathway, *daf-16*, were required for the suppression mediated by the *daf-7(lf)* mutation and the *daf-2(lf)* mutation, respectively (Figure 2.6C and 2.6D). These results are consistent with the finding that *daf-3* was also required for the ascaroside-mediated suppression of *daf-12(rh61)* (Figure 2.6B).

To determine whether the TGF-β and insulin signaling pathways act in parallel to modulate temporal cell fates, we tested for crosstalk between these pathways in the context of suppression of *daf-12(rh61)* phenotypes. Specifically, we determined whether *daf-16(lf)* could alter the suppression of *daf-12(rh61)* phenotypes by *daf-7(lf)*, and conversely, whether *daf-3(lf)* could alter the suppression of *daf-12(rh61)* phenotypes by *daf-2(lf)*. We found that *daf-16* was not required for *daf-7*-mediated suppression (Figure 2.6E, rows 5-6), and *daf-3* was not required for *daf-2*-mediated suppression (Figure 2.6E, rows 7-8), indicating that, similar to their regulation of dauer diapause, the TGF-β and insulin signaling

pathways act in parallel in the context of the L2d rewiring of the genetic regulatory pathways controlling larval cell fate progression.

Ligand-independent activity of *daf-12* is required for the ascaroside-mediated L2d rewiring of the pathways regulating temporal cell fates

Ascaroside (TGF-β) signaling and nutritional status (insulin) signaling converge to induce dauer larva arrest by down regulating DA production and hence reducing the levels of liganded DAF-12. Since we find that dauer-inducing conditions (ascarosides; loss of *daf-7* or *daf-2*) can suppress the heterochronic phenotypes of the DA-insensitive *daf-12(rh61)* mutant, it would appear that the TGF-β and insulin signaling pathways may regulate cell fate transitions by repressing a hypothetical DAF-12-independent function of DA. If that were the case, inhibiting or preventing DA production would mimic the effect of ascarosides and suppress the extra seam cell phenotype of *daf-12(rh61).* To test this possibility, we employed genetic ablation of DA production. *daf-9* encodes a CYP450 that is responsible for DA production (Motola *et al.*, 2006). Accordingly, *daf-9(lf)* mutants are dauer-constitutive due to lack of DA (Motola *et al.*, 2006). To test if ascarosides act by inhibiting DA production during L2d rewiring, we generated double mutants containing *daf-9(lf)* and *daf-12(rh61).* We observed that these double mutants lacking *daf-9* in the *daf-12(rh61)* background had an even stronger extra seam cell fate phenotype than *daf-12(rh61)* mutants (Figure 2.6F, row 1 vs 3), and that this phenotype was suppressed in the presence of ascarosides (Figure 2.6F, row 3 vs 4). These results indicate that the ascaroside-

induced L2d rewiring does not involve inhibition of DA biosynthesis, nor does rewiring require DA production or *daf-9* activity. The enhancement of the extra seam cell phenotype of *daf-12(rh61)* phenotype in the *daf-12(rh61)*; *daf-9(lf)* double mutant could reflect DAF-12-independent functions of DA or DA-independent functions of DAF-9.

The finding that ascaroside-mediated L2d rewiring did not involve the DA hormone raised the question as to whether the DA receptor, DAF-12 is required for the L2d rewiring. To determine whether *daf-12* is required for ascaroside-induced suppression of retarded seam cell phenotypes, we tested whether ascarosides could suppress the phenotypes of *daf-12(rh61rh411),* a *daf-12* null allele (Antebi *et al.*, 2000). *daf-12(rh61rh411)* animals display a milder extra seam cell phenotype than *daf-12(rh61)* (Antebi *et al.*, 2000), presumably because of a milder reduction of *let-7* family microRNAs compared to *daf-12(rh61)* (Hammell, Karp and Ambros, 2009). We observed that the ascaroside conditions that resulted in a very potent suppression of the extra seam cell phenotype of *daf-12(rh61)* animals resulted in only a very modest (albeit statistically significant) suppression of the *daf-12(rh61rh411)* phenotype (Figure 2.6G, compare changes in the average number of seam cells in row 1 vs 2 with 3 vs 4). This result suggests that ascaroside-induced L2d rewiring of the pathways regulating temporal cell fates largely requires *daf-12* function, and therefore represents a novel ligand-independent regulation of *daf-12* by TGF-β and insulin signaling.

The DAF-12 corepressor DIN-1S is not required for the ascaroside-mediated suppression of heterochronic phenotypes caused by insufficient expression of *let-7* family microRNAs

When DA is absent, DAF-12 interact with DIN-1S, and together they form a repressive complex that is necessary for dauer formation (Ludewig *et al.*, 2004; Motola *et al.*, 2006). *din-1S(lf)* suppresses heterochronic phenotypes of *daf-12(rh61)* (Ludewig *et al.*, 2004), likely by relieving repression of *let-7* family microRNA transcription. Therefore, it was possible that the ascaroside-mediated suppression of the heterochronic phenotypes of *daf-12(rh61)* could reflect ascaroside-induced down regulation of *din-1S* activity. To assess the potential involvement of *din-1S* in ascaroside-mediated suppression of the phenotypes caused by reduced *let-7* family microRNAs, we tested for ascaroside suppression of a compound mutant lacking *mir-48/241* and *din-1S* (Figure 2.6H). We observed that *din-1(lf)* did not prevent ascaroside suppression of the *mir-48/241* extra seam cell phenotypes (Figure 2.6H, row 3 vs 4), indicating that DIN-1S is not required for ascaroside-mediated L2d rewiring.

Heterochronic genes previously associated with the altered HBL-1 down-regulation program in post-dauer animals are required for the L2d suppression of heterochronic phenotypes caused by insufficient expression of *let-7* family microRNAs

In animals that arrested as dauer larvae and then later resumed development through post dauer larval stages, the genetic programming of

temporal cells fates differed substantially from animals that developed continuously (Karp and Ambros, 2012). In particular, proper cell fate progression through dauer larvae arrest and post-dauer development rests on an altered HBL-1 down-regulation program. These differences in HBL-1 down regulation include, 1) reallocation of roles for *lin-4* microRNA and *let-7* family microRNAs, and 2) alterations in the relative impacts of LIN-46 and the microRNA modulatory factor NHL-2 (Karp and Ambros, 2012). For example, animals deficient for *lin-4* exhibited stronger retarded developmental timing phenotypes when traversing developmental arrest followed by post-dauer development compared to animals of the same genotype that developed rapidly and continuously. Similarly, animals carrying loss of function mutations of *nhl-2* or *lin-46* exhibited enhanced retarded phenotypes after post dauer development. *nhl-2* encodes an RNA binding protein that functions as a microRNA positive modulator (Hammell *et al.*, 2009), and *lin-46* encodes a protein that acts downstream of the LIN-28 RNA binding protein (Pepper *et al.*, 2004). These results suggested that the rewiring of developmental cell fate progression in dauer-traversing larvae involves alterations in the post-transcriptional regulation of HBL-1 expression.

To confirm that L2d inducing conditions resulted in HBL-1 down-regulation, we tagged *hbl-1* at its endogenous locus with mScarlet-I using CRISPR/Cas9 genome editing (See Materials and Methods), and monitored the level of HBL-1::mScarlet-I expression in developing larvae. We compared HBL-1 expression in L2 and L3 stage *daf-12(rh61)* larvae to L2d and L3 stage larvae of the suppressed

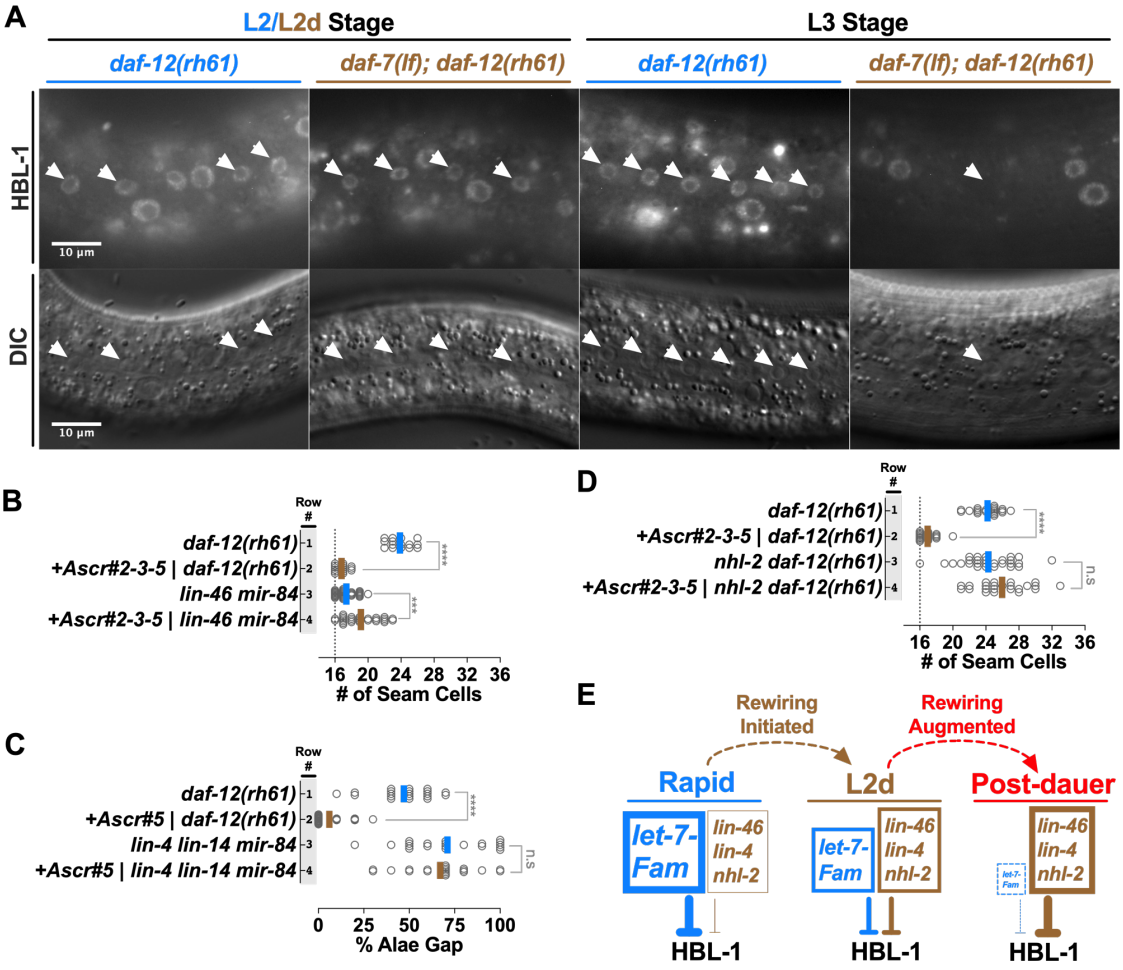

A
L2/L2d Stage
L3 Stage
daf-12(rh61)
daf-7(lf); daf-12(rh61)
daf-12(rh61)
daf-7(lf); daf-12(rh61)
HBL-1
DIC
10 µm
10 µm
B
Row #
daf-12(rh61)
+Ascr#2-3-5 | daf-12(rh61)
lin-46 mir-84
+Ascr#2-3-5 | lin-46 mir-84
16 20 24 28 32 36
# of Seam Cells
C
Row #
daf-12(rh61)
+Ascr#5 | daf-12(rh61)
lin-4 lin-14 mir-84
+Ascr#5 | lin-4 lin-14 mir-84
n.s.
0 25 50 75 100
% Alae Gap
D
Row #
daf-12(rh61)
+Ascr#2-3-5 | daf-12(rh61)
nhl-2 daf-12(rh61)
+Ascr#2-3-5 | nhl-2 daf-12(rh61)
n.s.
16 20 24 28 32 36
# of Seam Cells
E
Rewiring Initiated
Rewiring Augmented
Rapid
L2d
Post-dauer
let-7-Fam
lin-46
lin-4
nhl-2
HBL-1
HBL-1
HBL-1

Figure 2.7. Heterochronic Genes Associated with the Altered HBL-1 Downregulation Program in Post-Dauer Animals Are Required for the L2d Suppression of Heterochronic Phenotypes Caused by Insufficient Expression of *let-7* Family MicroRNAs

(A) Upper row: fluorescent images showing HBL-1 expression in L2/L2d and L3 stage hypodermal seam (white arrowheads) and hyp7 (all other) nuclei in *daf-12(rh61)* and *daf-7(lf); daf-12(rh61)* animals. Lower row: corresponding DIC images of the hypodermis. It should be noted that, consistent with the variability in the extra seam cell phenotype of *daf-12(rh61)* animals, HBL-1 expression at the L3-stage *daf-12(rh61)* animals displays variability across seam cells of individual worms. For example, HBL-1 expression may be present and absent in two neighboring seam cells, which presumably express L2 and L3 cell fates, respectively.

(B) Ascaroside conditions that suppress the extra seam cell phenotype of *daf-12(rh61)* enhance the extra seam cell phenotype of larvae lacking *lin-46* and *mir-84.*

(C) Ascaroside conditions that suppress the gapped alae (a consequence of retarded seam cell development that is manifested in young adults) phenotype do not suppress the gapped alae phenotype of *lin-4; lin-14; mir-84* animals.

(D) *nhl-2* activity is required for ascaroside-mediated suppression of *daf-12(rh61).*

(E) A model for the L2d rewiring and its potential augmentation during dauer arrest. Under L2d-inducing conditions, *let-7*-family microRNAs are downregulated and also become less important. The reduction in the let-7family level and importance is coupled to enhanced roles for the heterochronic genes previously associated with the altered HBL-1 downregulation program in post-dauer animals, involving *lin-46, lin-4,* and *nhl-2.* This shift in the reliance on the *let-7*-family microRNAs to the reliance on the alternative program for proper HBL-1 downregulation (hence, for proper L2-to-L3 cell-fate progression) constitutes the L2d rewiring. In post-dauer animals, consistent with an augmentation of the L2d rewiring program, the reliance on the altered HBL-1 downregulation program further increases while the *let-7*-family microRNAs become dispensable for proper HBL-1 downregulation. It should be noted that we do not know the mechanisms (e.g., elevated levels versus enhanced activities) of increased roles for *lin-46, nhl-2,* or *lin-4* during L2d or post-dauer development. The Student's t test is used to calculate statistical significance (p): n.s. (not significant) $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

daf-7(lf); daf-12(rh61) double mutants (Figure 2.7A). At the L2/L2d stage, HBL1 expression in *daf-12(rh61)* and *daf-7(lf); daf-12(rh61)* were comparable (Figure 2.7A, L2/L2d). At the L3 stage, however, whereas HBL-1 was over expressed in both seam and hyp7 cells of *daf-12(rh61)* animals, HBL-1 was absent in the seam cells of *daf-7(lf); daf-12(rh61)* animals (Figure 2.7A, L3). This downregulation of HBL-1 expression in seam cells of *daf-7(lf)*; *daf-12(rh61)* mutants is consistent with the suppression of extra seam cell phenotypes of *daf-12(rh61)* by *daf-7(lf)*.

To test whether the previously described genetic requirements for *lin-46*, *lin-4*, and *nhl-2* to downregulate HBL-1 in the dauer/post-dauer context (Karp and Ambros, 2012), also apply during L2d development, we examined the phenotypes of the relevant mutant strains during development through ascaroside-induced L2d, but in this case without dauer commitment or arrest. We observed that ascarosides failed to suppress the retarded phenotypes of animals that were lacking *lin-46 or lin-4* in combination with *mir-84(lf)* (to provide a sensitized background, blunting the expression of *let-7* family microRNAs), or that were lacking *nhl-2* in the *daf-12(rh61)* background. (Figure 2.7B-2.7D). Moreover, for doubly-mutant animals carrying both *mir-84(lf)* and *lin-46(lf)* mutations, ascaroside-induced L2d enhanced the retarded phenotypes (Figure 2.7B), similar to the enhancement reported for *mir-84(lf)*; *lin-46(lf)* animals that developed through dauer arrest and post-dauer development (Karp and Ambros, 2012). Similarly, ascarosides failed to suppress the gapped alae phenotype of animals lacking *lin-4* and *mir-84* (Figure 2.7C), consistent with the previous finding that this

phenotype of *lin-4(lf); mir-84(lf)* animals was enhanced for post dauer animals (Karp and Ambros, 2012). Lastly, ascarosides failed to suppress the extra seam cell phenotypes of *nhl-2: daf-12(rh61)* animals (Figure 2.7D), indicating that *nhl-2* is required for ascaroside-mediated suppression of *daf-12(rh61);* analogous to its role in post-dauer enhancement of the retarded phenotypes of *let-7* family microRNAs (Karp and Ambros, 2012). These results suggest that the L2d rewiring includes the activation of an alternative HBL-1 downregulation program, which involves *lin-46*, *lin-4* and *nhl-2*, and that this alternative program accounts for the reduced reliance on *let-7* family microRNAs for proper L2-to-L3 cell fate transition. These results also suggest that the genetic circuitry controlling cell fate progression via HBL-1 in larvae undergoing L2d development is similar to the circuitry associated with dauer larvae arrest, consistent with a rewiring mechanism that is initiated during L2d and augmented during dauer arrest (Figure 2.7E).

Discussion

Environmental and physiological stress signals can challenge the progression of *C. elegans* larval development, causing the larva to choose one of three distinct alternative developmental trajectories: 1) a rapid and continuous trajectory without the option for dauer arrest, 2) continuous development through an extended, bipotent L2d trajectory, wherein the option for dauer arrest is enabled, but not necessarily selected, and 3) development through the L2d trajectory followed by dauer larva arrest (Figure 2.8A). Regardless of which trajectory is chosen by the larva, the same sequence of stage-specific cell fates is

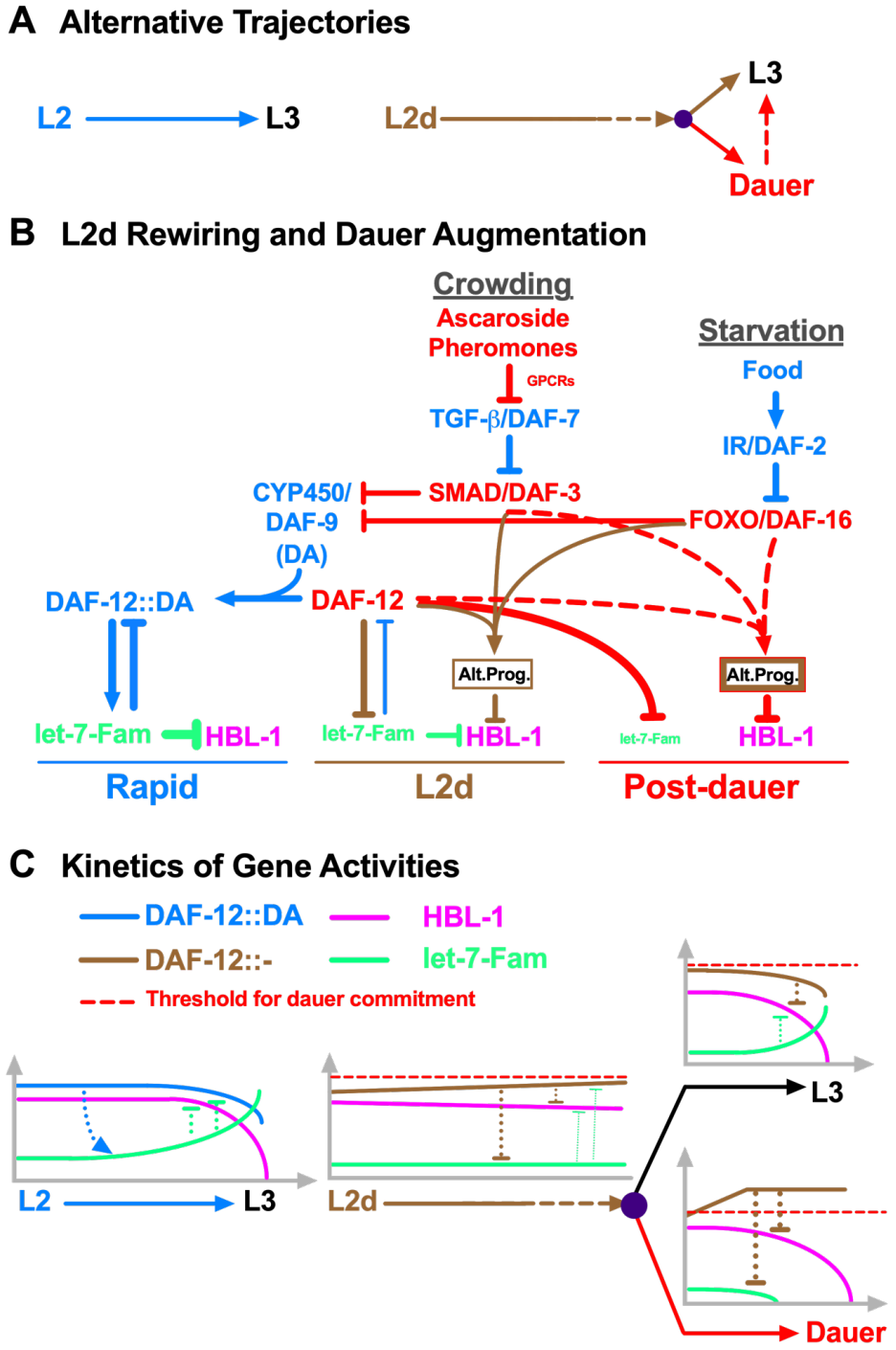

A Alternative Trajectories
L2
L3
L2d
L3
Dauer
B L2d Rewiring and Dauer Augmentation
Crowding
Ascaroside
Pheromones
GPCRs
TGF-β/DAF-7
Starvation
Food
IR/DAF-2
CYP450/
DAF-9
(DA)
SMAD/DAF-3
FOXO/DAF-16
DAF-12::DA
DAF-12
Alt.Prog.
Alt.Prog.
let-7-Fam
HBL-1
let-7-Fam
HBL-1
let-7-Fam
HBL-1
Rapid
L2d
Post-dauer
C Kinetics of Gene Activities
DAF-12::DA
HBL-1
DAF-12::-
let-7-Fam
Threshold for dauer commitment
L2
L3
L2d
L3
Dauer

Figure 2.8. Coordinate Regulation of Developmental Progression and Cell-Fate Transitions in *C. elegans* Larvae

(A) Alternative trajectories. The L2-to-L3 transition is rapid and deterministic (once committed to the L2 stage, the larvae do not have the dauer option), whereas the L2d-to-L3 transition is slower and bipotential. In both cases, HBL-1 is present throughout the L2/L2d stage, but it is downregulated by the beginning of the L3 stage.

(B) Pheromone and endocrine signals engage DAF-12 to initiate and regulate the rewiring of the HBL-1 downregulation. In response to crowding and starvation, TGF-β and insulin signaling pathways, respectively, modulate the ligand-dependent DAF-12 activity to repress the transcription of *let-7* family microRNAs and, at the same time, cooperate with DAF-12 in a ligand-independent manner to activate the alternative HBL-1 downregulation program (Alt. Prog.). The alternative program of L2d and dauer-interrupted trajectories are similar, but the alternative program of dauer-interrupted trajectory is stronger either due to an enhancement of the alternative program of L2d (depicted by thicker brown border) and/or due to employment of additional factors (depicted as bold red line) after the L2d larvae commit to dauer formation.

(C) DAF-12 ensures properly delayed but robust HBL-1 downregulation during L2d-to-L3 transition by coordinating the repression of *let-7* family microRNAs with the activation of the alternative HBL-1 downregulation program. During rapid, L2 development, DAF-12 activates the transcription of *let-7* family microRNAs, which in turn negatively regulate DAF-12, eliminating the dauer option. During slow, bipotential, L2d development, DAF-12 represses *let-7* family microRNAs, which otherwise would have prevented the accumulation of DAF-12. If the unliganded DAF-12 reaches a threshold, larvae commit to dauer formation; if not, larvae commit to continuous development. While mediating this decision, which necessitates the repression of *let-7* family microRNAs (for maintaining the dauer option) and delaying the downregulation of HBL-1 (for postponing L3 cell fates), DAF-12 cooperates with DAF-3 or DAF-16 to activate the alternative HBL-1 downregulation program (Alt. Prog.) to ensure robust HBL-1 downregulation during the L2d-to-L3 transition.

robustly expressed (Figure 2.1A and Figure 2.3 rows 1-3). The findings reported here illuminate how genetic regulatory networks that specify larval cell fate progression can accommodate these alternative life histories, and the physiological and environmental stresses that induce them.

Central to the coordination of temporal cell fates and life history choices in *C. elegans* is the nuclear hormone receptor transcription factor DAF-12 (Antebi *et al.*, 2000), and its ligand, dafachronic acid (DA) (Motola *et al.*, 2006). DAF-12 in the unliganded form is essential for the dauer larva trajectory, while ligand-bound DAF-12 inhibits the dauer larva program. At the same time, DAF-12 and DA control the L2-to-L3 cell fate transitions by regulating the expression of *let-7* family microRNAs, which are required to downregulate HBL-1 and thereby specify the proper timing of expression of L3 cell fates (Abbott *et al.*, 2005; Motola *et al.*, 2006; Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009).

The *let-7* family microRNAs also regulate the abundance of DAF-12 via a feedback loop that has been proposed to help ensure robust coordination of cell fates with dauer arrest (Hammell, Karp and Ambros, 2009). Under favorable conditions, when DA is abundant, DA-bound DAF-12 promotes continuous development, and also activates accumulation of *let-7* family microRNAs during the L2 stage, which in turn attenuate the accumulation of DAF-12 (thereby eliminating the dauer option) and also down regulate HBL-1 to enable rapid progression from L2 to L3 cell fates. Conversely, under unfavorable conditions, DA production is low and unliganded DAF-12 promotes the L2d/dauer program and

represses the expression of *let-7* family microRNAs. In turn the low level of *let-7* family microRNAs allows the accumulation of DAF-12 during L2d, maintaining the dauer option (Hammell, Karp and Ambros, 2009).

It was not previously clear how HBL-1 might be down regulated during L2d considering the repressed state of the *let-7* family microRNAs. We propose that the L2d program instigated by unliganded DAF-12 includes, in addition to the repression of *let-7* family microRNAs, also the activation of an alternative mechanism of HBL-1 downregulation (Figure 2.8B), which is responsible for the suppression of *daf-12(rh61)* by ascarosides (Figure 2.3, row 8), and by L2d-inducing mutants of *daf-7* and *daf-2* (Figure 2.3, rows 9 and 10). The L2d suppression of *daf-12(rh61)* phenotypes led us to propose that during wild type L2d, when DA is low, DAF-12 is unliganded and its activity is hence not unlike that of the ligand-binding defective mutant DAF-12(RH61). In this model, we propose that *daf-12(rh61)* animals constitutively run an “L2d-like program” for regulating temporal cell fates, which is discordant with rapid continuous development, but compatible with (and hence phenotypically suppressed by) the environmental and endocrine signals that induce the L2d trajectory.

Our conclusion that a DA-independent function of DAF-12 is required for the modulation of temporal cell fates during L2d is derived from our finding that the mild retarded phenotype of the *daf-12* null allele, *daf-12(rh61rh441),* was not suppressed by ascarosides, in contrast to the stronger retarded phenotype of *daf-12(rh61),* which is efficiently suppressed (Figure 2.6G). Consistent with our results

showing the requirement for *daf-12* for L2d rewiring, a *daf-2(lf)* mutant was reported to not suppress (indeed, to enhance) the extra seam cell phenotypes of *daf-12(lf)* (Huang, Zhang and Zhang, 2011).

We find that the DA-independent function of DAF-12 that modulates temporal cell fates during the L2d can be activated by either of the two upstream transcription factors, DAF-3, which mediates responses to TGF-β signaling from ascaroside-sensing neurons, and DAF-16, which mediates DAF-2/IGF signaling. We hypothesize that DAF-12 cooperates with DAF-3 or DAF-16 in a DA-independent manner to activate and modulate the alternative HBL-1 downregulation program (Figure 2.8B). Although the immediate action of DAF-12, together with DAF-3 or DAF-16, would likely be transcriptional, ultimately the alternative pathway for HBL-1 down regulation appears to be post-transcriptional, as we found that suppression of *daf-12(rh61)* or *mir-84(lf)* by L2d involves contributions from *lin-4* and *let-7* family microRNAs, in addition to the miRISC cofactor NHL-2 (Hammell *et al.*, 2009) and also LIN-46, which is thought to function post transcriptionally (Pepper *et al.*, 2004). Interestingly, the alternative HBL-1 downregulation program appears to be activated in seam cells but not in hyp7 cells (Figure 2.7A), which is distinctly different from the *let-7* family regulation of HBL-1 occurring both in the seam and hyp7 cells.

Prior to this study, it was not clear whether the inhibition of dauer larva formation by exogenously-supplied DA reflects prevention of L2d in addition to a block of dauer-commitment. We observed that exogenous DA hormone at levels

sufficient to prevent ascaroside-induced dauer formation does not prevent the extension of second larval stage development characteristic of L2d. Also, we observed that *daf-12(rh61)* animals, which are DA-insensitive and dauer-commitment defective nevertheless can readily undergo L2d. These observations suggest that L2d is initiated independently of DAF-12-DA activity, and are consistent with our model that a DA-independent function of DAF-12, together with activated DAF-3 or DAF-16, promote expression of the L2d program.

The studies described here involve animals that traverse the bi-potential L2d stage, but elect the option of resuming L3 development instead of dauer larva arrest. Previous studies showed that for animals that enter dauer diapause arrest and later are induced to emerge from dauer arrest and complete post-dauer L3 and L4 development, a similar change in the HBL-1 down-regulation program is evident (Karp and Ambros, 2012). Here we show that the genetic circuitry controlling cell fate progression via HBL-1 in larvae undergoing L2d development is similar to the circuitry previously identified for animals that arrest as dauer larvae (Karp and Ambros, 2012). In particular, we find that *lin-4* and NHL-2 have similarly prominent roles in both the L2d-only and the post-dauer trajectories. Moreover, for both the L2d-to-L3 trajectory, and the L2d-to-dauer-to-postdauer trajectory, an enhanced role for *lin-46* is evident, compared to the rapid continuous development trajectory. Our finding of an increased importance of LIN-46 during the L2d is consistent with a previous report that a *daf-2(lf)* mutation could enhance the retarded phenotype of *sea-2(lf)* (Huang, Zhang and Zhang, 2011), whose

phenotype presumably reflects elevated *lin-28* activity, and hence reduced *lin-46* activity.

These results, that the rewiring of HBL-1 down regulation for the L2d-only trajectory is largely similar to that for dauer-postdauer trajectory, are consistent with the fact that dauer larvae arrest is always preceded by L2d. Therefore, the rewiring associated with traversing post-dauer likely is initiated during L2d. The observation that suppression of the retarded phenotypes of certain mutants is more potent for the L2d-dauer-postdauer trajectory compared to the L2d-only trajectory (Figure 2.3, row 5 vs 6), suggests that rewiring may be partially implemented during L2d, and more fully engaged in association with dauer commitment (Figure 2.8B).

Our observations of the kinetics of HBL-1 down regulation during the L2 and L2d stages (Figure 2.9) suggest that HBL-1 levels are maintained at relatively high levels for much of the L2 or L2d stage, and down-regulated near the end of the stage (Figure 2.8C and Figure 2.9). This suggests that down-regulation of HBL-1, and hence commitment to L3 cell fate specification, may be gated by some event(s) coupled to the completion of the L2 or L2d stage. This would be consistent with a model wherein most of the L2d is occupied with rewiring of pathways upstream of HBL-1, followed by implementation of HBL-1 down regulation at the end of the L2d, in association with commitment to the L3 cell fate.

In summary, we show that environmental and endocrine stress signals that regulate developmental progression and alternative developmental trajectories

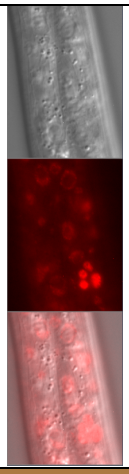
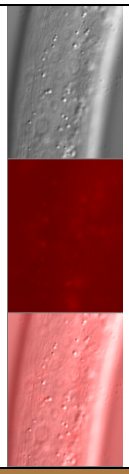
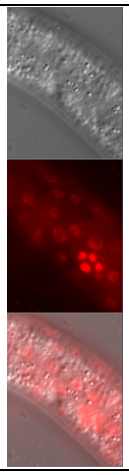
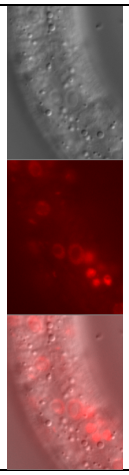
Time (Hours)	10	22	29	36	44
Genotype	*hbl-1(ma430[hbl-1::mScarlet-I)]*				
Stage	L1	Late L2	L3	L4	Adult
HBL-1 Expression (# of animals scored)	Yes (10/10)	Yes (10/10)	No (0/10)	No (0/10)	No (0/10)
Example Pictures	n/a			n/a	n/a
Genotype	*daf-7(e1372); hbl-1(ma430[hbl-1::mScarlet-I)]*				
Stage	L1	L2d	Late L2d	L3	L4
HBL-1 Expression (# of animals scored)	Yes (10/10)	Yes (10/10)	Yes (10/10)	No (0/10)	No (0/10)
Example Pictures	n/a				n/a

Figure 2.9. HBL-1 is present throughout the L2 or the lengthened L2d stage but it is absent at the post-L2 or post-L2d L3 stage.

Endogenously tagged HBL-1 expression was examined in wild-type and *daf-7(e1372)* backgrounds at 20°C. *daf-7(e1372)* animals form dauer larvae at 25°C but they develop continuously going through L2d at 20°C. For each time point and genotype ten animals were examined. In all animals at 22 hours, also in *daf-7(e1372)* animals at 29 hours, L2 stage specific V5p cell divisions were observed to have occurred (a cluster of four small and bright nuclei in each picture), indicating that the animals were at a late phase of the L2 stage. In L2d animals the lengthening of the second larval stage appeared to occur after the execution of these L2 stage cell divisions. HBL-1 is detected in late L2 and L2d larvae but it was not detected in post-L2 and post-L2d L3 stage larvae, indicating that the duration of HBL-1 expression is lengthened as the L2 stage is lengthened during L2d, and in both post-L2 L3 and post-L2d L3 animals HBL-1 is downregulated.

rewire the genetic regulatory network controlling temporal cell fate transitions during larval development of *C. elegans.* Our findings provide insight into how the regulation of temporal cell fate transitions can be adapted to accommodate stressful conditions that challenge developmental progression. Coordinate regulation of developmental progression and temporal cell fates seem to confer elasticity to *C. elegans* development, wherein the proper developmental schedule of cell fate transitions is unaffected by variations in developmental rate or developmental trajectory.

C. elegans larvae possess other life history options, in addition to the delayed developmental progression associated with the L2d-dauer trajectory. For example, appropriate pheromone signals and nutritional status can result in an acceleration of *C. elegans* development (MacNeil *et al.*, 2013; Aprison and Ruvinsky, 2016; Ludewig *et al.*, 2017). Moreover, larvae can temporarily suspend developmental progression at specific checkpoints in the late L2, L3, or L4 stages in response to acute starvation (Schindler, Baugh and Sherwood, 2014). We hypothesize that these signals that either accelerate development or result in programmed developmental arrest would also be associated with appropriate rewirings of the genetic regulatory pathways regulating temporal cell fate progression, so that cell fate transitions are appropriately synchronized with the dynamics of larval stage progression. Lastly, rewiring mechanisms induced by environmental signals, such as that described here, might also be adapted for the evolution of morphological plasticity or polyphenism observed in certain

Strain name	**Genotype**	**Related figure(s)**
VT1367	*maIs105[pCol-19::gfp] V*	Figure 2.2, 2.3, 2.6B, 2.6C, 2.6D
VT1453	*mir-48 mir-241(nDf51) maIs105 V; mir-84(n4037) X*	Figure 2.3
VT791	*maIs105 V; daf-12(rh61) X*	Figure 2.3, 2.4, 2.5, 2.6. 2.7
VT2962	*daf-7(e1372) III; maIs105 V; daf-12(rh61) X*	Figure 2.3, 2.6C, 2.6E
VT3568	*daf-2(e1370) III; maIs105 V; daf-12(rh61) X*	Figure 2.3, 2.6D, 2.6E
VT3135	*maIs105 V; srg-36srg-37(kyIR95) daf-12(rh61) X*	Figure 2.6A
VT2972	*maIs105 V; daf-3(mgDf90) daf-12(rh61) X*	Figure 2.6B, 2.6C
VT1308	*daf-7(e1372) III; maIs105 V*	Figure 2.6C
VT1755	*maIs105 V; daf-3(mgDf90) X*	Figure 2.6C
VT1747	*daf-7(e1372) III; maIs105 V; daf-3(mgDf90) X*	Figure 2.6C
VT2984	*daf-7(e1372) III; maIs105 V; daf-3(mgDf90) daf-12(rh61) X*	Figure 2.6C
VT1776	*daf-2(e1370) III; maIs105 V*	Figure 2.6D
VT3566	*daf-16(mgDf50) I; maIs105 V*	Figure 2.6D
VT3567	*daf-16(mgDf50) I; daf-2(e1370) III; maIs105 V*	Figure 2.6D
VT3569	*daf-16(mgDf50) I; maIs105 V; daf-12(rh61) X*	Figure 2.6D
VT3570	*daf-16(mgDf50) I; daf-2(e1370) III; maIs105 V; daf-12(rh61) X*	Figure 2.6D
VT3682	*daf-16(mgDf90) I; daf-7(e1372) III; maIs105 V*	Figure 2.6E
VT3681	*daf-2(e1370) III; maIs105 V; daf-3(mgDf90) X*	Figure 2.6E
VT3684	*daf-16(mgDf90) I; daf-7(e1372) III; maIs105 V; daf-12(rh61) X*	Figure 2.6E
VT3683	*daf-2(e1370) III; maIs105 V; daf-3(mgDf90) daf-12(rh61) X*	Figure 2.6E
VT2952	*maIs105 V; daf-9(m540) daf-12(rh61) X*	Figure 2.6F
VT3061	*wIs51[pScm::gfp] V; daf-12(rh61rh411) X*	Figure 2.6G
VT3057	*din-1(dh127) II; mir-48 mir-241(nDf51) maIs105 V*	Figure 2.6H
VT3894	*daf-12(rh61) hbl-1(ma430[hbl-1::mScarlet-I) X*	Figure 2.7A
VT3895	*daf-7(e1372) III; daf-12(rh61) hbl-1(ma430[hbl-1::mScarlet-I) X*	Figure 2.7A
VT2086	*lin-46(ma164) maIs105 V; mir-84(n4037) X*	Figure 2.7B
VT1065	*lin-4(e912); lin-14(n179) mir-84(n4037) X*	Figure 2.7C
VT3030	*nhl-2(ok818) III; maIs105 V; daf-12(rh61) X*	Figure 2.7D
VT3751	*maIs105 V; hbl-1(ma430[hbl-1::mScarlet-I]) X*	Figure 2.9
VT3893	*daf-7(e1372) III; hbl-1(ma430[hbl-1::mScarlet-I]) X*	Figure 2.9

Table 2.1. *C. elegans* strains used in Chapter II.

All gene, allele, and transgene names, and chromosome numbers are italicized (Genotype column). *maIs105* and *wIs51* are integrated extrachromosomal arrays expressing *pCol-19::gfp* and *pScm::gfp* to mark hypodermal cells of adult stage animals and hypodermal seam cells at all stages, respectively. All figures related to each strain are listed in the related figures column.

nematodes (Kiontke and Fitch, 2010; Susoy *et al.*, 2016) and other animals (Simpson, Sword and Lo, 2011).

Materials and methods

C. elegans culture conditions

C. elegans strains used in this study and corresponding figures in the chapter are listed in Table 2.1. *C. elegans* strains were maintained at 20°C on nematode growth media (NGM) and fed with the *E. coli* HB101 strain.

Dauer and L2d-inducing plates

For experiments involving the administration of ascarosides and/or DA, we adopted the protocol described by Butcher *et. al.* (Butcher *et al.*, 2008) with modifications. Namely, *C. elegans* was fed with the *E. coli* OP50 strain on plates containing 3 mL of 1% agarose (SeaKem® LE agarose, Cat#50004) with nematode growth media (NGM) without peptone. Synthetic ascarosides (kindly provided by the labs of Frank Schroeder and Jagan Srinivasan) were dissolved in ethanol (stock concentrations: ascr#2 5.69 mM, ascr#3 3.81 mM, and ascr#5 4.09 mM) and added to the melted agarose prior to plate-pouring to achieve the desired final concentration (3 μM, if not specified). Plates were seeded the next day with *E. coli* strain OP50 as follows: OP50 was grown in liquid Luria Broth (LB) media until the culture reached OD600 = 0.6-0.7. Then, the bacterial culture was pelleted by spinning at 3500 rpm for 10 min. The pellet was washed twice with a volume of sterile water equal to the LB culture volume. Finally, the pellet was resuspended in a volume of sterile water equal to one-fifth of the initial LB

culture volume. 50 µLs of this washed and 5x concentrated OP50 culture were used to seed ascaroside plates. To prepare ascaroside plates also containing Δ4-dafachronic acid (DA; Cayman Chemical, item no 14100; 1 mg of DA dissolved in ethanol) 50 µL of water containing DA at specified concentrations was added onto the lawn of bacteria.

Analysis of extra seam cell phenotypes

Gravid adult animals were washed off from NGM plates and collected in 2 mL of water. 0.84 mL of freshly prepared 2:1 mixture of bleach (6% sodium hypochlorite, Fisher Chemical SS2901) and 5N sodium hydroxide (prepared by dissolving Fisher S318500 in distilled water) was added onto the worms in 2 mL of water. Worms were incubated in this solution containing bleach and sodium hydroxide for 3 min. To pellet the released eggs, 8 mLs of water was added, and the tube was spun for 20 s at 600 x g. The supernatant is carefully removed and the pelleted eggs were washed three times with 10 mL of sterile M9 buffer. Eggs in M9 buffer were pipetted on control or treatment plates and cultured at 20°C (unless otherwise specified) until they reached the adult stage. The worms were scored at the young adult stage for the number of seam cells using fluorescence microscopy with the help of the *maIs105 [pCol-19::gfp]* transgene that marks the lateral hypodermal cell nuclei or the *wIs51[pScm::gfp]* transgene that marks the seam cell nuclei.

TaqMan assays for microRNA quantification

Synchronized L1 larvae of *daf-12(rh61)* were raised on control or ascr#2-3-5 plates at 20°C. Larvae that reached the L2/L2d-to-L3 molt were identified and picked under a dissecting microscope and collected in M9 media within two hours. For each experimental condition, three biological samples were collected, containing approximately 200 larvae per sample. Collected worms were snap-frozen and kept at −80°C until RNA extraction. RNA was extracted using the Trizol reagent (Invitrogen).

Two microliters of 30 ng/µL RNA samples were used for reverse transcription, and multiplex miR-Taqman reactions were carried out according to the manufacturer's instructions, and using an ABI 7900-HT Fast-Real Time PCR System. MicroRNAs were assayed in three technical replicates for each biological sample. Five highly expressed microRNAs that have not been reported to be environmentally regulated (*mir-1, lin-4, mir-52, mir-53, and mir-58*), were used as a control microRNA set. The average CT of the control microRNA set was used to normalize the CTs obtained from *let-7* family microRNAs.

Tagging of *hbl-1* at its endogenous locus

A mixture of plasmids encoding SpCas9 (pOI90, 70 ng/µL), and single guide RNAs (sgRNAs) targeting the site of interest (pOI89, 20 ng/µL) and the *unc-22* gene (pOI91, 10 ng/µL) as co-CRISPR marker (Kim *et al.*, 2014), a donor plasmid (pOI191, 20 ng/µL) containing the mScarlet-I sequence (Bindels *et al.*, 2016) flanked by homology arms, and a *rol-6(su1006)* containing plasmid

Primer #	Primer name	Primer Sequence (5' to 3')	Used for
Cloning of sgRNA guides			
1	Forward (pOI301)	gtttaagagctatgctggaaacagcatagcaagtttaaataaggctagtccg	To generate pOI83 by modifying the tracr sequence of pRB1017
2	Reverse (pOI302)	agagaccgagtaccggtttctc	
3	Forward (priOI316)	tcttgaagccagacaccaataatg	Cloning of pOI89 – *hbl-1(sgRNA)*
4	Reverse (priOI317)	aaaccattattggtgtctggcttc	
5	Forward (priOI323)	tcttggaacccgttgccgaatacac	Cloning of pOI91 – *unc-22(sgRNA)*
6	Reverse (priOI324)	aaacgtgtattcggcaacgggttcc	
Cloning of pOI191- HR template			
7	Forward (priOI670)	gaaggtctcatctggaggtggatctggaggtggatctggaggtggatctgtcagcaaggagaggcagttatc	To amplify *mScarlet* from pSEM91 and to fuse with a linker
8	Reverse (priOI671)	gaaggtctcacttgtagagctcgtccattcc	
9	Forward (priOI672)	gaaggtctcacaagtaatgaggacgtcctcgttaagg	To amplify a fragment containing the HR arms+ plasmid backbone from pOI115
10	Reverse (priOI673)	gaaggtctcacagattggtgtctggcttggtacat	
11	Forward (priOI705)	cccacaattcatgtacggatcccgtgccttcatcaagcacccagccg	To convert *mScarlet* to *mScarlet-I* using SDM (T74I = acc>atc)
12	Reverse (priOI706)	gagaggatgtcccaggagaat	
13	Left Arm-Forward (priOI342)	cgggaattcaaagatggcgaggtaagcgt	To clone the HR arms for the assembly of pOI115 (these primers define the ends of the HR arms)
14	Right arm – Reverse (priOI343)	gccggatccaacaagtattctgggggaggt	
Screening for HR			
15	Forward (priOI262)	tcatccggagacgaggagac	Screening of F1 progeny of CRISPR mix injected P0 worms for HR (hbl-1::mScarlet-I) events
12	Reverse (priOI706)	gagaggatgtcccaggagaat	
15	Forward (priOI262)	tcatccggagacgaggagac	Screening of F2 progeny of F1 worms positive for HR events – these primers flank the HR arms
16	Reverse (priOI228)	aaaagagcagcacgagttgg	

Table 2.2. Primers used in Chapter II.

(pOI124, 50 ng/µL) as co-injection marker was injected into the germlines of ten young adult worms. F1 roller and/or twitcher animals (around 200 worms) were cloned and screened by PCR amplification (Primers 12&15; Table 2.2) for the presence of the expected homologous recombination (HR) product. F2 progeny of F1 clones positive for the HR-specific PCR amplification product were screened for homozygous HR edits by PCR amplification of the locus using primers that flanked the HR arms used in the donor plasmid (Primers 15&16; Table 2.2). Finally, the genomic locus spanning the HR arms and mScarlet-I DNA was sequenced using Sanger sequencing. A single worm with a precise HR edited locus was cloned and backcrossed twice before used in the experiments. This HR edited allele, which contains a linker and the mScarlet-I sequence integrated in-frame with *hbl-1*, is named as *ma430* [*hbl-1::mScarlet-I*].

Plasmid DNA purification and microinjection of worms

Plasmid DNA for tagging *hbl-1* was purified using the ZR Plasmid Miniprep Kit (Zymo Research, Cat#: 11-308AC) with a modified protocol which includes a phenol:chloroform (Phenol:ChCl3::IAA, pH = 7.9, Ambion, Cat#AM9730) extraction step before loading the samples onto the columns. First, 600 µLs of the bacterial supernatant (Step 5 of the kit's protocol) was mixed with an equal volume of phenol:chloroform and vortexed for 10 s. Then, this mixture was centrifuged at 16.000xg for 5 min and 500 µLs of the aqueous (top, transparent) layer was transferred onto the columns. The plasmid DNA on the columns were washed with both endo-free wash and wash buffers as described in the kit's

protocol, and eluted with distilled water. Plasmid DNA was mixed at the final concentrations listed above for each plasmid in 20 µL of water. 3 µL of this injection mix was loaded into a glass injection needle using a microloader pipette tip (Eppendorf, 5242956003). Glass injection needles were pulled using a KOPF vertical pipette puller (model 720) and capillary glass tubes (FHC, Inc., borosil 30-30-0). Worms were immobilized on 2% agarose pads (by gently pressing the worms with a worm pick toward the agarose surface) covered with a drop of oil (Halocarbon, CAS#9002-83-9). Agarose pads were previously prepared by spreading a drop (~50-100 µLs) of 2% agarose (in water) between two cover slides (e.g., Fisherbrand 12-544E, 24X500-1.5). The immobilized worms were injected under a Zeiss Axiovert 35 inverted microscope equipped with a Leitz mechanical micromanipulator. Injected worms were washed off from agarose pads and transferred to NGM plates in M9 media using a P200 micropipette. Injected worms (P0) were cultured at 25°C in separate NGM plates for 2-3 days before F1 twitchers/rollers were cloned for culturing before genotyping.

Cloning of sgRNA plasmids

All plasmids in the injection mix had the same plasmid backbone which was derived from pRB1017 (Arribere *et al.*, 2014). sgRNA encoding plasmids were derived from pRB1017 (first to generate pOI83) by modifying the tracr encoding sequence to (F+E) form of the tracr (Chen *et al.*, 2013) (using the Q5 Site-Directed Mutagenesis kit, NEB Cat#E0554, and Primers 1&2; Table 2.2), which was reported to increase the CRISPR efficiency in *C. elegans* (Ward, 2015).

pOI89 and pOI91 sgRNA encoding plasmids were generated by cloning annealed primer pairs into the BsaI cloning site of pOI83 (Primers 3-6; Table 2.2).

Cloning of pOI191 HR template plasmid

The Golden Gate Assembly Kit (NEB Cat#E1600) is used to fuse two PCR fragments: *mScarlet* (PCR amplified from pSEM91 (El Mouridi *et al.*, 2017) using primers 7&8 in Table 2.2) and a DNA fragment containing left and right HR arms fused to the pRB1017 plasmid backbone (PCR amplified from pOI115 using primers 9&10 in Table 2.2., which was previously generated by assembling four PCR fragments containing a left HR arm, a GFP, a right HR arm, and the backbone of pRB1017). Single colony purified plasmid DNA was used to identify colonies containing the precise assembly of the HR arms and mScarlet. pOI191 was derived from this mScarlet clone (pOI186) by mutating a single nucleotide to convert mScarlet to mScarlet-I using the Q5 Site-Directed Mutagenesis kit (NEB Cat#E0554 and Primers 11&12; Table 2.2).

Microscopy imaging of *C. elegans* larva

All DIC and fluorescent images are obtained using a ZEISS Imager Z1 equipped with ZEISS Axiocam 503 mono camera, and the ZEN Blue software. Prior to imaging, worms were anesthetized with 0.2 mM levamisole in M9 buffer and mounted on 2% agarose pads. To be able to compare fluorescent signal intensities across different genetic backgrounds or larval stages, images of all larvae were taken using the same microscopy setting. These images were then

stitched together (e.g., Figure 2.7A) using the ImageJ Fiji software and the brightness and contrast of the montaged images were adjusted to enhance the visualization of the fluorescent signal.

Quantification and Statistical Analysis

Each circle on the genotype versus number of seam cells plots shows the observed number of seam cells on one side of a single young adult worm. ≥20 worms for each genotype or condition are analyzed and the average number of seam cells are denoted by lateral bars in the genotype versus number of seam cell plots. The Student's t test is used to calculate statistical significance when comparing different genotypes or conditions. The GraphPad Prism 8 software is used to plot the graphs and for statistical analysis.

CHAPTER III -- 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*

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Abstract

MicroRNAs target complementary mRNAs for degradation or translational repression, reducing or preventing protein synthesis. In *Caenorhabditis elegans*, the transcription factor HBL-1 (Hunchback-like 1) promotes early larval (L2)-stage cell fates, and the *let-7* family microRNAs temporally downregulate HBL-1 to enable the L2-to-L3 cell-fate progression. In parallel to *let-7*-family microRNAs, the conserved RNA-binding protein LIN-28 and its downstream gene *lin-46* also act upstream of HBL-1 in regulating the L2-to-L3 cell-fate progression. The molecular function of LIN-46, and how the *lin-28-lin-46* pathway regulates HBL-1, are not understood. Here, we report that the regulation of HBL-1 by the *lin-28-lin-46* pathway is independent of the *let-7*/*lin-4* microRNA complementary sites (LCSs) in the *hbl-1* 3′UTR, and involves stage-specific post-translational regulation of HBL-1 nuclear accumulation. We find that LIN-46 is necessary and sufficient to prevent nuclear accumulation of HBL-1. Our results illuminate that robust progression from L2 to L3 cell fates depends on the combination of two distinct modes of HBL-1 downregulation: decreased synthesis of HBL-1 via *let-7*-family microRNA activity, and decreased nuclear accumulation of HBL-1 via action of the *lin-28-lin-46* pathway.

Introduction

Precise and robust gene regulation is crucial for animal development. Optimal doses of developmental gene products expressed with spatiotemporal precision produce the wild-type body plan, whereas abnormally lower or higher

doses or ectopic expression of developmental genes can result in morphological defects that reduce the fitness of the individual. The proper spatiotemporal activity of developmental gene products is ensured by elaborate gene regulatory mechanisms, which often involve collaboration across semi-redundant mechanisms controlling the gene activity at different levels – transcriptional, translational and post-translational.

Caenorhabditis elegans development consists of an invariant set of cell division and differentiation events that produces the stereotyped adult body plan (Sulston and Horvitz, 1977). *C. elegans* developmental regulators are identified by loss-of-function or gain-of-function mutations that cause developmental lethality, or evident morphological defects. One class of developmental defects in *C. elegans* stems from changes in the order and/or timing of larval developmental events, controlled by the heterochronic gene pathway (Ambros and Horvitz, 1984). In this pathway, three transcription factors (LIN-14, HBL-1, LIN-29) control cell-fate transitions from earlier to later stages, and are temporally regulated – directly or indirectly – by certain microRNAs. In particular, LIN-14 is regulated by *lin-4* (Lee, Feinbaum and Ambros, 1993), HBL-1 (Hunchback-like-1) is regulated by three members of the *let-7* family (also known as the *let-7* sisters: *mir-48*, *mir-84* and *mir-241*) (Abbott *et al.*, 2005), and LIN-29 is post-transcriptionally regulated by the RNA-binding protein LIN-41 (Slack *et al.*, 2000) which is in turn regulated by *let-7* (Reinhart *et al.*, 2000). These microRNAs are dynamically expressed during larval development and they ensure proper

temporal downregulation of their targets, which is crucial for the proper program of stage-appropriate cell-fate transitions.

The conserved RNA-binding protein LIN-28 also plays key roles in the *C. elegans* heterochronic pathway. *lin-28* regulates early cell fates upstream of *lin-46* (Pepper *et al.*, 2004) and in parallel to *mir-48/84/241* (Abbott *et al.*, 2005), and regulates late cell fates upstream of the conserved microRNA *let-7* (Van Wynsberghe *et al.*, 2011; Vadla *et al.*, 2012). In larvae lacking *lin-28*, hypodermal stem cells (called seam cells) skip L2-stage proliferative cell fates and precociously express terminally differentiated adult cell fates (Ambros and Horvitz, 1984; Moss, Lee and Ambros, 1997), whereas the rest of the tissues, e.g. the gonad, are still juvenile and developing. *lin-46(lf)* suppresses these *lin-28(lf)* phenotypes (Pepper *et al.*, 2004): *lin-28(lf)*;*lin-46(lf)* double mutants are wild type for the phenotypes observed in *lin-28(lf)* animals. *lin-28(lf)* suppresses the heterochronic phenotypes of *mir-48/84/241* mutants (Abbott *et al.*, 2005), and *lin-46* is required for this suppression (Abbott *et al.*, 2005).

Animals lacking *lin-46* display weak heterochronic phenotypes that are enhanced when the larvae are cultured at low temperatures, such as 15°C, a condition that does not affect wild-type development (Pepper *et al.*, 2004). Although wild-type larval development is similarly robust against other stresses, such as population density pheromones or starvation (Ilbay and Ambros, 2019a), *lin-46(lf)* phenotypes are enhanced under these conditions (Ilbay and Ambros, 2019a) and when animals develop through a temporary diapause in

response to these stresses (Karp and Ambros, 2012). Interestingly, diapause-inducing conditions also repress *let-7* family microRNA expression (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009); which is thought to be important for an optimal diapause decision (Hammell, Karp and Ambros, 2009) and for prolonging HBL-1 expression in coordination with the rate of developmental stage progression (Ilbay and Ambros, 2019a). Therefore, LIN-46 activity is important for the proper downregulation of HBL-1 under physiological conditions where *let-7* family microRNA levels are reduced.

The molecular functions of LIN-46, and how these functions may relate to its role in the heterochronic pathway are not known. However, given the prominent involvement of microRNAs in the heterochronic pathway, in the context of regulating temporal cell fates, LIN-46 has been thought to act possibly by modulating (more precisely 'boosting') the activities of certain microRNAs, e.g. *lin-4* or *let-7* family microRNAs, perhaps by interacting with the miRISC (microRNA-induced silencing complex). By hypothetically boosting the activities of *lin-4* and/or *let-7* family microRNAs in response to environmental stress, LIN-46 could compensate for the reduced levels of these microRNAs. This model is supported by the correlated conservation of LIN-46 with the argonaute family proteins, suggesting a potential function for LIN-46 related to microRNA or small RNA pathways (Tabach *et al.*, 2013). Alternatively, LIN-46 could regulate HBL-1 activity by a mechanism independent of microRNAs.

Here, we report that deleting a genomic region encompassing the *let-7/lin-4* complementary sites (LCSs) in the *hbl-1* 3′UTR results in strong extra seam cell phenotypes, which is consistent with lack of *let-7/lin-4* microRNA regulation and a gain-of-function of HBL-1. Importantly, we find that *lin-28(lf)* suppresses, and *lin-46(lf)* enhances, the extra seam cell phenotype of *hbl-1(gf/ΔLCSs)*, indicating that regulation of HBL-1 by the *lin-28-lin-46* pathway is independent of the LCSs. Moreover, HBL-1, which normally localizes to the nucleus, accumulates in the cytoplasm of hypodermal seam cells in *lin-28(lf)*and *hbl-1(gf/ΔLCSs)* animals, and *lin-46* is required for this cytoplasmic accumulation of HBL-1. This cytoplasmic accumulation is accompanied by reduced nuclear accumulation of HBL-1, which also correlates with reduced HBL-1 activity. Lastly, we found that precocious expression of LIN-46 in L2-stage seam cells is sufficient to localize HBL-1 to the cytoplasm, reducing the nuclear accumulation of HBL-1, and thereby suppressing *hbl-1* gain-of-function phenotypes in *hbl-1(gf/ΔLCSs)* mutants. Our results indicate that the *C. elegans lin-28-lin-46* pathway regulates the temporal dynamics of nuclear accumulation of the HBL-1 transcription factor, acting in parallel with the translational repression exerted by the let-7-family microRNAs, to confer precision and robustness to the temporal downregulation of HBL-1 activity.

Results

Deletion of genomic regions encompassing the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR results in extra seam cell phenotypes

To explore whether *lin-46* acts downstream of *lin-28* in the heterochronic pathway by modulating the regulation of *hbl-1* by *let-7* family and/or *lin-4* microRNA, we sought to generate *hbl-1(gf)* alleles free from regulation by these microRNAs. The *hbl-1* 3′UTR contains ten *let-7* complementary sites (LeCSs) and a single *lin-4* complementary site (LiCS), collectively abbreviated as LCSs. In order to abrogate *let-7*- and *lin-4*-mediated regulation of *hbl-1*, we deleted a genomic region encompassing all LCSs in the *hbl-1* 3′UTR (*ma354*; Figure 3.1A; Table 3.1). We found that, similar to *mir-48/84/241(0)* mutants, *hbl-1(ma354[ΔLCSs])* animals have retarded seam cell defects, wherein L2-stage fates are reiterated at later stages, resulting in extra seam cells in young adult animals (Figure 3.1A).

Additionally, in our screens for large deletions in the progeny of CRISPR/Cas9-injected animals, we recovered smaller 3′UTR deletions of various sizes that removed several but not all LeCSs in the *hbl-1*3′UTR (Figure 3.1A; Table 3.1). We analyzed these smaller deletions along with the largest deletion (*ma354*), and found that most of these mutants also have extra seam cell phenotypes, although weaker than the *ma354* deletion (Figure 3.1A). 3′UTR

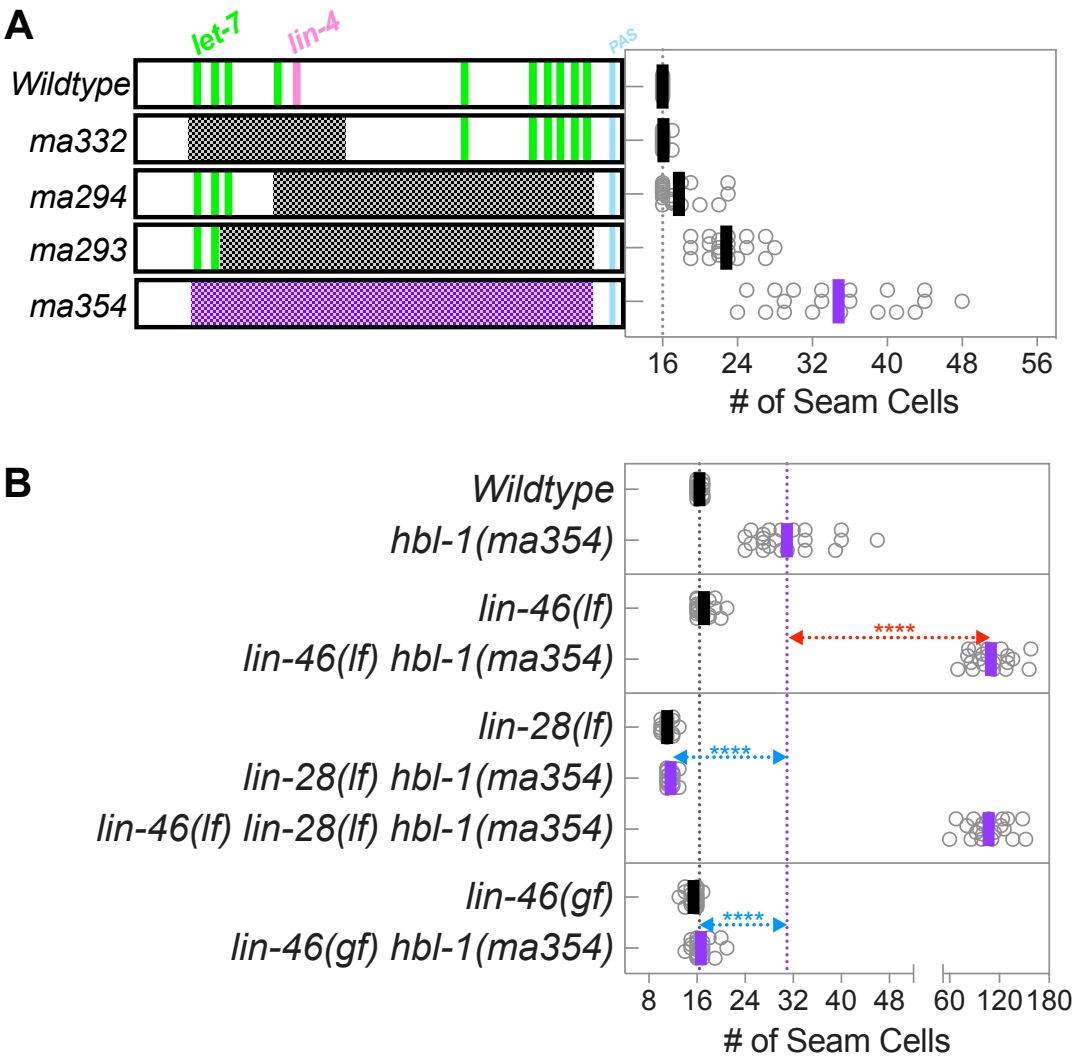

A
let-7
lin-4
PAS
Wildtype
ma332
ma294
ma293
ma354
16
24
32
40
48
56
# of Seam Cells
B
Wildtype
hbl-1(ma354)
lin-46(lf)
lin-46(lf) hbl-1(ma354)
lin-28(lf)
lin-28(lf) hbl-1(ma354)
lin-46(lf) lin-28(lf) hbl-1(ma354)
lin-46(gf)
lin-46(gf) hbl-1(ma354)
****
****
****
8
16
24
32
40
48
60
120
180
# of Seam Cells

Figure 3.1. The *C. elegans lin-28-lin-46* pathway regulates L2-to-L3 cell-fate transitions independently of the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR.

(A) Deletion of genomic regions encompassing the *let-7* and *lin-4* complementary sites in the *hbl-1* 3′UTR results in extra seam cell phenotypes. The *hbl-1* 3′UTR contains ten *let-7* (green bars) and one *lin-4* (pink bar) complementary sites. Wild-type *hbl-1* 3′UTR and four deletion alleles are depicted on the y-axis and the number of seam cells observed in young adults of animals bearing these alleles are shown on the x-axis. Each dot shows the number of seam cells observed in a single animal and the vertical bars show the average seam number in the group of animals observed for each allele. Note that the polyadenylation signal (PAS) in the *hbl-1* 3′UTR is not disrupted in the mutants. (B) Number of seam cells in single and compound mutants containing the *hbl-1(ma354)* allele are plotted. The *lin-46* null allele enhances the extra seam cell phenotype of *hbl-1(ma354). lin-28(lf)* suppresses the extra seam cell phenotype of *hbl-1(ma354)* and this suppression is lin-46 dependent. The *lin-46* gain-of-function allele similarly suppresses the extra seam cell phenotypes of *hbl-1(ma354).* In A and B, n=12 for wild type and n=20 for all other strains tested for the number of seam cells. Student's t-test was used to calculate statistical significance. ****P<0.0001.

Gene	Allele Name	Description	FP tagged	25 bp sequences flanking the deletion site and the size of the deletion
hbl-1	***ma322***	Deletes 4 LCSs in the 3'UTR	**No**	TCGTTAAGGAAACACTTCCCATAGC <-545 bp deleted-> ATTGTTTAACTATGCACACATTTGT
	ma294	Deletes 7 LCSs in the 3'UTR	**No**	ATCTAGAAGCAATTGTATACTGTTC <-892 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma293	Deletes 8 LCSs in the 3'UTR	**No**	ACTTGTTACTGTTTTCTTTTACCTC <-1051 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma430 ***ma475***	Deletes 8 LCSs in the 3'UTR	**Yes (mScarlet-I)**	TGTTACTGTTTTCTTTTACCTCTGA <-1048 bp deleted-> AAACTTCAGTGCGTTCTTCTGTCAT
	ma354	Deletes all LCSs in the 3'UTR	**No**	TTCTAATCATGGCCAGTTTCTTGCA <-1120 bp deleted-> GTGCGTTCTTCTGTCATCATGTACA
lin-46	***ma385***	Deletes all *lin-46* exons = *lin-46* null	**No**	AAACCAAGAATTGTATCAGTGGGAG <-1681 bp replaced with "AATTGT"> TACGCTTTGCATGAAAATTCACCAG
daf-12	***ma497***	Introduces a GFP tag at the C-terminal end of *daf-12*	**Yes (GFP)**	GCCAGGAGAATTTTTCAAAATCAAA <-agtaaaggagaa…864 bp of GFP …gaactatacaaa-> TAGACCTACTAGAAATCATCTAC(C/g)A Stop codon of daf-12, C/g = PAM mutation (Note: GFP tag contains a sense mutation = [TGATTTTAAAGAAGATGGAA[A/g]CATTCTTGGACACAAA TTGG])
	ma498	Introduces a linker::mScarlet-I tag (same as ma430) at the C-terminal end of *daf-12*	**Yes (mScarlet-I)**	GCCAGGAGAATTTTTCAAAATCAAA <- tctggaggtggatctggaggtggatctggaggtgga tct:: GTCAGCAAGGGAGAGGCAGTTATCA --rest of the 643 bp mScarlet-- CGGAGGAATGGACGAGCTCTACAAG-> TAGACCTACTAGAAATCATCTAC(C/g)A Linker, Stop codon of daf-12, C/g = PAM mutation

Table 3.1. New *hbl-1, lin-46, daf-12* alleles generated in Chapter III.

deletions that removed more LeCSs resulted in stronger extra seam cell phenotypes, which is consistent with the idea that these LeCSs are functional and they act partially redundantly.

lin-28(lf) suppresses and _lin-46(lf)_ enhances the extra seam cell phenotype of _hbl-1(ma354[ΔLCSs])_

Next, to test if the regulation of *hbl-1* by the *lin-28-lin-46* pathway was dependent on the *let-7* and *lin-4*complementary sites (LCSs) in the *hbl-1* 3′UTR, we generated compound mutants containing the *ma354* deletion, together with null alleles of *lin-28*, and/or *lin-46*. We found that *lin-28(lf)* suppresses and *lin-46(lf)* substantially enhances the extra seam cell phenotype of *hbl-1(ma354)* (Figure 3.1B), indicating that the regulation of *hbl-1* by *lin-28* does not require the LCSs in the *hbl-1* 3′UTR. We also found that *lin-46* is required for the suppression of *hbl-1(ma354)* by *lin-28(lf)* (Figure 3.1B). These results suggest that the *lin-28-lin-46* pathway regulates HBL-1 amount or activity through a mechanism independent of the *let-7/lin-4* regulation of HBL-1.

A _lin-46(gf)_ mutation can suppress the extra seam cell phenotypes of _hbl-1(ma354[ΔLCSs])_

LIN-46 is expressed precociously in *lin-28(lf)* animals (Ilbay, Nelson and Ambros, 2019; Chapter IV) and so suppression of *hbl-1(gf)* by *lin-28(lf)* could be solely due to precocious LIN-46, which is sufficient to inhibit L2 cell fates and promote transition to L3 and later cell fates (Ilbay, Nelson and Ambros, 2019).To

determine whether precocious LIN-46 expression alone is also sufficient to suppress the extra seam cell phenotypes of *hbl-1(ma354)* animals, we employed a *lin-46(gf)* mutation, *lin-46(ma467)*, that consists of a 12 bp deletion of *lin-46* 5′UTR sequences, and that results in precocious expression of LIN-46 and *lin-28(lf)*-like phenotypes (Ilbay, Nelson and Ambros, 2019). We generated double mutant animals carrying *hbl-1(ma354)* and *lin-46(ma467)*, and found that the gain-of-function allele of *lin-46* suppresses the extra seam cell phenotypes of *hbl-1(ma354*) (Figure 3.1B), suggesting that precocious LIN-46 expression is sufficient to suppress the *hbl-1(gf)* phenotypes. This result supports the interpretation of the suppression of *hbl-1(gf)* by *lin-28(lf)* as resulting from precocious LIN-46 expression.

Endogenously tagged HBL-1 is expressed in the nuclei of L1- and L2-stage hypodermal seam and hyp7 cells

The suppression and enhancement of the extra seam cell phenotypes of *hbl-1(ma354)* by *lin-28(lf)* and *lin-46(lf)*, respectively, could reflect changes in the level of HBL-1 protein. In order to test for changes in the levels of HBL-1 protein in *lin-28(lf)* or *lin-46(lf)* mutants, we tagged *hbl-1* at the endogenous locus with mScarlet-I using CRISPR/Cas9 (Ilbay and Ambros, 2019a). We observed that in wild-type L1 and L2 larvae HBL-1::mScarlet-I localizes exclusively to the nucleus (Figure 3.2A; Figure 3.5), which is consistent with HBL-1 functioning as a transcription factor (Fay *et al.*, 1999; Niwa *et al.*, 2009). Moreover, consistent with

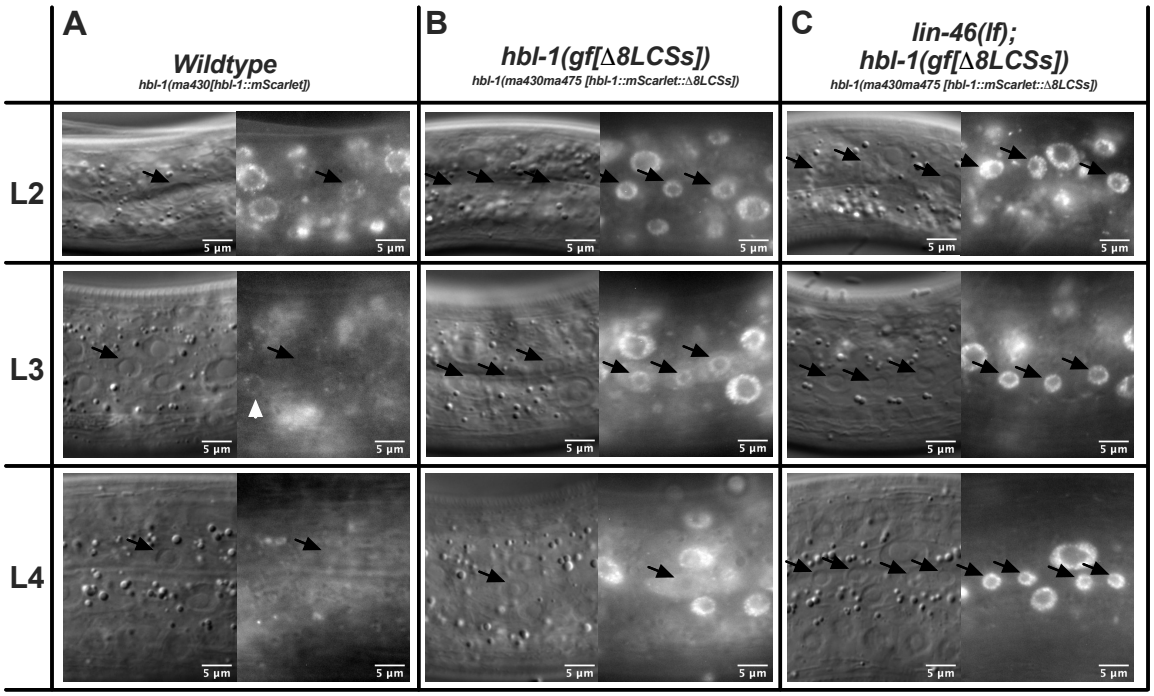

A
Wildtype
hbl-1(ma430[hbl-1::mScarlet])
B
hbl-1(gf[Δ8LCSs])
hbl-1(ma430ma475 [hbl-1::mScarlet::Δ8LCSs])
C
lin-46(lf);
hbl-1(gf[Δ8LCSs])
hbl-1(ma430ma475 [hbl-1::mScarlet::Δ8LCSs])
L2
L3
L4
5 µm

Figure 3.2. HBL-1 accumulates in the cytoplasm of L3- and L4-stage seam cells in larvae lacking LCSs in the *hbl-1* 3′UTR, and *lin-46* is required for this cytoplasmic accumulation of HBL-1.

All seam cell nuclei are marked with black arrows. (A) HBL-1 is expressed in hypodermal seam (black arrows) and hyp7 cells of L1- and L2-stage larvae but is not detected in L3- and L4-stage larvae. A rare occurrence of an L3-stage hyp7 cell expressing HBL-1 is marked with a white arrowhead. Note that HBL-1 is absent in all other nuclei, including the seam nucleus (black arrow) in the L3 stage panel. (B) In animals that lack a region of the *hbl-1* 3′UTR containing eight *let-7* complementary sites (LCSs), *hbl-1(ma430ma475),* HBL-1 is present in hypodermal seam (black arrows) and hyp7 cells at all stages (L2-L4 shown). In these animals, HBL-1 accumulates in the cytoplasm of seam cells, at the L3 and L4 stages. L3-stage seam cells still display a marked nuclear HBL-1 accumulation whereas L4-stage seam cells display almost an equal distribution of HBL-1 in both the nucleus and the cytoplasm. (C) In animals lacking lin-46 in addition to the eight LCSs in the *hbl-1* 3′UTR, HBL-1 does not accumulate in the cytoplasm of seam cells in L3- or L4-stage animals, rather HBL-1 accumulates in the nucleus of seam cells at all stages.

previous reports, endogenously tagged HBL-1 was expressed in the wild type in the hypodermal seam and hyp7 cells of L1- and L2-stage larvae and was not detected in L3- and L4-stage larvae (Abrahante *et al.*, 2003; Lin *et al.*, 2003; Ilbay and Ambros, 2019a) (Figure 3.2A; Figure 3.5).

HBL-1 is overexpressed and accumulates in the cytoplasm of L3- and L4-stage seam cells in larvae lacking LCSs in the *hbl-1* 3′UTR

To examine the impact of the loss of the LCSs in the *hbl-1* 3′UTR on the expression pattern of HBL-1, we deleted a region in the *hbl-1* 3′UTR of the mScarlet-I-tagged *hbl-1* allele, generating the *ma430ma475 [hbl-1::mScarlet-I::Δ8LeCSs]* allele. The *ma475* deletion encompasses eight LeCSs and the LiCS, similar to and only three base pairs shorter than the *ma293* deletion (Figure 3.1A; Table 3.1). Consistent with previous reports that utilized GFP reporters fused with wild-type *hbl-1* 3′UTR (Abrahante *et al.*, 2003; Abbott *et al.*, 2005), absence of these LeCSs resulted in HBL-1 expression that persisted in the L3- and L4-stage hypodermal cells (Figure 3.2B versus 3.2A). Interestingly, we observed that at the L3 and L4 stages, HBL-1 accumulates in the cytoplasm of the seam cells, which is accompanied by a reduction in the nuclear accumulation of HBL-1 (Figure 3.2B; Figure 4B). This observation suggested that perhaps the nuclear accumulation of HBL-1 is hindered (or cytoplasmic accumulation of HBL-1 is facilitated) in these L3/L4-stage seam cells.

***lin-28* is required for the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae**

lin-28(lf) animals skip L2-stage proliferative seam cell fates, suggesting that *lin-28* is required to support the activity of HBL-1 at the L2 stage. Moreover, loss of *lin-28* can suppress the extra seam cell phenotypes of *hbl-1* gain-of-function (gf) mutants (Figure 3.1B), indicating that *lin-28* is also required to support high and/or prolonged expression of HBL-1. We observed that in double mutants containing *hbl-1(ma430ma475)* and *lin-28(lf)*, HBL-1 accumulates primarily in the cytoplasm and is largely absent from the nucleus of the L2-stage seam cells (Figure 3.3B versus 3.3A), which explains the lack of HBL-1 activity and the suppression of *hbl-1(gf)* extra seam cell phenotypes in larvae lacking *lin-28*.

In order to test the possibility of an effect of the linker or the mScarlet-I tag on the localization of HBL-1, we also tagged another transcription factor, *daf-12* (nuclear accumulation of which is not affected by *lin-28(lf)*; Figure 3.4A), at its endogenous locus with the same linker and mScarlet-I and determined whether the localization of DAF-12 changed in *lin-28(lf)* animals (Figure 3.4). We observed that, unlike HBL-1, linker-mScarlet-I tagged DAF-12 did not accumulate in the cytoplasm of L2-stage seam cells in *lin-28(lf)* animals (Figure 3.4B), suggesting that the linker-mScarlet-I tag could not be the cause of cytoplasmic accumulation of HBL-1.

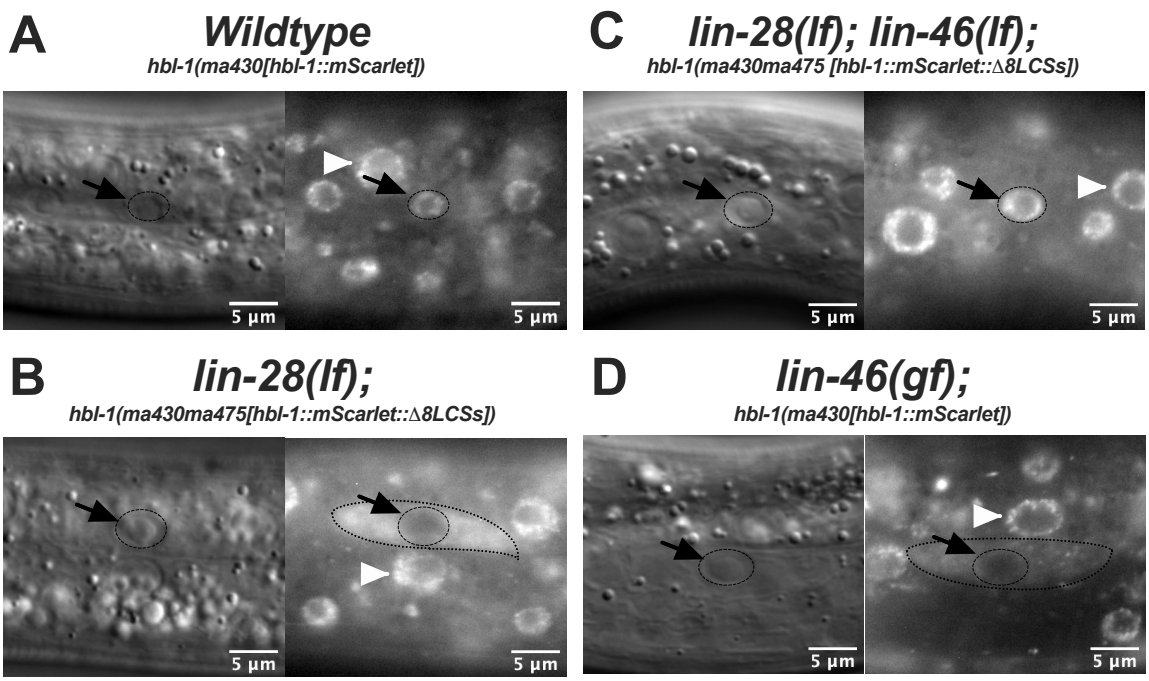

A
Wildtype
hbl-1(ma430[hbl-1::mScarlet])
C
lin-28(lf); lin-46(lf);
hbl-1(ma430ma475 [hbl-1::mScarlet::Δ8LCSs])
B
lin-28(lf);
hbl-1(ma430ma475[hbl-1::mScarlet::Δ8LCSs])
D
lin-46(gf);
hbl-1(ma430[hbl-1::mScarlet])
5 µm

Figure 3.3. The *lin-28-lin-46* pathway regulates nuclear accumulation of HBL-1. Differential interference contrast and fluorescent images of hypodermal (seam and hyp7) cells in L2-stage larvae. Black arrows and dashed circles show seam nuclei and white arrowheads show examples of hyp7 nuclei. (A) HBL-1 accumulates in the nucleus in wild-type animals. (B) HBL-1 is dispersed in the cytoplasm (outlined by dotted line) of L2-stage seam cells in lin-28(lf) animals, indicating that *lin-28* is required for the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae. Note that HBL-1 still accumulates in hyp7 nuclei (e.g. white arrowhead). (C) The *lin-28* target *lin-46* is required to prevent the nuclear accumulation of HBL-1 in L2-stage seam cells of *lin-28(lf)* larvae. (D) Precocious/ectopic LIN-46 expression is sufficient to reduce the nuclear accumulation of HBL-1 in the seam cells of L2-stage larvae. HBL-1 is present in the cytoplasm (outlined by dotted line) of the seam cell in the picture. Note that HBL-1 still accumulates in hyp7 nuclei (e.g. white arrowhead).

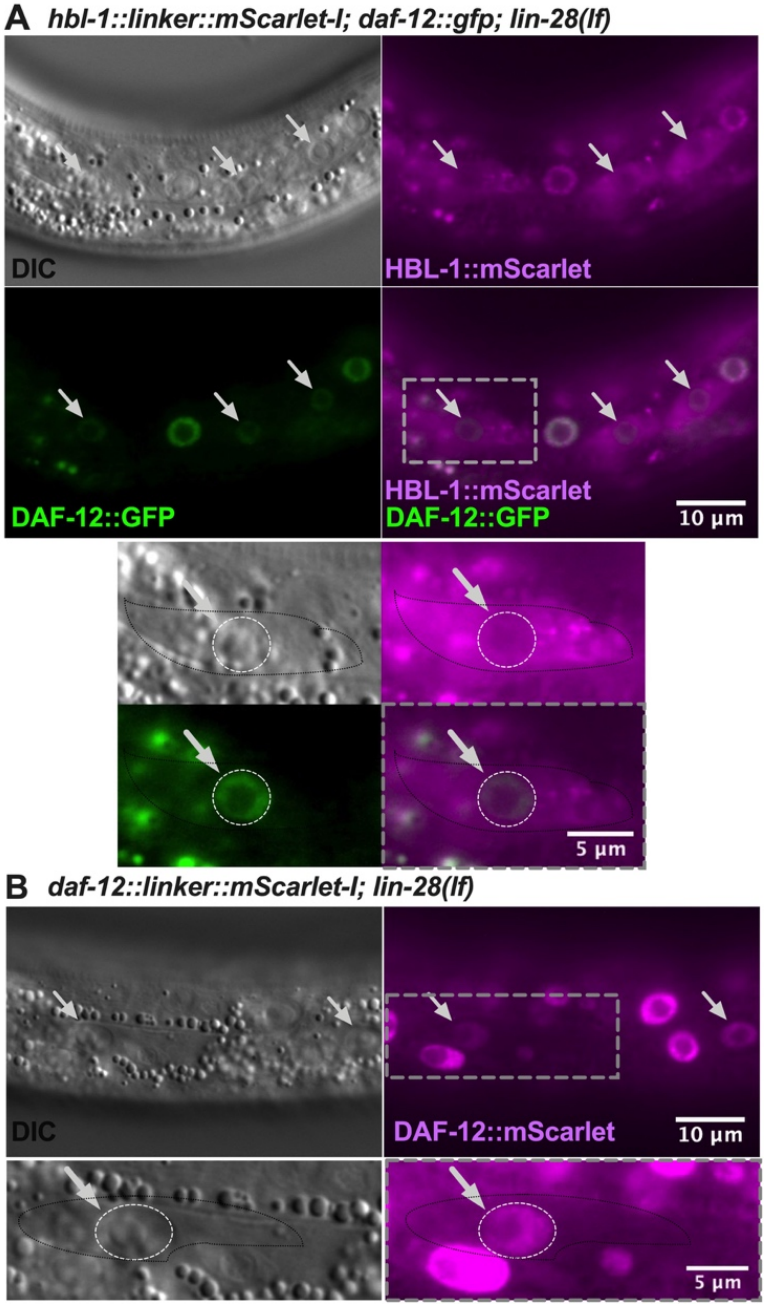

A hbl-1::linker::mScarlet-I; daf-12::gfp; lin-28(lf)
DIC
HBL-1::mScarlet
DAF-12::GFP
HBL-1::mScarlet
DAF-12::GFP
10 µm
5 µm
B daf-12::linker::mScarlet-I; lin-28(lf)
DIC
DAF-12::mScarlet
10 µm
5 µm

Figure 3.4. Nuclear localization of GFP or mScarlet-I tagged DAF-12 is not regulated by *lin-28*.

DIC and fluorescent images showing hypodermal seam and hyp7 nuclei of L2 stage *lin-28(lf)* larvae. White arrows indicate the seam cell nuclei. (A) In the absence of *lin-28*, HBL-1 is majorly excluded from the nuclei of L2 stage seam cells whereas nuclear localization of DAF-12 (GFP tagged) is not affected. (B) The linker and mScarlet-I tag that was used to tag *hbl-1* [*hbl-1(ma430)*] do not have an effect on nuclear localization of DAF-12 in the *lin-28(lf)* background.

lin-46 activity is required for the cytoplasmic accumulation of HBL-1 in both *hbl-1(ma430ma475)* and *lin-28(lf)* animals

HBL-1 accumulated both in the nucleus and the cytoplasm of L3- and L4-stage seam cells in *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* larvae (Figure 3.2B). When we combined *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* with *lin-46(lf)*, we did not observe cytoplasmic accumulation of HBL-1 at the L3 and L4 stages, rather HBL-1 accumulated in the nucleus at all stages (Figure 3.2C; Figure 3.5C), indicating that the L3/L4-stage cytoplasmic accumulation of HBL-1 requires *lin-46* activity.

HBL-1 accumulated primarily in the cytoplasm of L2-stage seam cells in *lin-28(lf)* larvae (Figure 3.3B). By contrast, in L2 larvae lacking both *lin-28* and *lin-46* HBL-1 no longer accumulated in the cytoplasm of L2 or later stage seam cells, rather HBL-1 accumulated in the nucleus of the seam cells at all stages (Figure 3.3C; Figure 3.5F).

In brief, *lin-46(lf)* resulted in the loss of cytoplasmic HBL-1 accumulation at all stages, accompanied by restoration of nuclear accumulation of HBL-1 (Figure 3.5).

Precocious LIN-46 expression is sufficient to reduce the nuclear accumulation of HBL-1

Lastly, we found that precocious LIN-46 expression, which is sufficient to suppress the extra seam cell phenotypes of *hbl-1(ma354)* (Figure 3.1B), is also sufficient to reduce the nuclear accumulation of HBL-1 (Figure 3.3D).

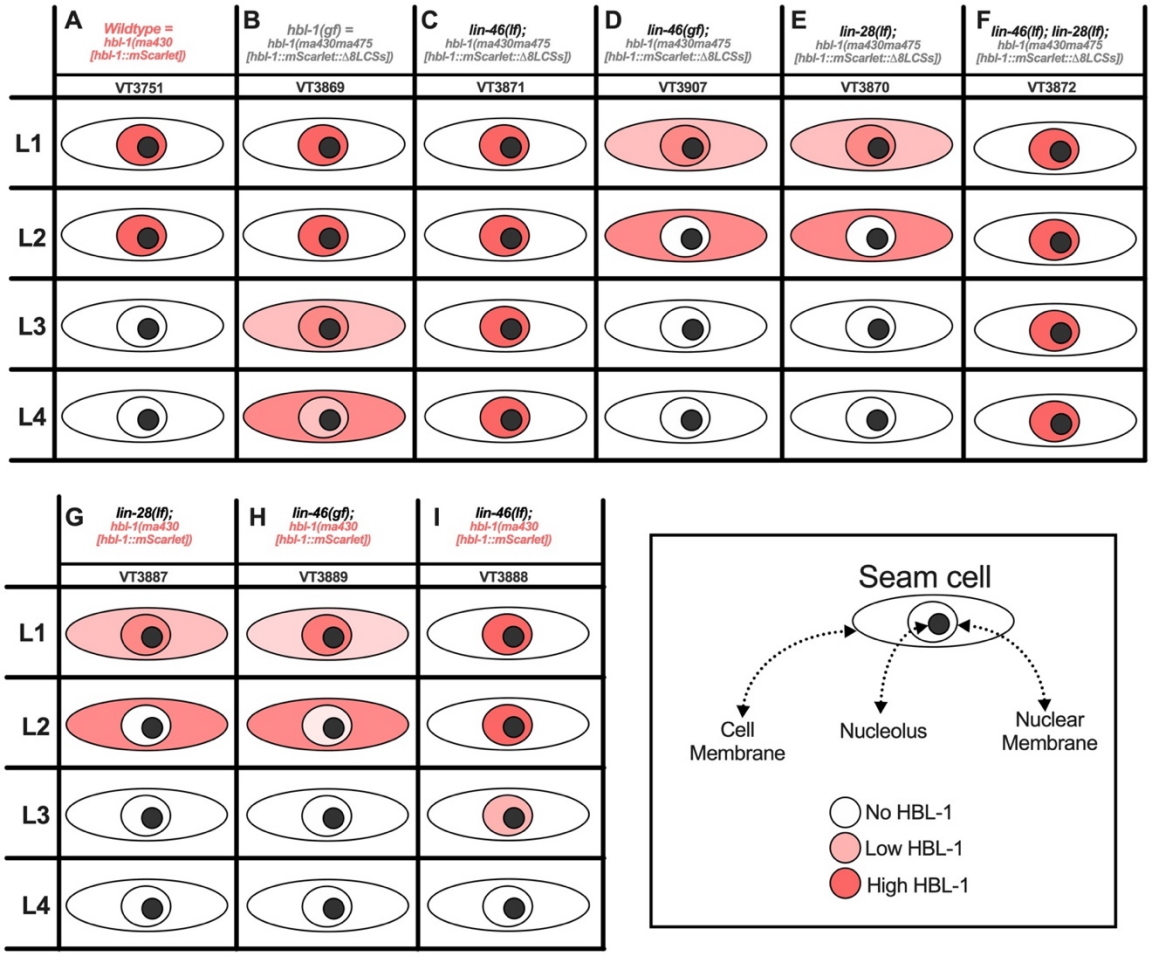

A
Wildtype =
hbl-1(ma430
[hbl-1::mScarlet])
VT3751
B
hbl-1(gf) =
hbl-1(ma430ma475
[hbl-1::mScarlet::Δ8LCSs])
VT3869
C
lin-46(lf);
hbl-1(ma430ma475
[hbl-1::mScarlet::Δ8LCSs])
VT3871
D
lin-46(gf);
hbl-1(ma430ma475
[hbl-1::mScarlet::Δ8LCSs])
VT3907
E
lin-28(lf);
hbl-1(ma430ma475
[hbl-1::mScarlet::Δ8LCSs])
VT3870
F
lin-46(lf); lin-28(lf);
hbl-1(ma430ma475
[hbl-1::mScarlet::Δ8LCSs])
VT3872
L1
L2
L3
L4
G
lin-28(lf);
hbl-1(ma430
[hbl-1::mScarlet])
VT3887
H
lin-46(gf);
hbl-1(ma430
[hbl-1::mScarlet])
VT3889
I
lin-46(lf);
hbl-1(ma430
[hbl-1::mScarlet])
VT3888
L1
L2
L3
L4
Seam cell
Cell
Membrane
Nucleolus
Nuclear
Membrane
No HBL-1
Low HBL-1
High HBL-1

Figure 3.5. Schematic representations of nucleo-cytoplasmic localization of HBL-1 in hypodermal seam cells across four larval stages and various genetic backgrounds.

HBL-1 expression (pink color) is denoted in nuclear/cytoplasmic compartments of seam cells for each larval stage and genotype. Ten animals for each larval stage and genotype were analyzed. Genotypes and corresponding strain names (VT#) are denoted at the top of each column.

These results, together with those presented above show that the suppression of *hbl-1(ma354)* phenotypes by *lin-28(lf)* or *lin-46(gf)* is accompanied by increased cytoplasmic accumulation of HBL-1 and a reduction in the nuclear accumulation of HBL-1. These findings suggest that LIN-46 negatively regulates HBL-1 activity by hindering its nuclear accumulation.

Spatiotemporal occurrence of cytoplasmic HBL-1 accumulation coincides with LIN-46 expression

Endogenously tagged *lin-46* is expressed in the seam cells of L3- and L4-stage larvae but not in the seam cells of L1- and L2-stage larvae (Ilbay, Nelson and Ambros, 2019). Therefore, the onset of LIN-46 expression coincides with the onset of cytoplasmic accumulation of HBL-1 in *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* animals (which are, unlike the wild type, not capable of properly downregulating HBL-1 at the end of the L2 stage owing to the lack of LCSs) (Figure 3.5). In *lin-28(lf)*animals, LIN-46 is expressed precociously (Ilbay, Nelson and Ambros, 2019) starting in mid L1-stage seam cells. This precocious onset of LIN-46 expression in *lin-28(lf)* animals also coincides with the precocious onset of the cytoplasmic accumulation of HBL-1 in *lin-28(lf)* animals (Figure 3.5).

We note that LIN-46 expression is detected in the seam cells but not in the hyp7 cells (Ilbay, Nelson and Ambros, 2019), and the presence/absence of LIN-46 expression in these two hypodermal cell types correlates with the presence/absence of cytoplasmic accumulation of HBL-1. For example, whereas HBL-1 accumulates in both the cytoplasm and the nucleus of seam cells L3/L4-

stage *hbl-1(ma430ma475[mScarlet-I::Δ8LCSs])* animals (Figure 3.2B), HBL-1 accumulates only in the nuclei of the hyp7 cells (Figure 3.2B). Similarly, whereas HBL-1 accumulates primarily in the cytoplasm of L2-stage seam cells of *lin-28(lf)* animals, HBL-1 still accumulates in the nuclei of hyp7 cells in these animals (Figure 3.3B, black arrow versus white arrowhead).

In brief, cytoplasmic accumulation of HBL-1 was observed in the hypodermal cells where (seam) and when [by the L3 stage in wild type and by the late L1 stage in *lin-28(lf)*] LIN-46 is expressed (Figure 3.5).

Discussion

Our results suggest that the *C. elegans lin-28-lin-46* pathway regulates the nuclear accumulation of HBL-1, a transcription factor that specifies L2-stage proliferative cell fates and opposes the progression to L3-stage self-renewal cell fates during *C. elegans* development. *lin-28* is required for the nuclear accumulation of HBL-1 in hypodermal seam cells at the L2 stage, which is, in turn, necessary for the execution of L2-stage proliferative cell fates. The *lin-28* target *lin-46* is responsible for preventing the nuclear accumulation of HBL-1 in *lin-28(lf)* animals, and in wild-type animals *lin-28*-mediated repression of the LIN-46 expression at the L1 and L2 stages (Ilbay, Nelson and Ambros, 2019) allows the nuclear accumulation of HBL-1 at those early larval stages. Using a *lin-46* 5′UTR mutation that renders LIN-46 expression poorly repressed by *lin-28*, we show that precocious LIN-46 expression in the seam cells of the L1/L2-stage larvae is sufficient to reduce the nuclear accumulation of HBL-1.

Furthermore, using *hbl-1* gain-of-function mutations with *let-7* and *lin-4* sites deleted from the *hbl-1* 3′UTR, we show that the *lin-28-lin-46* pathway acts in parallel with *let-7* family microRNAs. Hence, these two parallel pathways – the microRNA pathway controlling the rate of synthesis of HBL-1 through repression of *hbl-1* mRNA translation, and the *lin-28-lin-46* pathway controlling the nuclear accumulation of HBL-1 – function together to ensure the precision and robustness of stage-specific HBL-1 downregulation (Figure 3.6).

In wild-type animals, HBL-1 and LIN-46 are expressed at temporally distinct stages: HBL-1 is expressed at the L1 and L2 stages whereas LIN-46 is expressed at the L3 and L4 stages. In larvae of certain mutants, such as *hbl-1(gf)* L3 and L4 larvae, or *lin-28(0)* L1 and L2 larvae, LIN-46 and HBL-1 expression overlap, and cytoplasmic accumulation of HBL-1 is observed, accompanied by a reduction in the nuclear accumulation of HBL-1. Our data further show that the nucleus-to-cytoplasm displacement of HBL-1 in these contexts depends on *lin-46* activity. Therefore, one might have expected to observe cytoplasmic accumulation of HBL-1 after the L2-to-L3 transition in wild-type larvae, when LIN-46 begins to accumulate. Curiously, in wild-type animals, cytoplasmic accumulation of HBL-1 is not evident at any stage, despite the presence of LIN-46 in L3-adult animals. If LIN-46 causes cytoplasmic accumulation of HBL-1 in the wild type, why is HBL-1 not detected in L3 and L4 larvae? One explanation could be that in wild-type larvae, the post-translational repression of HBL-1 activity by LIN-46 (via cytoplasmic localization) functions

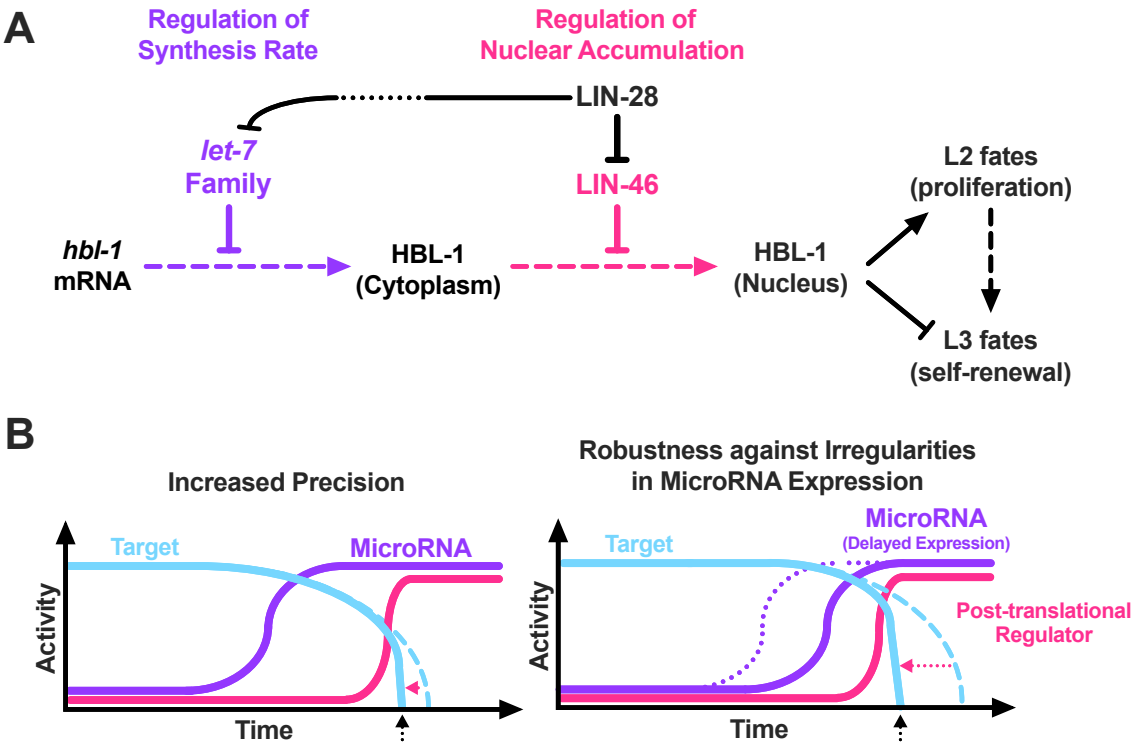

A
Regulation of Synthesis Rate
Regulation of Nuclear Accumulation
LIN-28
let-7 Family
LIN-46
hbl-1 mRNA
HBL-1 (Cytoplasm)
HBL-1 (Nucleus)
L2 fates (proliferation)
L3 fates (self-renewal)
B
Increased Precision
Robustness against Irregularities in MicroRNA Expression
Target
MicroRNA
Activity
Time
Target
MicroRNA (Delayed Expression)
Post-translational Regulator
Activity
Time

Figure 3.6. Regulation of gene activity through microRNA-mediated repression of translation accompanied by post-translational regulation of microRNA targets. (A) The conserved RNA-binding protein LIN-28 indirectly (indicated by the dotted line break) regulates the transcription (Tsialikas *et al.*, 2017) and activities (Nelson and Ambros, 2019) of *let-7*-family (*mir-48, mir-84, mir-241*) microRNAs, which inhibit the synthesis of HBL-1. LIN-28 also represses the expression of LIN-46 (Ilbay, Nelson and Ambros, 2019), which controls the nuclear accumulation of HBL-1. Temporal downregulation of LIN-28 at the end of the L2 stage allows LIN-46 to accumulate, which acts together with the let-7 family microRNAs to ensure precise and robust temporal downregulation of HBL-1 activity. (B) Hypothetical activity trajectories of a microRNA and its target(s) against time are plotted. Dashed blue lines represent the trajectory in the absence of the hypothetical post-translational regulator. Black arrows indicate a specific critical time when the target must be downregulated to permit normal development. A post-translational regulator of a microRNA target can increase the precision of temporal downregulation of the target (red arrow, left) or confer robustness against irregularities in microRNA expression (red arrow, right). This second scenario is similar to what is thought to happen in C. elegans larvae developing in the presence of pheromones or other L2d-inducing conditions: *let-7*-family expression is delayed (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009) and LIN-46 activity becomes more important for downregulating HBL-1 (Ilbay and Ambros, 2019a). Thus, LIN-46 confers robustness against a physiological delay in the expression of *let-7* family microRNAs.

semi-redundantly with the translational repression of HBL-1 by *let-7* family microRNAs. In this scenario, the microRNA pathway could exert the lion's share of HBL-1 downregulation at the L2-to-L3 transition, and the upregulation of LIN-46 at the L3 stage could play a secondary role to inhibit the nuclear accumulation of any residual HBL-1 protein. Indeed, in support of this idea, a low level of HBL-1 expression persists in the nuclei of L3-stage seam cells in *lin-46(lf)*animals (Figure 3.5). This scenario is also consistent with the differing strengths of the weaker retarded phenotypes of *lin-46(lf)* animals compared with *hbl-1(gf)* animals under standard culture conditions (Figure 3.1B).

Interestingly, conditions such as diapause-inducing stress signals that enhance *lin-46(lf)* phenotypes (Ilbay and Ambros, 2019a) also result in a reduction in the expression of *let-7* family microRNAs (Hammell, Karp and Ambros, 2009), resulting in a shift from primarily microRNA-mediated regulation of HBL-1 to primarily LIN-46-mediated regulation (Ilbay and Ambros, 2019a). In this context, where wild-type larvae experience diapause-inducing stress signals, and hence LIN-46-mediated cytoplasmic localization becomes the primary mode of HBL-1 downregulation, we expected to observe cytoplasmic accumulation of HBL-1 after the L2d-to-L3 transition. However, we could not detect any cytoplasmic HBL-1::mScarlet-I fluorescence in wild-type L3 larvae under L2d-promoting conditions. It is possible that the cytoplasmic HBL-1 in L3 larvae is unstable and/or dispersed such that the HBL-1::mScarlet-I signal is below the limit of detection in our fluorescence microscopy assays.

Sequence homology places LIN-46 into a conserved protein family, members of which include bacterial MOEA as well as human GPHN (gephyrin), which are implicated in molybdenum co-factor (MoCo) biosynthesis (Schwarz, Mendel and Ribbe, 2009). GPHN has also been reported to function as a scaffold protein that is required for clustering of neurotransmitter receptors (Feng *et al.*, 1998; Kneussel *et al.*, 1999), and has been shown to physically interact with several other proteins (Fritschy, Harvey and Schwarz, 2008), including tubulin (Kirsch *et al.*, 1991), dynein (Fuhrmann *et al.*, 2002) and mTOR (Sabatini *et al.*, 1999). It is not known whether LIN-46 possesses MOEA-related enzymatic activity and/or has scaffolding functions similar to GPHN, and, if so, how such activities could (directly or indirectly) contribute to inhibition of the nuclear accumulation of a transcription factor such as HBL-1.

Analysis of the HBL-1 amino acid sequence does not reveal a predicted nuclear localization signal (NLS) that could mediate HBL-1 nuclear transport. If HBL-1 has an unconventional or ‘weak’ NLS, it is possible that other unknown factors may be required to efficiently couple HBL-1 to the nuclear import machinery. LIN-46 might inhibit HBL-1 nuclear accumulation by binding or modifying a factor that is crucial for HBL-1 nuclear transport. Alternatively, LIN-46 could bind or modify HBL-1 directly in order to prevent its association with the nuclear import machinery. It is also possible that LIN-46 could act not by directly preventing nuclear import of HBL-1, but by causing HBL-1 to be trapped in the

cytoplasm, for example through the formation of LIN-46–HBL-1 complexes in association with a cytoplasmic compartment.

Regulation of nuclear accumulation in the context of temporal cell-fate specification during *C. elegans* development has not previously been reported. Other transcription factors, including LIN-14, DAF-12 (Antebi *et al.*, 2000) and LIN-29, play key roles in regulating temporal cell fates during *C. elegans* development. These other transcription factors are also regulated by microRNAs, like HBL-1, and regulation of their temporal abundances is important for the proper execution of stage-specific cell fates. Although we have no evidence that LIN-46 may also regulate the nuclear/cytoplasmic partitioning of other heterochronic pathway proteins (except for DAF-12; Figure 3.4), our findings suggest that similar post-translational mechanisms might be in place to function in parallel with the microRNA-mediated regulation and hence promote the robust temporal regulation of key developmental regulators.

Many *let-7* targets, as well as many targets of other microRNAs, in worms, flies and mammals are transcription factors (Ambros, 2004; Bartel, 2004; Enright *et al.*, 2004; John *et al.*, 2004). Therefore, similar mechanisms, whereby a transcription factor is regulated both by a microRNA and in parallel by a gene product that controls the nuclear accumulation of the same transcription factor, may be common. Additionally, the regulation of the *let-7* microRNA by LIN-28 is widely conserved. Targets of LIN-28 in other species may have roles, similar to LIN-46, in regulation of *let-7* targets, controlling their nuclear accumulation in

particular and their activities by means of post-translational interventions in general. A dual control of a gene product – its synthesis rate by microRNAs and activity by post-translational regulators – would allow more precise and/or more robust transitions between active and inactive states (Figure 3.6B).

Materials and Methods

C. elegans culture conditions

C. elegans strains used in this study and corresponding figures in the chapter are listed in Table 3.2. *C. elegans* strains were maintained at 20°C on nematode growth media (NGM) and fed with the *Escherichia coli* HB101 strain.

Assaying extra seam cell phenotypes

The worms were scored at the young adult stage (determined by the gonad development) for the number of seam cells using fluorescence microscopy with the help of the *maIs105* [*pCol-19::gfp*] transgene, which marks the lateral hypodermal cell nuclei, or the *wIs51*[*pScm::gfp*] transgene, which marks the seam cell nuclei.

Each circle on the genotype versus number of seam cells plots shows the observed number of seam cells on one side of a single young adult worm. Twenty worms (except for wild type, *n*=12) for each genotype were analyzed and the average number of seam cells are denoted by vertical bars in the genotype versus number of seam cell plots. Student's *t*-test was used to calculate statistical significance when comparing different genotypes. GraphPad Prism 8 software was used to plot the graphs and for statistical analysis.

Microscopy

All differential interference contrast and fluorescent images were obtained using a ZEISS Imager Z1 equipped with a ZEISS Axiocam 503 mono camera, and ZEN Blue software. Prior to imaging, worms were anesthetized with 0.2 mM levamisole in M9 buffer and mounted on 2% agarose pads. ImageJ Fiji software was used to adjust the brightness and contrast of the images to enhance the visualization of the fluorescent signal. All images were taken using the same microscopy settings and a standard exposure time for all larval stages and genetic background, but because the brightness and contrast of the individual images were enhanced separately, the signal intensities do not represent the relative expression levels and cannot be used to compare expression levels across different larval stages of genetic backgrounds.

Generation of new alleles using CRISPR/Cas9

CRISPR/Cas9 genome editing tools were used to generate the *hbl-1* 3′UTR deletion alleles, the *lin-46* open-reading frame (ORF) deletion allele, and to tag the *daf-12* gene with GFP and mScarlet-I at its endogenous locus.
For the *hbl-1* 3′UTR deletions and the *lin-46* ORF deletion (for alleles, see Table 3.1), a mixture of plasmids encoding SpCas9 (pOI90), and a pair of single guide RNAs (sgRNAs, expressed from pOI83; (Ilbay and Ambros, 2019a) targeting both sites of interest (for primers, see Table 3.3) and the *unc-22* gene (pOI91) as co-CRISPR marker (Kim *et al.*, 2014), and a *rol-6(su1006)*-containing plasmid (pOI124) as co-injection marker was injected into the gonads of young adult

worms. F1 roller and/or twitcher animals (~50 or more worms until the desired allele was detected) were cloned and screened by PCR amplification (for primers, see Table 3.3) for the presence of the expected size PCR product consistent with deletion of the genomic region spanning between the sites targeted by the pair of guides.

To tag *daf-12* at the endogenous locus with the same linker and mScarlet-I sequence as the *hbl-1*(ma430) allele, a homologous recombination (HR) donor plasmid (pOI193) and sgRNA plasmid (pOI93) were included in the CRISPR mix, which contained plasmids pOI90 (spCas9), pOI91 (*unc-22* guide) and pOI124 (*rol-6*). The HR plasmid pOI193 contains the C-terminal end of the *daf-12* gene fused in-frame with the linker and mScarlet-I sequence, subcloned from pOI191, which was used to tag *hbl-1* to generate the *ma430* allele. To tag *daf-12* with GFP, instead of pOI193, an HR donor plasmid (pOI122) that contained the GFP sequence flanked by HR sequences was included in the CRISPR mix.
In all new CRISPR alleles, genomic regions spanning the deletion site or the HR arms and the tags introduced were sequenced using Sanger sequencing. For each allele, a single worm with a precise (HR) edited locus was cloned and backcrossed twice before being used in the experiments.

Strain name	Genotype	Related figures
VT1367	*maIs105 V*	3.1A&B
VT3307	*maIs105 V; hbl-1(ma294) X.*	3.1A
VT3336	*maIs105 V; hbl-1(ma293) X.*	3.1A
VT3399	*maIs105 V; hbl-1(ma332) X*	3.1A
VT3500	*wIs51 V; hbl-1(ma354) X*	3.1A&B
VT3593	*lin-46(ma385) I; maIs105 V.*	3.1B
VT3696	*lin-46(ma385) wIs51 V; hbl-1(ma354) X*	3. 1B
VT790	*lin-28(n719) I; maIs105 V*	3.1B
VT3571	*lin-28(n719) I; maIs105 V; hbl-1(ma354) X*	3.1B
VT3698	*lin-28(n719) I; lin-46(ma385) wIs51 V; hbl-1(ma354) X*	3.1B
VT3855	*lin-46(ma467) maIs105 V*	3.1B
VT3891	*lin-46(ma467) maIs105 V; hbl-1(ma354) X*	3.1B
VT3751	*maIs105 V; hbl-1(ma430[hbl-1::mScarlet-I]) X*	3.2A&3A, 3.5A
VT3869	*wIs51 V; hbl-1(ma430ma475[hbl-1::mScarlet-I::UTRdel]) X*	3.2B, 3.5B
VT3871	*wIs51 lin-46(ma385) V; hbl-1(ma430ma475[hbl-1::mScarlet-I::UTRdel]) X*	3.2C, 3.5C
VT3870	*lin-28(n719) I; maIs105 V; hbl-1(ma430ma475) X*	3.3B, 3.5E
VT3872	*lin-28(n719) I; wIs51 lin-46(ma385) V; hbl-1(ma430ma475) X*	3.3C, 3.5F
VT3889	*lin-46(ma467) maIs105 V; hbl-1(ma430) X*	3.3D, 3.5H
VT3907	*lin-46(ma467) maIs105 V; hbl-1(ma430ma475) X*	3.5D
VT3887	*lin-28(n719) I; maIs105 V; hbl-1(ma430) X; syIs(ajm-1::gfp)*	3.5G
VT3888	*lin-46(ma385) maIs105 V; hbl-1(ma430) X; syIs(ajm-1::gfp)*	3.5I
VT3922	*lin-28(n719) I; daf-12(ma497[daf-12::gfp] hbl-1(ma430[hbl-1::mscarlet-I]) X*	3.4A
VT3924	*lin-28(n719) I; daf-12(ma498[daf-12::mScarlet-I] X*	3.4B

Table 3.2. *C. elegans* strains used in Chapter III.

Cloning/ PCR	Primer Name	Primer Sequence	Plasmid name and/or purpose
Cloning: Annealed primer-pairs that are cloned into pOI83 to express sgRNAs	priOI250F	tcttggaacgcactgaagtttgagg	pOI104& pOI138 are injected together to delete a region of *hbl-1* 3'UTR encompassing all *let-7*-complementary sites.
	priOI251R	aaaccctcaaacttcagtgcgttcc	
	priOI421F	tcttgaggtgtacgtgcaagaaac	
	priOI422R	aaacgtttcttgcacgtacacctc	
	priOI362F	tcttgcgtagatcaaccacgtctc	pOI113&pOI160 are injected together to delete the *lin-46* coding sequences (all exons).
	priOI363R	aaacgagacgtggttgatctacgc	
	priOI517F	tcttgtcaatccaatgagttcttc	
	priOI518R	aaacgaagaactcattggattgac	
	priOI175F	tcttgattgaaggcatggcatcgtt	pOI93 is used to tag *daf-12* with gfp or mScarlet-I.
	priOI176R	aaacaacgatgccatgccttcaatc	
Cloning: HR Template to tag daf-12 with gfp (pOI122)	priOI346F	cgggaattctttaggttcttcggtttcttcgac	5-prime HR arm, PCR amplified using N2 DNA as template. pOI346F contains an EcoRI cut site (underlined).
	priOI349R	attctcctggcagctcttcg	
	priOI232F	ggtgaagccgaagagctgccaggagaatttttcaaaatcaaaagtaaaggagaagaacttttc	To amplify GFP from pCM1.53. Primers contain tails complementary to *daf-12* 5-prime and 3-prime HR arms to allow PCR stitching of HR arms with GFP.
	priOI239R	ggaggcaatatagaatcaagttgtgaggtattgaaggcatggcatcgtttcgtagatgatttctagtaggtctatttgtatagttcgtccatgccatg	
	priOI348F	aaacgatgccatgccttcaatac	3-prime HR arm, PCR amplified using N2 DNA as template. pOI346F contains a HindIII cut site (underlined).
	priOI347R	gccaagcttggctaggctgcatgaatcac	
Cloning: HR Template to tag daf-12 with linker::mScarlet-I (pOI193)	priOI670	gaaggtctcatctggaggtggatctggaggtggatctggaggtggatctgtcagcaaggagaggcagttatc	To amplify the linker+mScarlet sequences from pOI191, which is the plasmid that was used as the HR template to tag *hbl-1* with mScarlet. Primers contain Golden Gate (NEB) cloning tails (underlined).
	priOI671	gaaggtctcacttgtagagctcgtccattcc	
	priOI741	ggctacggtctcccagatttgattttgaaaaattctcctggc	To amplify 5-, 3-primer HR arms and the backbone of pOI122 to fuse with the linker+mScarlet sequence. Primers contain Golden Gate (NEB) cloning tails (underlined).
	priOI742	ggctacggtctcccaagtagacctactagaaatcatctac	
PCR Primers	priOI147F	tgcaaaccgacctagtgcat	These primers flank the *hbl-1* 3'UTR. They are used to detect large deletions in the F1/F2 progeny of pOI104& pOI138 (in a CRISPR mix) injected animals.
	priOI148R	aaagtagccagtcccctcgt	
	priOI515F	accatactgctgaaatcccaa	These primers flank the *lin-46* exons. They are used to detect the ORF deletion in the F1/F2 progeny of pOI113&pOI160 (in a CRISPR mix) injected animals.
	priOI516R	taagtacgcaaacacgctgc	
	priOI3	gaggcgtttcgtcaaagttg	These primers flank the HR arms in pOI122&pOI193. They are used to detect GPF or mScarlet integration in F1/F2 progeny of animals injected with CRISPR mixes containing these plasmids together with pOI93.
	priOI114	cccttatgggttggctgaga	

Table 3.3. Cloning and PCR Primers used in Chapter III.

CHAPTER IV -- *C. elegans lin-28* Controls Temporal Cell-Fate Progression by Regulating *lin-46* Expression via the 5'UTR of *lin-46* mRNA

This chapter is published in bioRxiv as a preprint and is under review in *Cell Reports.*

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', *bioRxiv*, p. 697490. doi: 10.1101/697490.

Experiments to determine *lin-46* mRNA levels as presented in Figure 4.3 was conducted by Charles Nelson.

Abstract

Human Lin28 is a conserved RNA-binding protein that promotes proliferation and pluripotency and can be oncogenic. Lin28 and *C. elegans* LIN-28 bind to precursory RNAs of the conserved – cellular differentiation-promoting – microRNA *let-7* and inhibits its maturation. Lin28/LIN-28 also binds to and regulates many mRNAs in various cell types. However, the determinants and consequences of these LIN-28-mRNA interactions are not well understood. Here, we report that LIN-28 in *C. elegans* represses the expression of LIN-46, a downstream protein in the heterochronic pathway, via the 5' UTR of the *lin-46* mRNA. We show that both LIN-28 and the 5'UTR of *lin-46* are required to prevent LIN-46 expression and that precocious LIN-46 expression is sufficient to skip L2 stage proliferative cell-fates, resulting in heterochronic defects similar to the ones observed in *lin-28(0)* animals. We hypothesize that the *lin-46* 5'UTR mediates LIN-28 binding to and repression of the *lin-46* mRNA. Our results demonstrate that precocious LIN-46 expression alone can account for *lin-28(0)* phenotypes, demonstrating the biological importance of regulation of target mRNAs by LIN-28.

Introduction

Animal development involves complex cell lineages within which different cell-fates are executed in specific orders and at a pace that is in synchrony with

overall developmental rate. Expressing symmetric cell-fates that allow cell proliferation or asymmetric cell-fates that enable both self-renewal and the generation of new cell types, a single totipotent cell (embryo) and its progeny generate the populations of specified cells that form the diverse tissues and organs of the animal body. Gene regulatory networks control the levels and spatiotemporal expression patterns of developmental genes so that proper cell-fates are acquired at the right time and place during development.

C. elegans develops through four larval stages (L1-L4). Each larval stage is comprised of an invariant set of cell division and cell fate specification events (Sulston and Horvitz, 1977). The order of cell-fates and the timing of cell-fate transitions within individual cell lineages are regulated by genes in the heterochronic pathway (Ambros and Horvitz, 1984; Ambros, 2011). In this pathway, three major temporal regulatory transcription factors control the transitions from earlier to later cell fates. These transcription factors are directly or indirectly regulated by microRNAs and/or RNA binding proteins, thereby facilitating proper cell-fate transitions during larval development.

One of the transcription factors in the heterochronic pathway, Hunchback-like-1 (HBL-1), promotes L2-stage symmetric cell divisions and prevents progression to L3-stage asymmetric cell divisions (Abrahante *et al.*, 2003; Lin *et al.*, 2003). HBL-1 is expressed at the L1 and L2 stages and is downregulated during the L2-to-L3 transition (Abrahante *et al.*, 2003; Ilbay and Ambros, 2019a). Proper temporal regulation of HBL-1 activity is characterized by specification of L2

cell-fates at the L2 stage and progression to L3 cell-fates concomitant with the L2-to-L3 stage progression. Mutations that reduce HBL-1 activity during the L1 and L2 stages result in the skipping of L2 cell-fates, whereas mutations that cause ectopic HBL-1 activity at the L3 and L4 stages lead to reiterations of L2 cell-fates at these later larval stages. During the L2-to-L3 transition HBL-1 is regulated by *let-7*-family microRNAs (*mir-48/84/241*) (Abbott *et al.*, 2005) as well as by *lin-28* (Abbott *et al.*, 2005; Vadla *et al.*, 2012), which acts on *hbl-1* indirectly via a protein coding gene *lin-46* (Pepper *et al.*, 2004; Ilbay and Ambros, 2019b).

LIN-28 is a conserved RNA-binding protein first identified as a heterochronic gene product in *C. elegans* (Ambros and Horvitz, 1984). In *C. elegans* larvae lacking *lin-28* activity, hypodermal stem cells skip L2-stage specific symmetric cell divisions and precociously transition to later stage cell-fates leading to premature terminal differentiation of the hypodermis (Ambros and Horvitz, 1984). LIN-28 inhibits the maturation of the conserved microRNA *let-7* (Van Wynsberghe *et al.*, 2011), which is required for this hypodermal differentiation at the end of the L4 stage (Reinhart *et al.*, 2000). Curiously, although *let-7* is expressed precociously at the early stages in *lin-28(0)* larvae, and *let-7* function is required for the precocious terminal differentiation of hypodermal cells in *lin-28(0)* animals, *let-7* is not required for the skipping of L2 stage cell fates observed in *lin-28(0)* (Vadla *et al.*, 2012). Instead, loss of a protein coding gene, *lin-46,* suppresses both early and late stage *lin-28(0)* phenotypes (Pepper *et al.*, 2004) without repressing precocious *let-7* expression (Vadla *et al.*, 2012) . This suggests that LIN-46 might be mis-

regulated in *lin-28(0)* animals and could be responsible for *lin-28(0)* phenotypes irrespective of *let-7*.

Previous studies shown that LIN-28 binds to the *lin-46* mRNA (Stefani *et al.*, 2015) but the consequence of this binding is not known. Additionally, *lin-46* encodes a protein related to a bacterial molybdenum cofactor biosynthesis enzyme and the mammalian protein Gephyrin (Feng *et al.*, 1998; Fritschy, Harvey and Schwarz, 2008), and although the molecular functions of LIN-46 are not clear, our recent findings suggest that LIN-46 affects temporal cell-fates by inhibiting the nuclear accumulation of the transcription factor HBL-1 (Ilbay and Ambros, 2019b). Therefore, it is possible that LIN-28 could promote L2 fates by restricting the expression of LIN-46 thereby preserving the nuclear activity of the L2-fate-promoting factor HBL-1.

C. elegans' LIN-28 and its homologs in mammals (Lin28) have conserved functions: LIN-28/Lin28 inhibit *let-7* expression (Newman, Thomson and Hammond, 2008; Rybak *et al.*, 2008; Viswanathan, Daley and Gregory, 2008; Van Wynsberghe *et al.*, 2011), bind to and regulate mRNAs (Cho *et al.*, 2012; Wilbert *et al.*, 2012; Hafner *et al.*, 2013; Stefani *et al.*, 2015), and promote proliferation and pluripotency (Moss, Lee and Ambros, 1997; Yu *et al.*, 2007; Yuan *et al.*, 2012). Similar to *C. elegans* development, during mammalian embryogenesis LIN-28 is expressed at early/pluripotent stages and is downregulated at later/differentiated stages (Moss, Lee and Ambros, 1997; Moss and Tang, 2003; Yang and Moss, 2003). LIN-28 down-regulation in differentiating tissues allows *let-7* microRNA to

accumulate resulting in further *let-7*-driven cellular differentiation through the repression of pluripotency and self-renewal promoting genes (Shyh-Chang and Daley, 2013), including *lin-28*. Lastly, LIN-28 expression is associated with many types of cancers and poor prognosis (Viswanathan *et al.*, 2009; Thornton and Gregory, 2012), and conversely, *let-7* is known to act as a tumor suppressor by repressing oncogenes (Johnson *et al.*, 2005, 2007; Lee and Dutta, 2007; Sampson *et al.*, 2007).

While certain phenotypes observed in Lin28-deficient mammalian cells can be attributed to increased *let-7* expression and consequent repression of *let-7* targets (Rybak *et al.*, 2008; Melton, Judson and Blelloch, 2010), there are *let-7* independent functions of Lin28 (Balzer *et al.*, 2010; King *et al.*, 2011; Vadla *et al.*, 2012), some of which could be explained by mis-regulation of specific mRNA targets of Lin28 (Polesskaya *et al.*, 2007; Xu and Huang, 2009; Xu, Zhang and Huang, 2009; Qiu *et al.*, 2010). Lin28 can regulate the translation of target mRNAs either positively (Hafner *et al.*, 2013) or negatively (Cho *et al.*, 2012), perhaps in a cell-type specific manner. Regardless, LIN-28 tends to bind to multiple sites on its mRNA targets (Cho *et al.*, 2012). However, the contribution of each respective binding site to mRNA regulation, whether LIN-28 binding at certain regions require prior LIN-28 binding to other regions, and/or whether LIN-28 binding to different regions along the transcripts (5’ or 3’UTR or exons) may exert different effects on the mRNA such as repression, activation, localization is unclear. Moreover, while the LIN-28-bound mRNA regions are enriched for certain motifs (e.g. GGAG),

these sequence motifs are neither required nor sufficient for LIN-28 binding to its targets (Cho *et al.*, 2012; Hafner *et al.*, 2013). In brief, the rules and consequences of LIN-28/lin-28 binding to mRNAs are not well understood.

Here we show that the critical target of LIN-28 in *C. elegans*, LIN-46, is expressed only at the L3 and L4 stages in a temporal profile that is the inverse of LIN-28, which is expressed at the L1 and L2 stages. We find that LIN-46 is expressed precociously at the L1 and L2 stages in *lin-28(0)* animals, supporting the idea that LIN-28 represses LIN-46 expression at these early larval stages. We also find that, similar to *lin-28(0)*, mutations in the 5'UTR of *lin-46* result in precocious LIN-46 expression in the hypodermal seam cells, and that this ectopic LIN-46 expression at the L1 and L2 stages is sufficient to result in skipping of L2 stage symmetric seam cell divisions and precocious expression of L3-adult fates. Endogenously tagged LIN-46 is also expressed in the vulval precursor cells (VPCs) and LIN-46 is precociously expressed in the VPCs both in *lin-28(0)* and *lin-46* 5'UTR mutants. Ectopic LIN-46 expression in the VPCs in *lin-46* 5'UTR mutants is sufficient to accelerate cell-fate transitions in these cells, which results in protruding vulva phenotypes similar to *lin-28(0)* animals. Due to the phenotypic similarity between *lin-28(0)* and *lin-46* 5'UTR mutants, we hypothesize that the *lin-46* 5'UTR mediates LIN-28 binding to the *lin-46* mRNA, which results in the repression of LIN-46 expression from the *lin-46* mRNA. Our results demonstrate that precocious LIN-46 expression alone, which is observed in *lin-28(lf)* animals

and is sufficient to suppress L2 cell-fates and to induce precocious transition to L3 cell-fates, can account for majority of the *lin-28(lf)* phenotypes.

Results

lin-28 represses LIN-46 expression during early larval stages

The developmental expression patterns of *lin-28* and *lin-46* were previously identified using transcriptional reporters (transgenes expressing a fluorescent protein driven by the promoter of interest) and translational reporters (transgenes expressing the open reading frame of a gene of interest fused with a fluorescent protein driven by the promoter of the gene) (Moss, Lee and Ambros, 1997; Pepper *et al.*, 2004). The expression of such transgenes does not necessarily represent and accurate readout of expression and spatiotemporal patterns of the endogenous genes. To more accurately determine the expression patterns of LIN-28 and LIN-46, we used CRISPR/Cas9 genome editing to tag *lin-28* and *lin-46* with fluorescent proteins at their endogenous loci (Figure 4.1A). Phenotypic analyses determined that both of the endogenously tagged loci were fully functional. We found that endogenously tagged LIN-28::GFP expression is comparable to previous reports (Moss, Lee and Ambros, 1997): LIN-28 is highly expressed in the embryos and at the L1 and L2 stage larvae and is diminished at subsequent stages (Figures 4.1B and 4.1C). The expression pattern of endogenously tagged LIN-46::mCherry differs from the pattern observed in previous reports

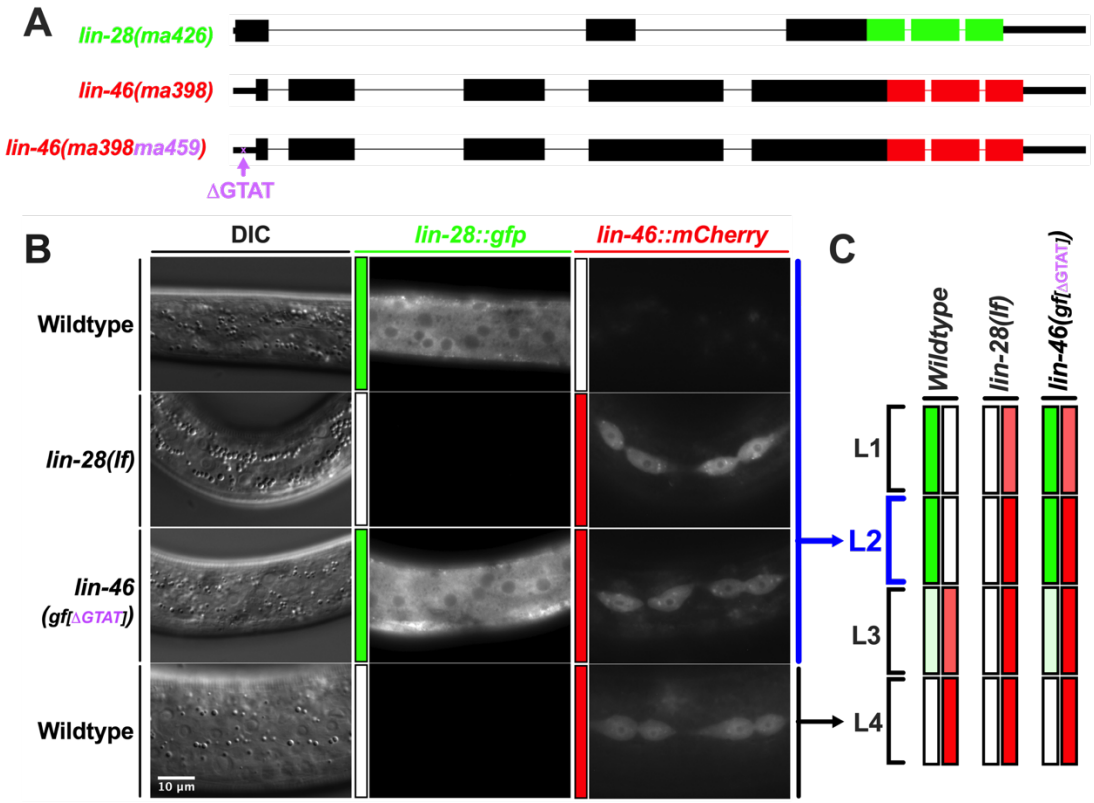

A
lin-28(ma426)
lin-46(ma398)
lin-46(ma398ma459)
ΔGTAT
B
DIC
lin-28::gfp
lin-46::mCherry
Wildtype
lin-28(lf)
lin-46 (gf[ΔGTAT])
Wildtype
10 μm
C
Wildtype
lin-28(lf)
lin-46(gf[ΔGTAT])
L1
L2
L3
L4

Figure 4.1. *lin-28* and *lin-46* 5'UTR prevent LIN-46 expression at the L1 and L2 stages.

(A) Schematic views of the *C. elegans lin-28* and *lin-46* genes and the CRISPR-mediated integration of GFP (green) or mCherry (red) coding sequences at the C-termini. Thick bars represent the exons, thin bars represent the 5'UTRs (left) and 3'UTRs (right). Lines between the exons represent the introns. *Ma426* denotes the endogenously GFP-tagged *lin-28* allele and *ma398* denotes the endogenously mCherry-tagged *lin-46* allele. The *ma398ma459* allele harbors the "GTAT" deletion in the 5'UTR of *lin-46* that is also tagged with mCherry at the C-terminus. (B) DIC and fluorescent images showing LIN-28 and LIN-46 expression in wildtype L2 (first row) and L4 (last row) stage larvae, and in *lin-28(lf)* and *lin-46(gf/[ΔGTAT])* L2 stage larvae. Fluorescent images of different larvae are taken using the same microscopy setting. All images are then stitched together using the ImageJ software to adjust the brightness and contrast uniformly across the images for enhanced visualization. (C) Schematic representation of the LIN-28 and LIN-46 expression observed during L1-L4 larval stages of wildtype, *lin-28(lf),* and *lin-46(gf/[ΔGTAT])* animals. Note that LIN-46 is precociously expressed at the L1&L2 stages in both *lin-28(lf)* and *lin-46(gf[ΔGTAT])* mutants.

(Pepper *et al.*, 2004): LIN-46::mCherry was detected predominately in hypodermal seam cells and in the ventral hypodermal vulval precursor cells at the L3 and L4 stages (Figure 4.1B&C and Figure 4.3A). Our finding shows that LIN-46::mCherry expression is restricted to the L3 and L4 stages whereas a previous report indicated that *lin-46* transcription occurs at all larval stages (Pepper *et al.*, 2004), which suggests that *lin-46* is post-transcriptional regulated.

The expression pattern of endogenously tagged LIN-46 reveals that LIN-28 and LIN-46 are expressed in a temporally mutually exclusive manner: LIN-28 is expressed early (L1 and L2 stages) and LIN-46 is expressed late (L3 and L4 stages). This mutually exclusive expression pattern suggested that LIN-28 could potentially repress LIN-46 expression during the L1 and L2 larval stages. To test this, we examined the effect of loss of *lin-28* on the expression pattern of LIN-46. We found that LIN-46 is expressed precociously at the L1 and L2 stages in *lin-28(0)* animals (Figures 4.1B and 4.1C), consistent with the conclusion that LIN-28 represses LIN-46 expression at these early larval stages.

Mutations in the *lin-46* 5'UTR result in *lin-28(0)*-like phenotypes and precocious LIN-46 expression

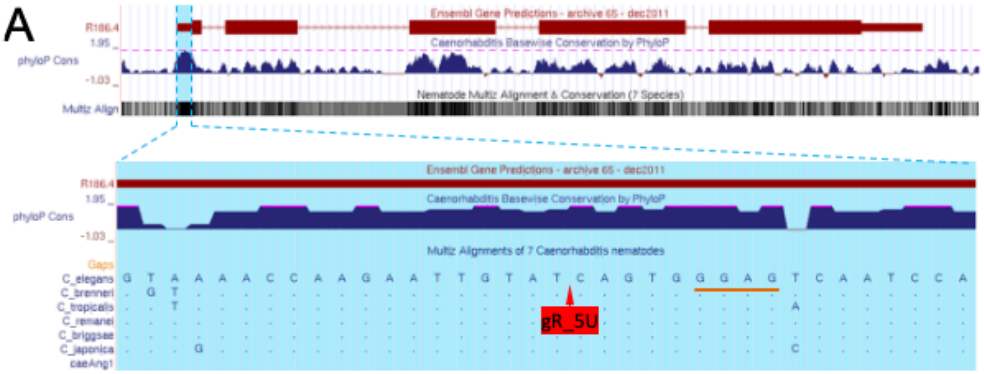
The finding that LIN-46 is expressed precociously at the L1 and L2 stages in *lin-28(0)* animals suggests two non-mutually exclusive hypotheses: 1) that LIN-28 might directly repress LIN-46 expression, and 2) that precocious LIN-46 expression could contribute to the precocious developmental phenotypes of *lin-28(0)*. The latter hypothesis is supported by previous findings that *lin-46(0)*

suppresses the precocious development of *lin-28(0)* (Pepper *et al.*, 2004). However, while *lin-46* activity was shown to be necessary for the precocious phenotypes of *lin-28(0),* it is not known if LIN-46 expression is sufficient for precocious development. The possibility that LIN-28 could directly repressed LIN-46 expression by binding to the *lin-46* mRNA in *C. elegans* is supported a previous study in which LIN-28 crosslinked to *lin-46* mRNA (Stefani *et al.*, 2015).

We sought to test the above hypotheses by mutation of putative LIN-28-interacting sequences in the *lin-46* mRNA sequence and assaying for precocious developmental phenotypes. While the previous study indicated that LIN-28 binds to both the 5' and 3' UTRs as well as all five exons of the *lin-46* mRNA (Stefani *et al.*, 2015), we noticed that the *lin-46* 5'UTR exhibits unusually high sequence conservation among nematodes (Figure 4.2A) and contains a GGAG motif that is often associated with LIN-28 binding (Figures 4.2A and 4.2B). Therefore, we targeted the *lin-46* 5'UTR using a CRISPR guide (Figures 4.2A and 4.2B) and observed frequent *lin-28(0)*-like phenotypes in the F1/F2 progeny of the injected P0 animals (Figure 4.2C). We genotyped several of these F1/F2 progeny, and we found a range of *lin-46* 5'UTR deletions varying in size (2-19 bp) in animals expressing *lin-28(0)*-like phenotypes (Figure 4.2D).

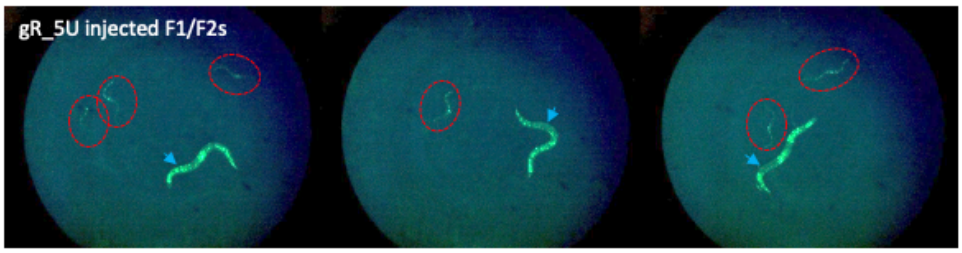
To determine the effects of the *lin-46* 5'UTR mutations on the expression of LIN-46 we injected the CRISPR mix containing a guide targeting the *lin-46* 5'UTR into animals carrying the *lin-28::gfp [lin-28(ma426)]* and *lin-46::mCherry [lin-46(ma398)]* alleles (Figure 4.2A), and generated *lin-46* 5'UTR deletion mutations,

A



B

C



D

	Allele Name	P0 Strain Injected - Strain name and genotype
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma457*	VT3737 = *lin-28(ma426) II; lin-46(ma398) V*
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma458*	VT3737 = *lin-28(ma426) II; lin-46(ma398) V*
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma459*	VT3737 = *lin-28(ma426) II; lin-46(ma398) V*
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma460*	N2 = wildtype
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma461*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma462*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma463*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma464*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma465*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma466*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma467*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma468*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma469*	VT3741 = mals105 (Pcol19::gfp) V
GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA	*ma472*	VT3741 = mals105 (Pcol19::gfp) V

Figure 4.2. CRISPR/Cas9 mutagenesis of the conserved 5'UTR of *lin-46*.

*(*A) Genome browser view of the *C. elegans lin-46* gene (top) and the magnified 5'UTR sequence (bottom). Genome browser tracks: Ensemble Gene Predictions (top), PhyloP conservation (middle), Nematode Multiz Alignment (bottom). Note that the phyloP and Multiz tracks show the high conservation in the 5'UTR among the seven nematode species listed in the figure. The gR_5U guide cut-site and the GGAG in the 5'UTR of *lin-46* are marked. (B) *lin-46* 5'UTR flanked by the TTTCAG splice acceptor and the *lin-46* ORF as well as the gR_5U and the GGAG are shown. (C) Examples of F1/F2 animals among the progeny of gR_5U injected P0s that express precocious *Pcol-19::gfp* are marked with red circles. F1/F2 progeny displaying wildtype *Pcol-19::gfp* pattern are marked with blue arrowheads. (D) Various mutations in the 5'UTR of *lin-46* that are detected in the F1/F2 progeny of the gR_5U injected animals displaying precocious *Pcol-19::gfp* or protruding vulva phenotypes are listed in the left column. Red fonts indicate the deleted nucleotides in each allele. Allele names given to these mutations and the P0s strains injected are given in the middle and right columns, respectively.

which resulted in precocious LIN-46::mCherry expression at the L1 and L2 stages (Figures 4.1A-4.1C). This result shows that an intact *lin-46* 5'UTR is required for proper LIN-46 expression. Importantly, our CRISPR mutagenesis of the *lin-46* 5' UTR did not affect the expression of LIN-28 (Figures 4.1B and 4.1C). Thus LIN-46 is expressed precociously in *lin-46* 5'UTR mutants despite the presence of LIN-28, indicating that the *lin-46* 5'UTR likely mediates LIN-28 binding to, and hence the repression of, the *lin-46* mRNA.

lin-46 mRNA is expressed at the L1 and L2 stages, fluctuating within a range of approximately two-fold in wild-type animals (Figure 4.3, black line). In *lin-28(0)* and *lin-46* 5'UTR mutants, the *lin-46* mRNA is also expressed at the L1 and L2 stages at similar levels to wild-type; nonetheless, at certain time points, *lin-46* mRNA level is slightly but statistically significantly higher or lower in the mutants (Figure 4.3, red and blue lines). Importantly, these fluctuations in the mRNA levels in wild-type or mutant animals do not correlate with the LIN-46 protein accumulation pattern (Figures 4.1B and 4.1C). Namely, LIN-46 is never detected at the L1 and L2 stages in wild-type animals, whereas LIN-46 is always present throughout the late L1 and L2 stages in *lin-28(0)* and *lin-46* 5'UTR mutants. Therefore, the precocious LIN-46 expression in *lin-28(0)* and *lin-46* 5'UTR mutants are likely due to an increase in the translatability of the *lin-46* mRNA in these mutants rather than an increase in the *lin-46* mRNA level.

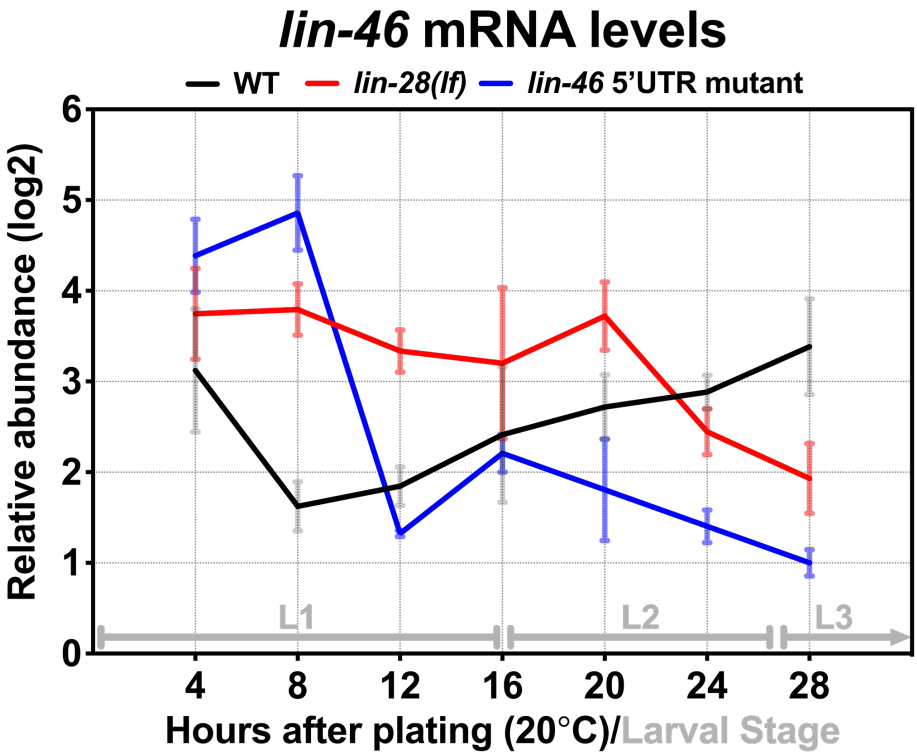

lin-46 mRNA levels
WT
lin-28(lf)
lin-46 5'UTR mutant
Relative abundance (log2)
0
1
2
3
4
5
6
L1
L2
L3
4
8
12
16
20
24
28
Hours after plating (20°C)/Larval Stage

Figure 4.3. Temporal dynamics of *lin-46* mRNA expression at the L1 and L2 stages. qRT-PCR analysis of *lin-46* mRNA levels in samples of total RNA from staged populations of synchronously developing WT, *lin-28(lf)*, and *lin-46(5'UTRΔGTAT)* animals at 20^{O}C. n = three biological replicates. Data are mean ± s.d.

Precocious LIN-46 expression causes precocious cell-fate transitions in hypodermal seam and vulval cell lineages

To compare in detail the heterochronic phenotypes of three different *lin-46* 5'UTR mutants, *ma461, ma467,* and *ma472*, that are deleted for six, 12, and 19 nucleotides, respectively to those of *lin-28(0)* mutants, we assessed the number of seam cells, the timing of *Pcol-19*::*gfp* expression–a reporter for terminal differentiation of hypodermal cells, the timing of the formation of an adult-specific cuticle structure called alae, and the penetrance of the protruding vulva (Pvl) phenotype (Figure 4.4).

First, all three *lin-46* 5'UTR mutants had fewer seam cells than wildtype (Figure 4.4A), which indicates that *lin-46* 5'UTR mutations, presumably as a consequence of consequent precocious LIN-46 expression, skip the L2-stage symmetric seam cell divisions. The severity of this seam cell phenotype varies for each mutant and is weaker than *lin-28(0)* (Figure 4.4A). The variability in the number of seam cells is due to variation in cell-fate decisions across the seam cells of each larva; namely, in *lin-28(0)* animals almost all seam cells skip L2 cell-fates whereas in the 5'UTR mutants of *lin-46* not all but only some (and a varying number of) seam cells skip L2 stage cell-fates. It also worth noting that while the majority of the *ma472* animals had fewer than sixteen seam cells, some *ma472* animals had more than sixteen seam cells (Figure 4.4A) indicating that at least certain

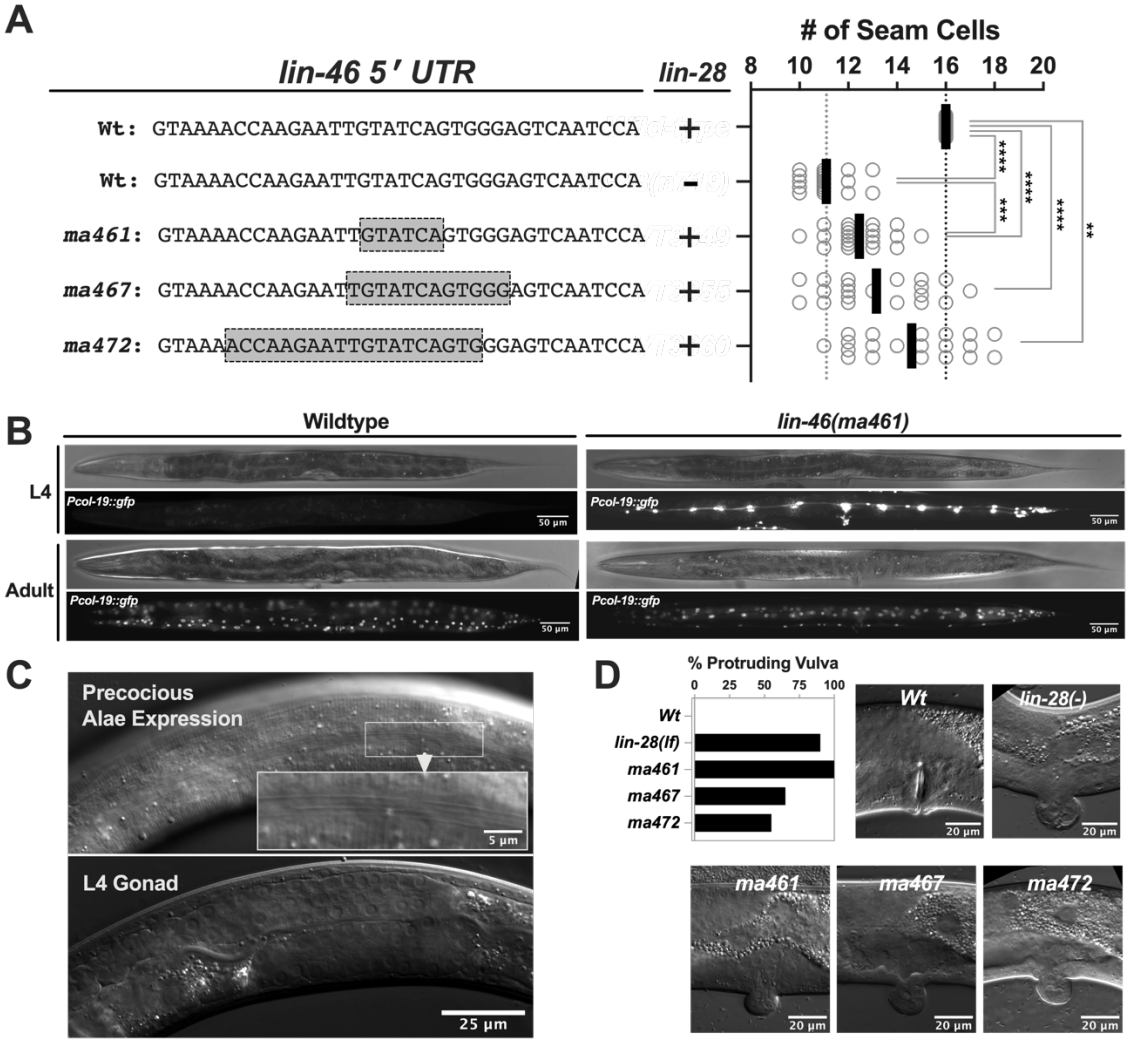

A
lin-46 5′ UTR
lin-28
Wt: GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA +
Wt: GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA -
ma461: GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA +
ma467: GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA +
ma472: GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCA +
# of Seam Cells
8 10 12 14 16 18 20
****
***
****
****
**
B
Wildtype
lin-46(ma461)
L4
Adult
Pcol-19::gfp
50 µm
C
Precocious Alae Expression
5 µm
L4 Gonad
25 µm
D
% Protruding Vulva
0 25 50 75 100
Wt
lin-28(lf)
ma461
ma467
ma472
Wt
lin-28(-)
ma461
ma467
ma472
20 µm

Figure 4.4. Precocious LIN-46 expression causes precocious cell-fate transitions in hypodermal seam and vulval cell lineages.

(A) Number of seam cells observed in young adults of wildtype, *lin-28(lf),* and three *lin-*46 5'UTR mutants (*ma461, ma467, and ma472*). Each dot in the plot on the right represents the number of seam cells observed on one lateral side of a single worm. Vertical black bars indicate the average number of seam cells observed for each genotype. (B) DIC and fluorescent images of L4 and adult stage wildtype and *lin-46(ma461*) animals. The adult specific *Pcol-19::gfp (maIs105)* is normally expressed in the hypodermal seam and hyp7 cells at the adult stage of both wildtype and *lin-46(ma461)* animals. However, unlike in wildtype larvae, *Pcol-19::gfp* is also precociously expressed in the seam cells of L4 stage *lin-46(ma461)* larvae. (C) DIC images showing the (precocious) adult-specific cuticle structure called alae (upper panel) on the cuticle of a larva at the L4 stage indicted by the developmental stage of the gonad (lower panel). (D) Percent protruding vulva (Pvl) phenotype observed in wildtype, *lin-28(lf),* and three *lin-46* 5'UTR mutants and DIC images showing normal vulva or Pvl morphology observed in each genotype.

seam cells reiterated L2 cell-fates at later stages, which might be due to reduced *lin-46* expression in those seam cells. This variation in the number of seam cells that skip or reiterate L2 fates in *lin-46* 5'UTR mutants could reflect a variability in the timing or level of LIN-46 expression across seam cells due to the mis-regulation of *lin-46* translation.

Second, similar to what is observed in *lin-28(0)* animals, seam cells in *lin-46* 5'UTR mutants precociously express adult fates during larval stages as demonstrated by the precocious expression of *Pcol-19::gfp*, (Figure 4.4B) and by the expression of an adult alae in L4 stage larvae (Figure 4.4C).

Lastly, we quantified the percent animals that display protruding vulva (Pvl) phenotypes in young adults (a characteristic of precocious vulval development in *lin-28(0)* animals): all three *lin-46* 5'UTR mutants displayed Pvl phenotypes similar to *lin-28(0)* animals (Figure 4.4D).

LIN-46 is expressed in the vulval precursor cells (VPCs) and precocious LIN-46 expression leads to precocious onset of vulva development

During *C. elegans* larval development, stem cells of the ventral hypodermal lineages P3-P8 divide during the L1 stage and give rise to the six P3.p-P8.p VPCs (Sulston and Horvitz, 1977). After their birth in the L1 stage, the VPCs temporarily arrest in the G1 phase of the cell cycle until the L3 stage when they undergo a single round of cell division (Figure 4.5A) (Susan Euling and Ambros, 1996). Concomitant with this cell division, three of the six VPCs (P5.p, P6.p, and P7.p)

undergo additional rounds of cell divisions, giving rise to 22 cells that progressively differentiate and form the adult vulva (Figure 4.5A). The timing of the first VPC divisions in the mid-L3 stage is controlled by genes in the heterochronic pathway, including *lin-28* (Ambros and Horvitz, 1984; Susan Euling and Ambros, 1996). In *lin-28(0)* mutants, the first VPC divisions precociously take place in the L2 stage; the VPC progeny subsequently continue to precociously divide and differentiate, resulting in precocious vulva development, evidenced by an abnormally formed, protruding vulva. Loss of *lin-46* suppresses the Pvl phenotype caused by *lin-28(0).* However, because LIN-46 expression in the VPCs had not been previously detected using transgenic reporters (Pepper *et al.*, 2004), it was not clear how loss of *lin-46* could affect the Pvl phenotype of *lin-28(0)* animals.

We found that endogenously tagged LIN-46 is expressed in the VPCs at the L3 and L4 stages (Figure 4.5A), which coincides with the period when the VPCs develop into adult vulva. In *lin-28(0)* mutants, LIN-46 is precociously expressed in the L2-stage VPCs, which coincides with its precocious VPC development (Figure 4.5B, left). Similarily, in *lin-46* 5'UTR mutants, LIN-46 is precociously expressed in the VPCs at the L2 stage coinciding with their VPCs precocious development in these mutants (Figure 4.3B, right). This indicates that precocious LIN-46 expression is sufficient to alter the timing of vulva development inferring that precocious LIN-46 expression is likely responsible for precocious vulva development

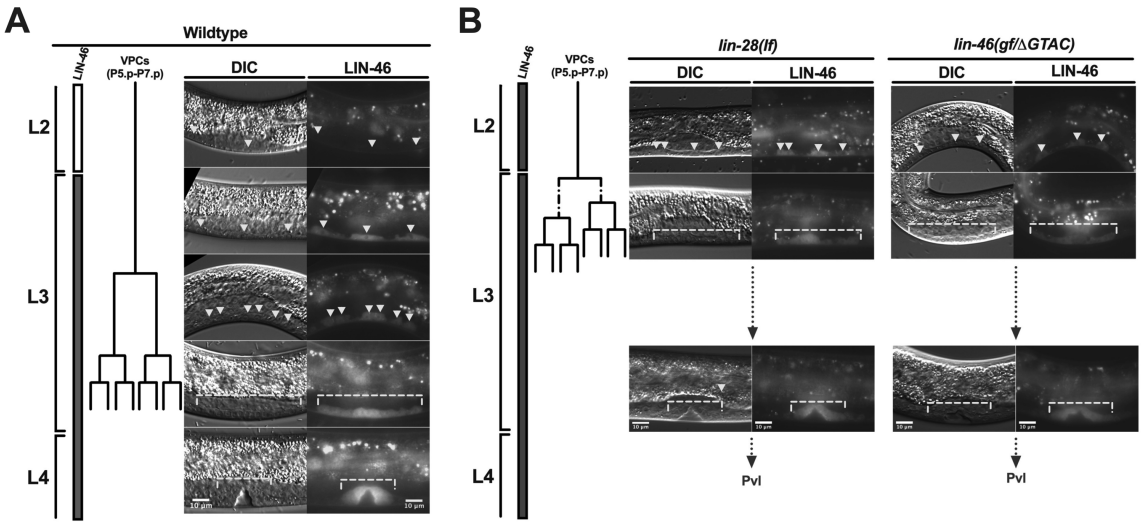

A
Wildtype
LIN-46
VPCs
(P5.p-P7.p)
DIC
LIN-46
L2
L3
L4
10 µm
10 µm
B
LIN-46
VPCs
(P5.p-P7.p)
lin-28(lf)
DIC
LIN-46
lin-46(gf/ΔGTAC)
DIC
LIN-46
L2
L3
L4
Pvl
Pvl

Figure 4.5. LIN-46 is expressed in the vulval precursor cells (VPCs) and precocious LIN-46 expression leads to precocious onset of vulva development. (A) Wildtype larval stages and the cell lineage diagram of the vulval precursor cells (VPC), and DIC and fluorescent showing LIN-46 expression in the VCPs (indicated by arrowheads or brackets). (B) Larval stages (according to gonad morphology), precocious LIN-46 expression and VPC development in *lin-28(0)* and *lin-46(ΔGTAT)* animals are shown. Importantly, the synchrony among P5.p, P6.p and P7.p and among their progeny are lost in *lin-28(0)* and *lin-46(ΔGTAT).* Accordingly, due to the variability (indicated by dotted lines in the lineage) in the timing of the VPC divisions, the distinct temporal patterns of vulva development observed in the wildtype animals from early to late L3 stage (as in Panel A) is also lost.

in *lin-28(0)* animals. Altogether, our results show that the LIN-28 target LIN-46 suppresses L2 cell-fates to promote the transition to L3 cell-fates in both seam cells and vulval precursor cells, and that precocious LIN-46 expression causes precocious cell-fate transitions, which is likely responsible for the majority of the phenotypes observed in *lin-28(0)* animals.

The architecture and conservation of the *lin-46* 5'UTR

Most *C. elegans* transcripts are trans-spliced resulting in the fusion of a 22-nt splice-leader (SL) RNA to the 5' end of the transcripts (Blumenthal, 2012). The presence of an upstream splice acceptor "TTTCAG" (Figures 4.6 and 4.2B) and expressed sequence tag (EST) clones that contain the *SL1-lin-46*-5'UTR fusion sequence (e. g. GenBank: FN875625.1) indicate that *lin-46* mRNA is trans-spliced. We used the RNAfold Webserver (Gruber *et al.*, 2008) to predict the structure of the *SL1-lin-46*-5'UTR fusion sequence (Figure 4.6A and 4.7A). The predicted structure of the *SL1-lin-46*-5'UTR chimeric RNA shows base-pairing between *SL1* and the first eight nucleotides at the 5' end of the *lin-46* 5'UTR (Figure 4.6A, "*SL1*-complementary"). These eight nucleotides are highly conserved across *Caenorhabditis* species (Figure 4.6B); and a nucleotide variation at position four that is found in five different species preserve the predicted base-pairing (A-U to G-U) with the *SL1* sequence, which supports the biological relevance of the predicted structure (Figure 4.6). Perturbing this *SL1*-complementary region in

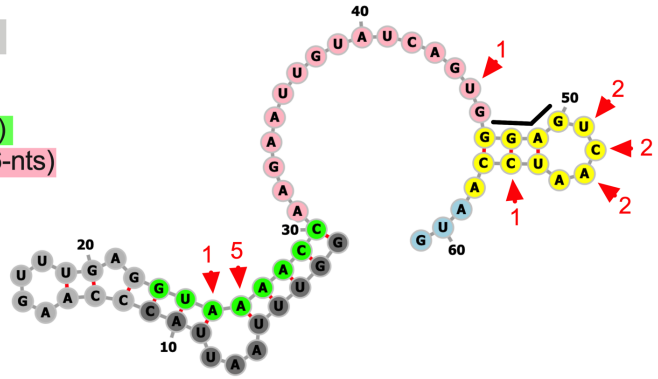
A

Splice leader (SL1, 22-nts)

***lin-46* 5'UTR (36-nts):**
SL1-complementary (8-nts)
Single-stranded stretch (16-nts)
Stem-loop (12-nts)

AUG, the 1st Codon

Trans-spliced 5'UTR of *lin-46* + *first codon (AUG)*



B

***Caenorhabditis* Species**	Splice acceptor	SL1-complementary	Single-stranded Stretch	Stem-loop
C. elegans:	TTTCAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. remanei:	TTTCAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. latens:	TTTCAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. briggsae:	TT**A**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. doughertyi:	TT**A**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. brenneri:	TT**C**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. nouraguensis:	TT**C**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. sp29:	TT**C**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. sp26:	TTTCAG	GTA**G**AACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. sp40:	TT**G**CAG	GTA**G**AACC	AAGAATTGTATCAGTG	GGAGTCAATCCAATG
C. sp34:	TTTCAG	GTA**G**AACC	AAGAATTGTATCAG**A**G	GGAGTCAATCCAATG
C. japonica:	TTTCAG	GTA**G**AACC	AAGAATTGTATCAGTG	GGAG**C**CAATCCAATG
C. sinica:	TT**A**CAG	GTA**G**AACC	AAGAATTGTATCAGTG	GGAGT**AC**ATCCAATG
C. afra:	TT**G**CAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGTC**G**ATCCAATG
C. kamaaina:	TTTCAG	GTAAAACC	AAGAATTGTATCAGTG	GGAGT**T**AAT**T**CAATG
C. tropicalis:	TT**C**CAG	GT**T**AAACC	AAGAATTGTATCAGTG	GGAG**A**CAATCCAATG

Figure 4.6. The architecture and conservation of the *lin-46* 5'UTR.
(A) The predicted structure of the trans-spliced *lin-46* 5'UTR and the annotation of distinct structural regions. Red arrowheads indicate nucleotides that are mutated in nematode species (as listed in panel B); numbers denote the number of times a mutation is found at the position indicated. (B) An alignment of the genomic sequences encoding the *lin-46* 5'UTR in various *Caenorhabditis* species. The *lin-46* 5'UTR is highly conserved and the conservation pattern is consistent with the conservation of the base-pairing interactions in the predicted structure: base-pairing nucleotides are more conserved or nucleotide changes preserve base-pairing (e.g. A:U to G:U).

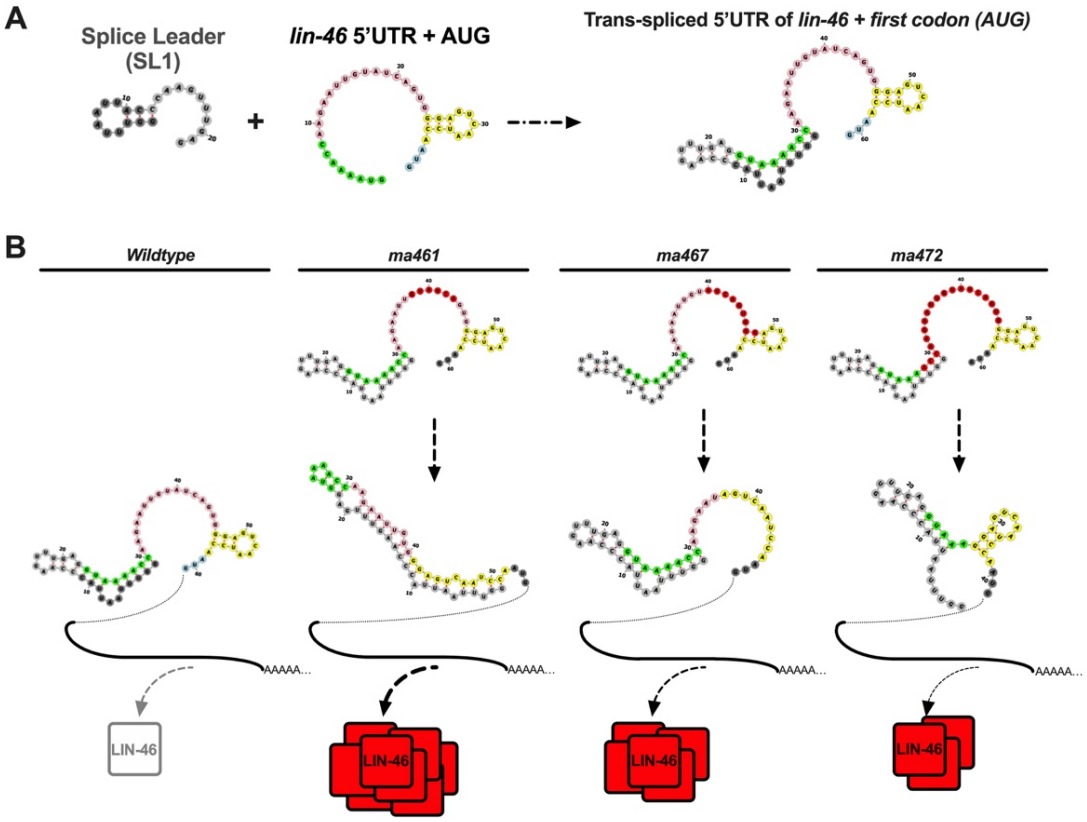

A
Splice Leader (SL1)
+
lin-46 5'UTR + AUG
Trans-spliced 5'UTR of lin-46 + first codon (AUG)
B
Wildtype
ma461
ma467
ma472
AAAAA...
LIN-46

Figure 4.7. The effects of 5'UTR mutations on the folding of the trans-spliced 5'UTR of *lin-46* and corresponding LIN-46 expression levels inferred from the strength of the gain-of-function phenotypes. (A) Predicted folding of the splice leader (SL1) and *lin-46* 5'UTR before and after trans-splicing. (B) Predicted changes in the folding of the trans-spliced *lin-46* 5'UTR in three different mutants and schematic representation of LIN-46 expression inferred from the phenotypes (e.g. Figure 4.4A) observed in animals carrying each mutation.

addition to mutating more downstream sequences in the 5'UTR resulted in a weaker phenotype (*ma461* versus *ma472* in Figure 4.4A, 4.4D, 4.7B), which suggests that these first eight nucleotides or their base-pairing with the *SL1* sequences has a positive impact on LIN-46 expression.

Sixteen nucleotides that follow the *SL1*-complementary region are conserved among all *Caenorhabditis* species analyzed here and constitute a "single-stranded stretch" region (Figure 4.6A) with the exception of *C. sp34* that has a single nucleotide change in this region (Figure 4.6B). This single-stranded stretch was the region primarily targeted by our CRISPR guide used to generate the 5'UTR mutants (Figure 4.2B). Mutations of various sizes (Figure 4.2D) in this region alone displayed precocious LIN-46 expression (Figure 4.1, *ma459*) and strong *lin-28(0)*-like phenotypes (Figure 4.4A, *ma461*). Interestingly, in certain *lin-46* 5'UTR mutants, such as the *ma459,* that result in strong precocious phenotypes, the predicted RNA structure is entirely altered (Figure 4.7B), which may indicate a causative relationship between loss of all structural elements in the *lin-46* 5'UTR and a strong loss of LIN-46 repression.

The last 12 nucleotides in the *lin-46* 5'UTR contains a GGAG sequence that is located in the stem of a predicted stem-loop structure (Figure 4.6A, stem-loop). The sequence conservation pattern in this region supports the biological relevance and significance of the predicted structure: 1) a C to T nucleotide change in the stem preserves base-pairing (G-C to G-U), and 2) mutations in the nucleotides in

the loop region, which are not contributing to the hairpin stability, appear to be more tolerated (Figure 4.6). The GGAG motif is found to be enriched in LIN-28 bound RNA regions (Stefani *et al.*, 2015), however, here the GGAG sequence or the stem-loop in the *lin-46* 5'UTR alone is not sufficient to confer repression of LIN-46 expression (see *ma472* in Figure 4.42A and Figure 4.7). Moreover, perturbing the GGAG sequence in addition to the single-stranded stretch sequences did not enhance but rather weakened the precocious phenotypes (*ma467* vs *ma461* in Figures 4.4A, 4.4D and 4.7B), which suggests that this GGAG-containing loop, rather than an having an inhibitory role, can positively affect LIN-46 expression.

Discussion

Our results provide insights into how the conserved RNA-binding protein LIN-28 regulates its critical mRNA target, *lin-46*, in *C. elegans,* and demonstrate that *lin-46* mis-regulation is likely responsible for the phenotypes observed in *lin-28(0)* animals. Our results suggest that LIN-28 controls temporal cell-fate progression by regulating LIN-46 expression via the 5'UTR of *lin-46* mRNA.

The temporally mutually exclusive expression pattern between LIN-28 and LIN-46 (revealed by the endogenously tagged alleles of *lin-28* and *lin-46*) and the effect of loss-of-function of *lin-28* on the LIN-46 expression led us to conclude that *lin-28* represses LIN-46 expression at early stages (Figure 4.8). Our results also suggest that the 5'UTR of *lin-46* prevents LIN-46 expression, which is likely via mediating LIN-28 binding to and repression of the *lin-46* mRNA. We showed that LIN-46 is precociously expressed in *lin-28(0)* animals; with the help of the *lin-46*

5'UTR mutants, which uncouple precocious LIN-46 expression from the loss-of-function of *lin-28,* we showed that precocious LIN-46 expression alone is sufficient to suppress L2 cell-fates and to promote precocious transitions to L3 cell-fates (Figure 4.4). Lastly, endogenously tagged LIN-46 is expressed in the VPCs, which had not been reported before, and *lin-28* and portions of the *lin-46* 5'UTR are required to repress LIN-46 expression in the VPCs. Precocious onset of LIN-46 expression in the VPCs is sufficient to stimulate the precocious onset of vulva development (Figure 4.5). These results demonstrate that precocious LIN-46 expression in seam cells and VPCs in *lin-28(0)* mutants is responsible for the two major heterochronic phenotypes observed in the *lin-28(0)* animals; skipping of L2 stage seam cell proliferation and precocious onset of vulva development.

We hypothesize that the *lin-46* 5'UTR contains a LIN-28-binding element that is required for LIN-28-mediated repression of LIN-46 expression from the *lin-46* mRNA. The evidence that supports this hypothesis include: 1) The phenotypic similarities between *lin-28(0)* and the 5'UTR mutants of *lin-46* reported here; 2) LIN-28 binding to the *lin-46* mRNA (including the *lin-46* 5'UTR) reported previously (Stefani *et al.*, 2015), 3) The existence of a putative LIN-28 interacting sequence, the "GGAG", in the 5'UTR of *lin-46* (Figure 4.7). However, because previously LIN-28 was shown to interact with the *lin-46* mRNA at multiple sites across the entire *lin-46* mRNA in addition to the 5'UTR (Stefani *et al.*, 2015), it is surprising that mutations of the *lin-46* 5'UTR are sufficient to cause a phenotype that is consistent with an almost total loss of LIN-28-mediated regulation of the *lin-46* mRNA.

Nonetheless, at least two models could reconcile a potential total loss of LIN-28-mediated repression of LIN-46 expression by mutating the 5'UTR of *lin-46* alone while leaving other LIN-28 binding sites on the *lin-46* mRNA intact. The first model is that the binding of LIN-28 to the *lin-46* 5'UTR would be required for inhibiting LIN-46 expression whereas LIN-28 binding to other sites on the *lin-46* mRNA would not inhibit LIN-46 expression. It is known that translation initiation is highly regulated (Sonenberg and Hinnebusch, 2009); and the 5'UTRs harbor sequence elements, such as upstream open reading frames (uORFs), or structural elements, such as highly structured RNA (including G-quadruplexes and pseudoknots) or specific RNA structures that serve as binding sites for RNA-binding proteins (Hentze *et al.*, 1987), which can interfere with or inhibit the translation initiation (Hinnebusch, Ivanov and Sonenberg, 2016; Leppek, Das and Barna, 2018). In the second model, among all the LIN-28 binding sites on the *lin-46* mRNA, the *lin-46* 5'UTR (and particularly the single-stranded stretch region) might have the highest affinity for LIN-28 and might be required to initiate a sequential binding of multiple LIN-28 proteins to the *lin-46* mRNA, leading to the formation of a repressive LIN-28-*lin-46*-mRNA mRNP (messenger ribonucleoprotein) complex. In support of this model, in *in vitro* assays, LIN28 is shown to preferentially bind to single stranded RNA and more than one LIN28 proteins are shown to bind to long (longer than 30-nts) RNA in a sequential manner after the first LIN28 binds to a predicted single-stranded loop (Hafner *et al.*, 2013).

Precocious cell-fate transition phenotypes observed in *lin-46* 5'UTR mutants are not as strong as the phenotypes observed in *lin-28(0)* mutants. Moreover, the severity of the *lin-28(0)*-like phenotypes vary among different *lin-46* 5 'UTR alleles. The severity of these phenotypes does not correlate with the size of the *lin-46* 5'UTR deletions; and in some cases, larger deletions result in not stronger but more moderate phenotypes (Figure 4.4). These findings are consistent with a model where the *lin-46* 5'UTR harbors multiple cis-regulatory elements that can either positively or negatively affect LIN-46 expression. Accordingly, mutants that inactivate a negative regulatory element (the presumed LIN-28 binding site) without disturbing a positive regulatory element result in higher LIN-46 expression and hence stronger precocious phenotypes.

A putative positive regulatory element in *lin-46* 5'UTR could be the first eight nucleotides of the 5'UTR that are predicted to base-pair with the *SL1* sequence (*SL1*-complementary, Figure 4.6). The *lin-46* mRNA is trans-spliced, which results in the fusion of the *SL1* RNA to the 5' end of the transcript (Figures 4.6 and 4.7). A small stem-loop structure in the *SL1* sequence (Figure 4.7A) has been shown to enhance translation in nematodes (Wallace *et al.*, 2010). In the predicted folding of the *SL1-lin-46*-5'UTR chimeric RNA (Figure 4.7A), the nucleotides that form the stem-loop in the *SL1* alone base-pair with the first eight nucleotides of the *lin-46* 5'UTR (Figure 4.7). This *SL1*-5'UTR base-pairing is lost in the *lin-46(ma472)* (Figure 4.7) and the phenotype of this larger deletion is weaker than the two other, smaller deletions (Figure 4.4A and 4.7), which is consistent with the idea that base-

pairing of *SL1* to the *lin-46* 5'UTR has a positive impact on the translatability of the *lin-46* mRNA.

The *C. elegans lin-28-lin-46* pathway acts in parallel to *let-7*-family microRNAs (Abbott *et al.*, 2005; Vadla *et al.*, 2012) and regulate the nuclear localization, hence the activity, of a critical *let-7*-family target and a transcription factor, HBL-1 (Ilbay and Ambros, 2019b). *lin-46* activity becomes more important in preventing L3/L4-stage HBL-1 activity at low temperatures (Pepper *et al.*, 2004), or when animals develop through a temporary diapause (Karp and Ambros, 2012), or merely when animals experience an extended L2 (called L2d) in the presence of diapause-inducing pheromones or starvation stress (Ilbay and Ambros, 2019a). L2d/Dauer-inducing conditions also result in the repression of *let-7*-family microRNAs (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009). Therefore, LIN-28-mediated regulation of *lin-46* mRNA translation might play a role in controlling a compensation mechanism against environmentally-induced reduction in *let-7*-family levels, perhaps by regulating the level of LIN-46 accumulation at the early L3 stage.

Remarkably, precocious LIN-46 expression conferred by the *lin-46* 5'UTR mutants can fully compensate for the loss of all ten *let-7*-complementary sites in the *hbl-1* 3'UTR (Ilbay and Ambros, 2019b), which otherwise causes a severe extra seam cell phenotype due to ectopic HBL-1 activity at L3/L4 stages (Ilbay and Ambros, 2019b). Therefore, one way to compensate for reduced *let-7*-family levels by LIN-46 activity would simply be the induction of LIN-46 expression in response

to environmental conditions that repress *let-7*-family microRNAs. Such an induction mechanism that regulates LIN-46 levels at the L3 stage to match the level of *let-7*-family repression at the L2 stage may utilize LIN-28/5'UTR-mediated regulation of LIN-46 expression as a gate to uncouple *lin-46* mRNA accumulation from LIN-46 protein accumulation, which can provide a control over the rate of LIN-46 accumulation at the early L3 stage. In this hypothetical model, during a lengthened L2 (L2d), *lin-46* mRNA may accumulate in proportion to the length of the L2d stage (which is thought to correlate with the severity of the environmental conditions as well as the degree of *let-7*-family repression). At the L2/L2d stage *lin-46* mRNA cannot be translated due to LIN-28-mediated inhibition; however, at the L3 stage, when LIN-28 expression is diminished, the L2/L2d-accumulated pool of *lin-46* mRNA would be translated. Thus, LIN-46, expressed from a *lin-46* mRNA pool whose size negatively correlates with the reduction in *let-7*-family levels, can accumulate fast enough to sufficiently inhibit the residual or ectopic HBL-1 at the post-L2d L3 stage, compensating for the reduced *let-7*-family microRNA levels by an appropriately matching level of LIN-46.

Lastly, 3'UTR- and microRNA-mediated mechanisms and their roles in controlling temporal dynamics of gene expression have extensively been studied in the context of the *C. elegans* heterochronic pathway. However, the involvement of 5'UTRs in the heterochronic pathway was not known and the identities and roles of cis-regulatory elements in the *C. elegans* 5'UTRs are largely unknown. Conservation in the 5'UTRs is not widespread, but interestingly, the 5'UTRs of

many heterochronic and developmental genes in *C. elegans* appear to be evolutionary conserved, which may provide a platform to further explore the functions of mRNA cis-regulatory elements and the roles of trans-acting RNA binding proteins in regulating stage specific gene expression, and developmental progression as well as other important developmental processes.

In summary, we provide evidence indicating that LIN-28 represses the expression of its critical mRNA target in *C. elegans*, an intact *lin-46* 5'UTR is required LIN-28-mediated repression of *lin-46* expression, and precocious LIN-46 expression alone is likely responsible for the majority of *lin-28(lf)* phenotypes. Our findings highlight the biological importance of the mRNA targets of LIN-28 (*C. elegans* LIN-28 and its orthologs), which may have important functions in regulating pluripotency, reprogramming, or oncogenesis in humans and various other organisms.

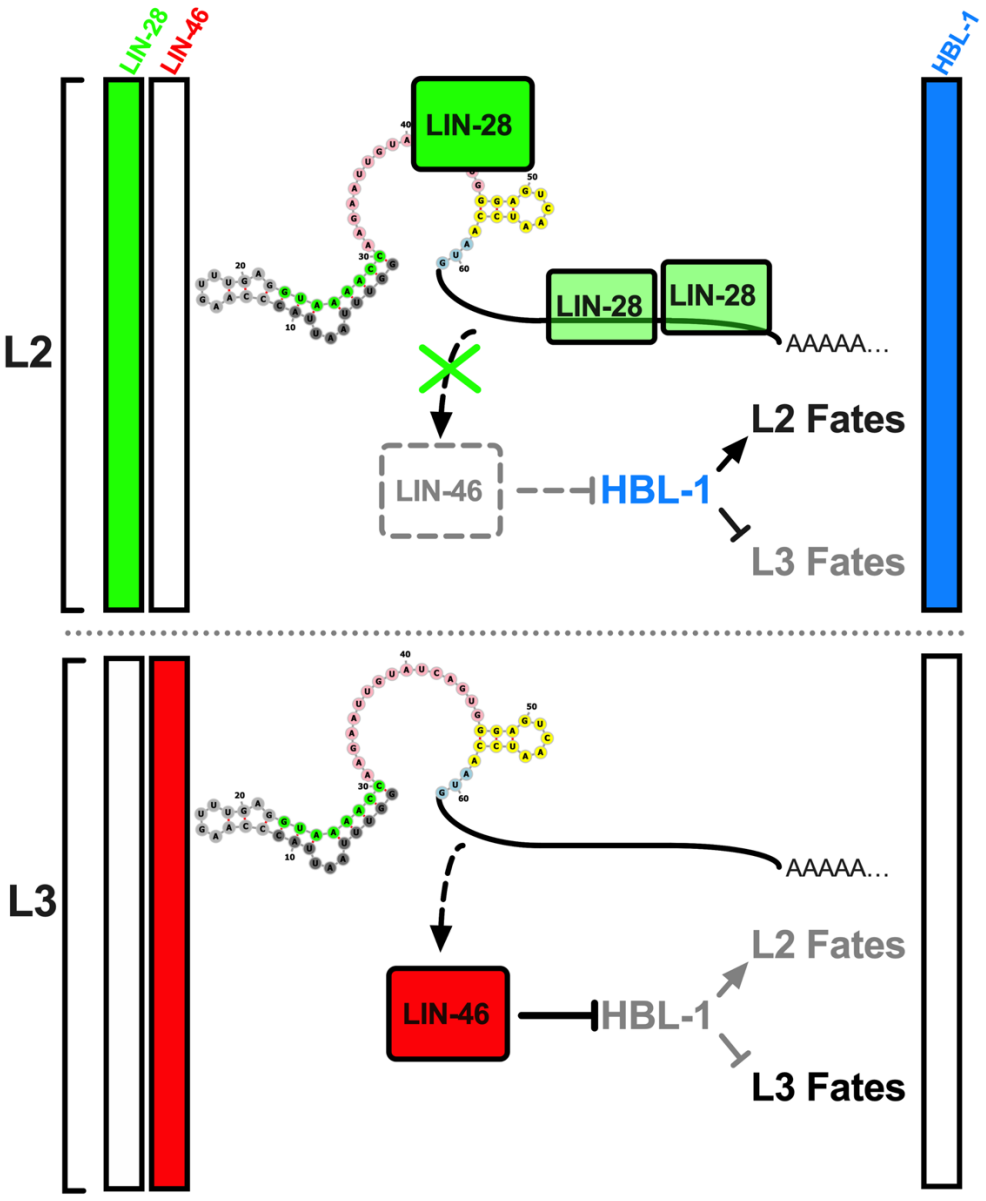

LIN-28
LIN-46
HBL-1
L2
LIN-28
LIN-28
LIN-28
AAAAA...
LIN-46
HBL-1
L2 Fates
L3 Fates
L3
AAAAA...
LIN-46
HBL-1
L2 Fates
L3 Fates

Figure 4.8. Model: LIN-28 controls temporal cell-fate progression by regulating LIN-46 expression via the 5'UTR of *lin-46* mRNA.

At the L2 and L3 stages LIN-28 is highly expressed and although the *lin-46* mRNA is transcribed it cannot be translated due to LIN-28-mediated inhibition of its translation. Inhibition of LIN-46 expression at the L2 stage permits HBL-1 to function: HBL-1 promotes L2 cell-fates and prevents L3 cell-fates. At the L2 to L3 molt, LIN-28 expression is diminished, and this allows LIN-46 expression. LIN-46 opposes HBL-1 activity, thereby preventing expression of L2 cell-fates at the L3 stage.

Materials and Methods

C. elegans culture conditions

C. elegans strains used in this study and corresponding figures in the paper are listed in Table 4.1. *C. elegans* strains were maintained at 20°C on nematode growth media (NGM) and fed with the *E. coli* HB101 strain.

Assaying extra seam cell and Pvl phenotypes

The worms were scored at the young adult stage (determined by the gonad development) for the number of seam cells using fluorescence microscopy with the help of the *maIs105* [*pCol-19::gfp*] transgene that marks the lateral hypodermal cell nuclei and/or for protruding vulva phenotype (Pvl) by examining the vulva morphology (as given in Figure 4.4D).

Each circle on the genotype versus number of seam cells plots shows the observed number of seam cells on one side of a single young adult worm. A minimum of 20 worms for each genotype are analyzed and the average number of seam cells (denoted by lateral bars in the genotype versus number of seam cell plots); percent Pvl values are calculated and represented using a bar graph. The Student's t test is used to calculate statistical significance when comparing different genotypes. The GraphPad Prism 8 software is used to plot the graphs and for statistical analysis.

Microscopy

All DIC and fluorescent images are obtained using a ZEISS Imager Z1 equipped with ZEISS Axiocam 503 mono camera, and the ZEN Blue software. Prior to

Strain name	Genotype	Related figures
VT3737	*lin-28(ma426[lin-28::gfp] I; lin-46(ma398[lin-46::mCherry] V*	Figure 1A-C, 3A
VT3652	*lin-28(n719) I; lin-46(ma398[lin-46::mCherry] V*	Figure 1A-C, 3B
VT3847	*lin-28(ma426[lin-28::gfp] I; lin-46(ma398ma459[ΔGTAT::lin-46::mCherry] V*	Figure 1A-C, 3B
VT1367	*maIs105 (Pcol-19::gfp) V*	Figure 2A, B, D, S1C
VT790	*lin-28(n719) I; maIs105 V*	Figure 2A, D
VT3849	*lin-46(ma461) maIs105 V*	Figure 2A-D
VT3855	*lin-46(ma467) maIs105 V*	Figure 2A, D
VT3860	*lin-46(ma472) maIs105 V*	Figure 2A, D

Table 4.1. *C. elegans* strains used in Chapter IV.

imaging, worms were anesthetized with 0.2 mM levamisole in M9 buffer and mounted on 2% agarose pads. The ImageJ Fiji software is used to adjust the brightness and contrast of the images to enhance the visualization of the fluorescent signal. All images are taken using the same microscopy settings and a standard exposure time for all larval stages and genetic background for each reporter (*lin-28::gfp* and *lin-46::mCherry*). To enhance the visualization of the fluorescent signals in the figures and to allow comparison of signal intensities in larvae of different genetic backgrounds, fluorescent images of larvae from different backgrounds are stitched together using the ImageJ software and the brightness and contrast of these montaged images were adjusted (in Figure 4.1B and 4.5).

Tagging of lin-28 and *lin-46* using CRISPR/Cas9

A mixture of plasmids encoding SpCas9 (pOI90, 70 ng/µL), and single guide RNAs (sgRNAs) targeting the site of interest (60 ng/µL of pSW65 for *lin-28* or pOI113 for *lin-46*) and the *unc-22* gene (pOI91, 30 ng/µL) as co-CRISPR marker, a donor plasmid (20 ng/µL of pOI173 for lin-28 or pOI167 for lin-46) containing the gfp or mCherry sequence flanked by gene-specific homology arms, and a *rol-6(su1006)* containing plasmid (pOI124, 30 ng/µL) as co-injection marker was injected into the germlines of ten young adult worms. F1 roller and/or twitcher animals (100-200 worms) were cloned and screened by PCR amplification (Table 4.2) for the presence of the expected homologous recombination (HR) product. F2 progeny of F1 clones positive for the HR-specific PCR amplification product were screened for homozygous HR edits by PCR amplification of the locus using

primers that flanked the HR arms used in the donor plasmid (Table 4.2). Finally, the genomic locus spanning the HR arms and *gfp* or *mCherry* DNA was sequenced using Sanger sequencing. A single worm with a precise HR edited *lin-28* or *lin-46* locus was cloned and backcrossed twice before used in the experiments.

CRISPR/Cas9-mutagenesis of the *lin-46* 5'UTR

A mixture of plasmids encoding SpCas9 (pOI90, 70 ng/µL), and gR_5U single guide RNA (sgRNAs) targeting the *lin-46* 5'UTR (Figure S1; pOI193 60 ng/µL) was injected into the germlines of young adult worms expressing the adult onset *gfp* transgene (Table 4.1; VT1357). F1 or F2 animals displaying precocious cell-fate phenotypes, which were consisted of precocious *Pcol-19::gfp* expression in the seam cells (Figure 4.2) and protruding vulva morphology (Figure 4.4D), were cloned and genotyped for in-del events at the gR_5U targeting site (Figure 4.2D).

Quantitative PCR

Samples of total RNA were pre-treated with turbo DNase (Invitrogen). cDNA was synthesized using SuperScript IV (Invitrogen) following the manufacturer's instructions, using the RT oligonucleotide "oligo (dT)". qPCR reactions were performed using CoWin Biosciences FastSYBR Mixture (Low Rox) following the manufacturer's instructions, using a Viia 7 Real Time PCR System (Applied Biosystems). ΔCTs were calculated by normalizing samples to *gpd-1* (*GAPDH*). ΔCTs were then inverted so that greater values reflect greater RNA levels, and were normalized to set the value of the least abundant sample to one. For each biological replicate, the average of three technical replicates was used.

Cloning/PCR primers	Primer name	Primer Sequence	Plasmid name and/or purpose
Cloning: Annealed primer-pairs that are cloned into pOI83 to express sgRNAs	priSW224	tcttgtagatgattctattcatcag	pSW65, sgRNA expressing plasmid is used to tag *lin-28* with gfp
	priSW225	aaacctgatgaatagaatcatctac	
	priOI362	tcttgcgtagatcaaccacgtctc	pOI113, sgRNA expressing plasmid is used to tag *lin-46* with mCherry
	priOI363	aaacgagacgtggttgatctacgc	
	priOI724	tcttgaaaccaagaattgtatcag	pOI193, sgRNA gR_5U expressing plasmid is used to mutagenize the lin-46 5'UTR (Figure S1)
	priOI725	aaacctgatacaattcttggtttc	
Cloning: Primers that are used to clone the HR template to tag *lin-28* with gfp (pOI173)	priOI551	agaaccccaaacggacggaattctccccgggct agcggtgcgagcggatcgagcag	Primer pair used to amplify the blasmid backbone. Primers contain tails to allow Gibson Assembly with 5' and 3' HR arms.
	priOI552	accataagcaaagtttctctcgcaggtaccaagct tggatcgacgagagcagcgc	
	priOI553	tgcgagagaaactttgcttatggt	Primer pair used to amplify the 5' HR arm using N2 (wildtype *C. elegans*) DNA as template.
	priOI554	ttcatcagaggaattactattcttt	
	priOI555	aagaatagtaattcctctgatgaaAgtaaaggag aagaacttttcactg	Primer pair used to amplify gfp sequence (similar to but modified version of the gfp sequence found in pCM1.53; modification is insertion of a loxP site into the last intron of gfp).
	priOI556	ctctatcaatattctcagtgtctagatgattctatttgta tagttcgtccatgccatg	
	priOI557	tctagacactgagaatattgatagagaaataatgc aatatatggtctcaaatag	Primer pair used to amplify the 3' HR arm using N2 (wildtype *C. elegans*) DNA as template.
	priOI558	gacaattccgtccgtttggggttct	
Cloning: Primers that are used to clone the HR Template to tag and *lin-46* with mCherry (pOI167)	priOI574F	ggtggtggtggtggtgtctcaaagggtgaaga agataacatgg	Primer pair used to amplify mCherry sequence and to add a 6xGly linker.
	priOI575R	cttatacaattcatccatgccacc	
	priOI576F	ggtggcatggatgaattgtataagtgaaaattcac cagtatcaatatttcc	Primer pair used to amplify 5' and 3' HR arms and a plasmid backbone from a plasmid (pOI120) that contained HR template containing gfp instead of mCherry
	priOI577R	ctttgagacaccaccaccaccacctgcaaagcgt agatcaaccacgtctcc	
Cloning: primers used in cloning of pOI120 and define the ends of the HR arms in pOI167	priOI353F	gccggatccgggaagtagctaaaacgttga	Used to amplify the 5' and 3' HR arm in cloning of the pOI120 plasmid; define the ends of the HR arms in pOI120 and pOI167. Primers contain restriction enzyme cut sites (underlined).
	priOI354R	gccaagcttagaaaacgccatgttttggaaga	
PCR Primers used for screening and validating HR events.	priOI559F	cgaatggaaaaggtagagaagc	priOI559 and priOI560 flank the homologous recombination (HR) arms in the HR template plasmid (pOI173). Primer pairs priOI559-pri373R and pri372F-priOI560R were used to screen F1 progeny for *lin-28::gfp* integration events, and priOI559-priOI560R pair is used to detect *lin-28::gfp* alleles in F2 progeny and to validate the precise edit of the locus using sanger sequencing.
	priOI373R (GFP_R)	ccatctaattcaacaagaattgggacaac	
	priOI372F (GFP_F)	ggtccttcttgagtttgtaac	
	priOI560R	agcggagaatcagaagacgttg	
	priOI223F	acgaacggctgcaagttttg	priOI223 and priOI226 flank the homologous recombination (HR) arms in the HR template plasmid (pOI167). Primer pairs priOI223F-pri588R and priOI586F-pri226R were used to screen F1 progeny for *lin-46::mCherry* integration events, and priOI223F-priOI226R pair is used to detect *lin-46::mCherry* alleles in F2 progeny and to validate the precise edit of the locus using sanger sequencing.
	priOI588R (mCherry_R)	tgcggtttgtgttccctcat	
	priOI586F (mCherry_F)	atgagggaacacaaaccgca	
	priOI226R	actcctcagtttgtctctggc	

Table 4.2. Cloning and PCR Primers used in Chapter IV.

CHAPTER V -- Pre-diapause rewiring of the *C. elegans* heterochronic circuit regulating *hbl-1* activity: altered roles for *let-7*-family microRNAs, potentiation of compensatory posttranslational inhibition, and integration of stress-response genes

Abstract

Under favorable conditions, the nematode *Caenorhabditis elegans (C. elegans)* develops rapidly through four larval stages (L1-L4). At each larval stage specific cell-fate programs are executed; the order and temporal progression of these cell-fate programs are controlled by genes in the heterochronic pathway. Certain environmental and physiological stress signals cause a lengthened L2 stage, referred to as the L2d. The L2d is a developmentally bipotential stage that can be followed by either of two distinct post-L2 trajectories: direct development through the L3 and L4 stages, or developmental diapause termed the dauer larva.

L2d-inducing conditions result in a rewiring of the heterochronic pathway (called the L2d rewiring) (Ilbay and Ambros, 2019a), which includes a reduction in the reliance on three *let-7*-family microRNAs (*mir-48, mir-84, mir-241*) for temporal downregulation of a transcription factor, Hunchback-like-1 (HBL-1). The levels of these microRNAs are reduced during L2d, and although certain heterochronic genes, such as *lin-4*, *lin-46*, and *nhl-2*, are known to be required to compensate for the reduced levels of *mir-48, mir-84, and mir-241*, the L2d alternative configuration of the heterochronic pathway is not well understood. In particular, it is not known 1) whether other members of the *let-7*-family microRNAs, namely, *let-7*, *mir-793, mir-794,* and *mir-795* are involved in the L2d alternative heterochronic pathway, 2) whether the 3'UTR of *hbl-1*, which harbors *let-7*- and *lin-4*-complementary sites (LCSs), is required for the L2d

compensation, 3) whether genes and pathways that are not known to function in the heterochronic gene network -- but perhaps are related to stress response mechanisms -- could also be involved in the L2d alternative heterochronic pathway.

In the studies reported in this Chapter, we found that none of the *let-7*-family members -- when singly mutated -- nor the LCSs in the *hbl-1* 3'UTR were essential for the L2d alternative configuration of the heterochronic pathway. On the other hand, we found that certain genes that were not previously known to be functioning in the heterochronic pathway, namely, the autophagy genes, *lgg-1* and *atg-18*, as well as the DEAD box helicase gene, *vbh-1*, are involved in the L2d alternative heterochronic pathway. We further determined that *lgg-1* and *vbh-1* regulate temporal cell-fates under L2d-inducing conditions but not under normal conditions.

Introduction

Animal development is remarkably robust against many perturbations, including unavoidable environmental or physiological stresses such as crowding or starvation. How do developmental programs adapt to various challenges, maintaining the fidelity to wildtype phenotypes? Developmental robustness is in large part conferred by complex gene regulatory networks that are wired to integrate many (potentially interfering) stress signals in order to execute proper developmental programs at the right time and place and in coordination with developmental decisions that affect the developmental trajectory.

The nematode *Caenorhabditis elegans (C. elegans)* develops through four larval stages, L1-L4. Importantly, each larval stage consists of an invariant set of cell division and differentiation events, leading to the production of a defined number of cells with defined cell identities that constitute the tissues of adult worms (Sulston and Horvitz, 1977). Owing to the simplicity and the invariance of stage-specific developmental events, *C. elegans* has been an excellent model organism to elucidate various aspects of animal development and to study developmental robustness. For example, studying *C. elegans* development revealed a network of heterochronic genes controlling the timing and order of developmental events (Ambros and Horvitz, 1984)(Ambros, 2000).

The *C. elegans* heterochronic gene pathway includes three transcription factors (TFs; LIN-14, HBL-1, LIN-29) that specify stage-specific cell-fates (Ambros and Horvitz, 1987; Rougvie *et al.*, 1995; Abrahante *et al.*, 2003; Lin *et al.*, 2003) and regulator gene products that help shape temporal expression patterns of these TFs with high precision and robustness. Different microRNAs directly or indirectly regulate these heterochronic TFs: *lin-4* microRNA regulates LIN-14 (Lee, Feinbaum and Ambros, 1993; Wightman, Ha and Ruvkun, 1993); three *let-7*-family microRNAs (*mir-48, mir-84, mir-241* or *mir-48/84/241)* regulate HBL-1 (Abbott *et al.*, 2005); and *let-7* regulates LIN-29 indirectly by directly regulating LIN-41(Reinhart *et al.*, 2000) -- a repressor of *lin-29.* In parallel with the translational repression exerted by microRNAs*,* HBL-1 (Hunchback-like 1) is also regulated by the *lin-28-lin-46* pathway (Pepper *et al.*, 2004), which controls

the nuclear accumulation and hence the activity of HBL-1 (Ilbay and Ambros, 2019b).

HBL-1 is expressed at the L1 and L2 stages and promotes L2 stage-specific symmetric divisions of hypodermal seam cells (Abrahante *et al.*, 2003; Lin *et al.*, 2003), which results in an increase in the number of these cells from ten to sixteen on each lateral side of the worm. *Mir-48/84/241* and *lin-46* activities are required for downregulating HBL-1 at the end of the L2 stage in order to permit progression to L3 cell-fates (Pepper *et al.*, 2004; Abbott *et al.*, 2005). When *mir-48/84/241* are mutated (Abbott *et al.*, 2005), or when the *let-7*-complementary sites (LCSs) in the *hbl-1* 3'UTR are deleted (Ilbay and Ambros, 2019b), L2 cell-fates are reiterated at the later stages, leading to extra seam cells at the L3 and later stages (Abbott *et al.*, 2005; Ilbay and Ambros, 2019b). Loss of *lin-46* also results in a similar but less severe extra seam cell phenotype (Pepper *et al.*, 2004). Therefore, the microRNA-mediated repression is considered to be the major mode of HBL-1 downregulation at the end of L2 stage; and LIN-46 -- which is expressed only at the L3 and L4 stages (Ilbay, Nelson and Ambros, 2019) -- functions to reinforce HBL-1 downregulation by inhibiting the nuclear accumulation of any residual HBL-1 at the L3 and L4 stages (Ilbay and Ambros, 2019b).

Interestingly, both the reliance on *mir-48/84/241* for proper HBL-1 downregulation (Ilbay and Ambros, 2019a) and the expression levels (Hammell, Karp and Ambros, 2009) of these microRNAs are reduced when the worms go

through the pre-diapause L2d stage in the presence of diapause-inducing pheromones or starvation signals. Under such L2d-inducing conditions (diapause-inducing conditions that do not reach the diapause commitment threshold) components of an alternative heterochronic pathway that was previously described in post-diapause animals (Karp and Ambros, 2012) (which is not well understood except that it involves *lin-4, nhl-2, lin-46)* become more important for HBL-1 downregulation and hence for L2-to-L3 cell-fate progression (Ilbay and Ambros, 2019a). The overall response to L2d-inducing conditions in the context of temporal cell-fate regulation is called *the L2d rewiring*; and it includes 1) reduced levels of and reliance on *mir-48/84/241* and 2) increased reliance on the alternative heterochronic genes, which compensates for the reduced levels of *mir-48/84/241,* at least in part via posttranslational inhibition of HBL-1 by LIN-46.

The L2d rewiring is controlled by the nuclear hormone receptor, DAF-12, which represses the transcription of *let-7*-family microRNAs (Bethke *et al.*, 2009; Hammell, Karp and Ambros, 2009) and is required for the activation of the alternative heterochronic pathway under L2d-inducing conditions (Ilbay and Ambros, 2019a). An allele of *daf-12*, called *rh61*, constitutively (regardless of the presence or absence of the L2d-inductive signals) represses the expression of *let-7*-family microRNAs (Hammell, Karp and Ambros, 2009). Thus, in *daf-12(rh61)* animals under favorable conditions, because the L2d alternative heterochronic pathway is not activated and *let-7*-family microRNAs are

inappropriately reduced, retarded, extra seam cell phenotypes are expressed. Importantly, L2d-inducing conditions -- without elevating *let-7*-family levels -- almost completely suppress the extra seam cell phenotypes of *daf-12(rh61)* animals (Ilbay and Ambros, 2019a), which is presumably as a result of the activation of the L2d alternative heterochronic pathway. These L2d-incuding conditions include ascarosides, and a partial loss of function allele of the TGF-β receptor (*daf-7*), called *e1372* (Ilbay and Ambros, 2019a).

The roles of *lin-4, nhl-2*, and *lin-46* within the L2d alternative configuration of the heterochronic pathway are not yet clear. However, under normal conditions, we know that *lin-46* inhibits HBL-1 post-translationally and independently of *let-7*-family microRNAs (Abbott *et al.*, 2005; Ilbay and Ambros, 2019b); *lin-4* regulates *lin-28* (Moss, Lee and Ambros, 1997; Abrahante *et al.*, 2003; Lin *et al.*, 2003; Karp and Ambros, 2012), which represses *lin-46* translation; and *nhl-2* modulates activities of microRNAs (Hammell *et al.*, 2009), which include *let-7*-family and *lin-4* . Therefore, during L2d, potentiation of the activity of any of these three genes, *lin-46, lin-4,* or *nhl-2,* could lead to an enhanced downregulation of HBL-1 either by increased LIN-46 activity and/or *let-7*-family activity, resulting in the suppression of extra seam cell phenotypes.

There are many questions that remain unanswered regarding the L2d alternative configuration of the heterochronic pathway. In particular, it is not known whether the rest of the *let-7*-family microRNAs, namely *let-7*, and the recently discovered members *mir-793, mir-794,* and *mir-795* (Ruby *et al.*, 2006)

are involved in the compensation against reduced mir-48/84/241 levels. Moreover, whether direct regulation of *hbl-1* by *lin-4* or *let-7*-family microRNAs through the 3'UTR of *hbl-1*, which harbors *let-7*- and *lin-4*-comlementary sites (LCSs), is essential in the context of the L2d alternative configuration of the heterochronic pathway is unknown. Lastly, it is not known whether additional, perhaps stress response related pathways, could be involved in the L2d alternative heterochronic pathway.

We found that null alleles of *mir-793, mir-794,* or *mir-795,* which singly do not cause evident phenotypes, can enhance the extra seam cell phenotypes in combination with *mir-48/241* mutants, suggesting that *mir-793, mir-794,* and *mir-795* also contribute to L2-to-L3 cell-fate progression and presumably to HBL-1 downregulation. Moreover, we generated doubly-mutant strains containing a null allele of each of the seven *let-7*-family microRNAs combined with *daf-12(rh61)*, and found that in each case, the retarded *daf-12(rh61)* phenotypes were to varying degrees suppressed by L2d-inducing pheromones. This indicates that no single *let-7*-family microRNA is essential for the ascaroside-mediated suppression of the heterochronic phenotypes of *daf-12(rh61).* Nonetheless, the loss of certain *let-7*-family members, such as *mir-84, mir-793, and mir-794*, had a greater impact on the degree of suppression than others. Surprisingly, *mir-48*, which is the main family member that is required for L2-to-L3 cell-fate progression under favorable conditions, was less critical than all other *let-7*-family members (except *mir-795*) for the suppressibility of *daf-12(rh61)* by ascarosides.

These results illustrate that the L2d rewiring involves a redistribution of roles among certain *let-7*-family members, mainly, a reduction the importance for *mir-48* and an elevation in the importance for *mir-84*, *mir-793*, and *mir-794*. We also found that the extra seam cell phenotypes of the *hbl-1* 3'UTR mutant lacking all LCSs *[hbl-1(ma354)]* (Ilbay and Ambros, 2019b) were partially suppressed in the presence of L2d-inducing pheromones. This result indicates that the LCSs in the *hbl-1* 3'UTR are still at play but are not essential for L2d rewiring; and *let-7-/lin-4*-independent factors are involved in the L2d alternative configuration of the heterochronic pathway.

Lastly, in order to investigate whether potential L2d-induced genes -- that were not previously known to be temporal cell-fate regulators -- could be potentially play roles in the L2d alternative heterochronic pathway, we performed a pilot RNAi screen. We found that indeed the autophagy genes, *lgg-1* and *atg-18*, and the vasa- and belle-like DEAD box helicase, *vbh-1*, were required for the L2d suppression of the heterochronic defects of *daf-12(rh61)* by the L2d-inducing *daf-7(e1372)* mutation. Moreover, RNAi knockdown of *lgg-1* and *vbh-1* resulted in heterochronic defects only under L2d-inducing conditions and not under normal conditions indicating that these genes are integrated into the heterochronic pathway during L2d rewiring.

Results

The extended *let-7*-family, *mir-48, mir-84, mir-241*, *mir-793, mir-794,* and *mir-795,* contribute to L2-to-L3 cell-fate progression.

let-7 is a widely conserved microRNA that was first identified in *C. elegans* by loss-of-function mutations that cause a developmental lethality phenotype associated with altered developmental timing (Reinhart *et al.*, 2000). Six additional members of the *C. elegans let-7*-family, *mir-48, mir-84, mir-241,* (Lau *et al.*, 2001; Ambros and Lee, 2004), and *mir-793, mir-794,* and *mir-795* (Ruby *et al.*, 2006), were identified by molecular cloning and sequencing of *C. elegans* small RNAs. *mir-795* can be found in other nematode species, while *mir-793* and *mir-794* appear to be *C. elegans* inventions. The functions of *mir-48/84/241* were already determined (Abbott *et al.*, 2005) before *mir-793/794/795* were identified (Ruby *et al.*, 2006); and the functions of these newer *let-7*-family microRNAs remained unknown for many years. Moreover, null alleles of *mir-793* and *mir-795* were not available.

First, we mutated the genomic loci encoding *mir-793* and *mir-795* microRNAs using CRISPR/Cas9 genome editing. Loss of *mir-793, mir-794* (we used the *tm4915* allele)*,* or *mir-795* did not reveal any heterochronic developmental defects*.* In the context of temporal downregulation of HBL-1 and controlling L2-to-L3 cell-fate progression, *mir-84*, *mir-48* and *mir-241* function redundantly (Abbott *et al.*, 2005)*.* Loss of *mir-48* alone results in mild heterochronic phenotypes (Abbott *et al.*, 2005), and *mir-84(0)* and/or *mir-241*(0) enhances these *mir-48(0)* phenotypes (Abbott *et al.*, 2005).

In order to test for redundancy between *mir-48* and the new members of the *let-7*-family, we generated strains doubly mutant for *mir-48* and each of the

new members. We analyzed these doubly mutant strains and compared them to *mir-48*(0) alone, and to the *mir-48(0); mir-84(0)* and *mir-48(0) mir-241(0)* double mutants at 15ºC, 20ºC, and 25ºC (Figure 5.1A). We found that *mir-241(0),* as previously reported (Abbott *et al.*, 2005), enhances the extra seam cell phenotypes of *mir-48(0)* mutants, but null mutations of *mir-793, mir-794,* or *mir-795* did not enhance *mir-48(0)* phenotypes. Therefore, it appears that if *mir-793, mir-794,* or *mir-795* function redundantly with *mir-48*, and their individual contributions are relatively minor compared to *mir-241.*

In order to test whether a contribution to *hbl-1* regulation by *mir-793, mir-794,* or *mir-795* could be revealed in the absence of both *mir-48* and *mir-241*, we generated triply mutant strains that are null for *mir-48* and *mir-241* and for each of *mir-793, mir-794,* or *mir-795*. We found that similar to *mir-84*, loss of *mir-793, mir-794,* or *mir-795* enhanced the extra seam cell phenotypes of *mir-48(0) mir-241(0)* double mutants (Figure 5.1B), suggesting that each of these three microRNAs acts redundantly with *mir-48* and *mir-241***.**

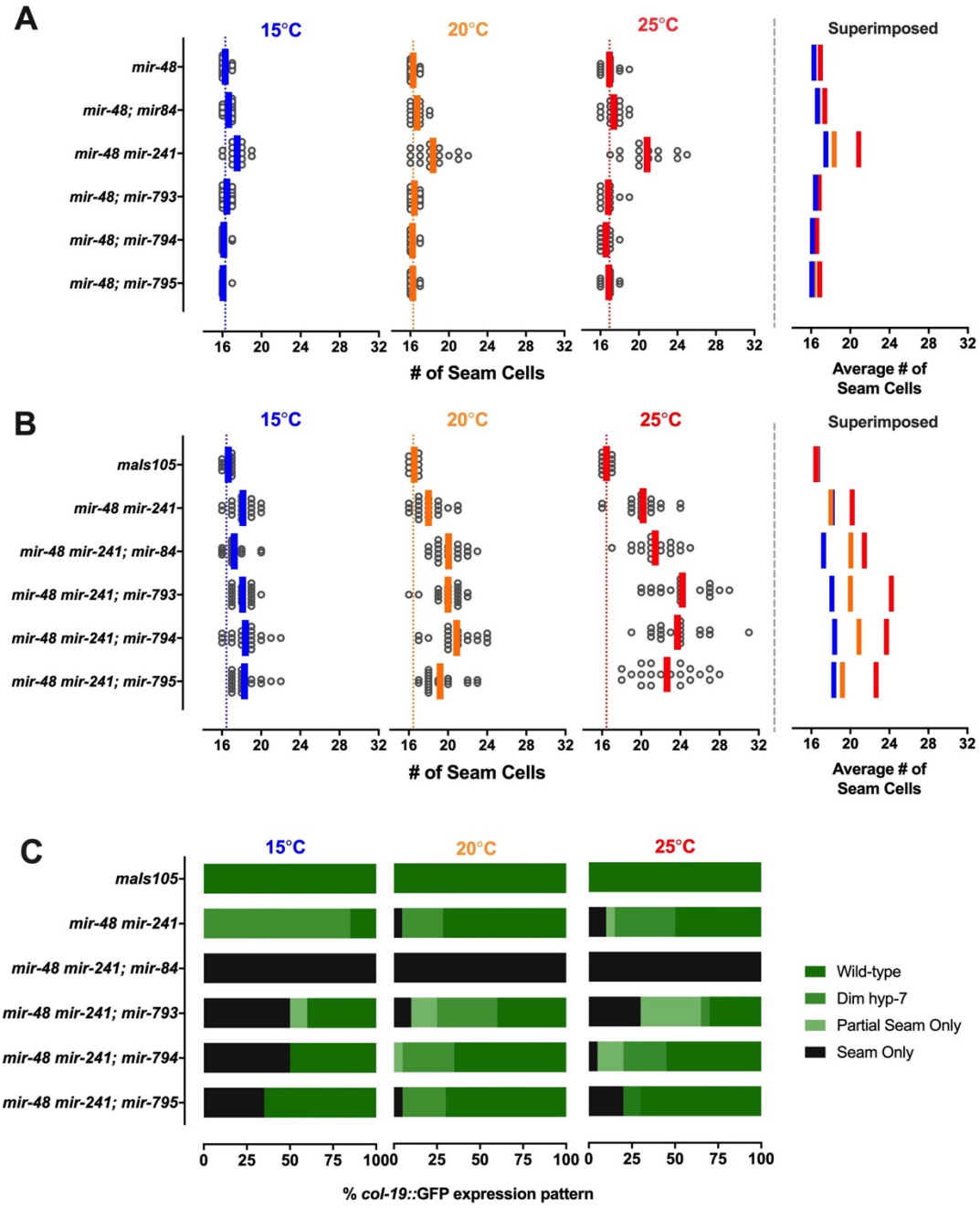

A
15°C
20°C
25°C
Superimposed
mir-48
mir-48; mir84
mir-48 mir-241
mir-48; mir-793
mir-48; mir-794
mir-48; mir-795
16 20 24 28 32
# of Seam Cells
Average # of Seam Cells
B
15°C
20°C
25°C
Superimposed
mals105
mir-48 mir-241
mir-48 mir-241; mir-84
mir-48 mir-241; mir-793
mir-48 mir-241; mir-794
mir-48 mir-241; mir-795
16 20 24 28 32
# of Seam Cells
Average # of Seam Cells
C
15°C
20°C
25°C
mals105
mir-48 mir-241
mir-48 mir-241; mir-84
mir-48 mir-241; mir-793
mir-48 mir-241; mir-794
mir-48 mir-241; mir-795
Wild-type
Dim hyp-7
Partial Seam Only
Seam Only
0 25 50 75 100
% col-19::GFP expression pattern

Figure 5.1. All *let-7*-sisters, *mir-48, mir-84, mir-241*, *mir-793, mir-794,* and *mir-795,* contribute to L2-to-L3 cell-fate progression.

(A) Number of seam cells observed in double mutants of *let-7* family microRNAs containing *mir-48(0)* and a null allele of each remaining family member at three different temperatures are shown. Vertical bars indicate average number of seam cells for each strain and they are superimposed in the graph on the right. (B) Number of seam cells observed in triple mutants of *let-7* family microRNAs containing *mir-48(0) and mir-241(0)*, and a null allele of each remaining family member at three different temperatures are shown. Vertical bars indicate average number of seam cells for each strain and they are superimposed in the graph on the right. (C) For each strain in panel B, percent *pCol-19::gfp* -- which is expressed both in seam and hpy7 cells in adult stage wildtype worms -- expression pattern in young adult worms are shown at three different temperatures.

These results suggest that, in the context of controlling L2-to-L3 cell-fate progression, all four microRNAs, *mir-84*, *mir-793, mir-794, and mir-795,* act redundantly with *mir-48* and *mir-241* and contribute to the regulation of L2-to-L3 cell-fates at similar degrees. Interestingly, for all compound mutants, the extra seam cell phenotypes were enhanced at higher temperatures (Figure 5.1A and 5.1B).

We also examined the effects of loss of *mir-84, mir-793, mir-794, and mir-795* on the pattern of adult onset *col-19::gfp* expression (Figure 5.1C) and found that *mir-84* robustly enhanced the retarded *col-19::gfp* expression phenotype in *hyp7* cells of young adult animals at all three temperatures (Figure 5.1C), whereas loss of *mir-793*, *mir-794*, or *mir-795* resulted in variable degrees of retarded *col-19::gfp* phenotypes, ranging from wildtype to mild. These results suggest that *mir-84* has a relatively more potent contribution than do *mir-793, mir-794,* or *mir-795* to regulating the larval to adult cell-fate transitions in hyp7 cells.

Redistribution of roles among *let-7*-family microRNAs during L2d rewiring

daf-12(rh61) mutants display heterochronic phenotypes due to reduced levels of *let-7*-family microRNAs (Hammell, Karp and Ambros, 2009). Interestingly, whereas the *let-7*-family expression levels in *daf-12(rh61)* larvae are insufficient for specifying proper L2-to-L3 cell fate transitions during rapid development (hence retarded heterochronic phenotypes), these reduced *let-7*-family levels are sufficient during L2d development when the L2d alternative

heterochronic pathway is activated by ascarosides (Ilbay and Ambros, 2019a) (Figure 5.2, second row versus first row). Thus, we reasoned that the reduced levels of *let-7*-family microRNAs in *daf-12(rh61)* animals represent the normal *let-7*-family levels during L2d in wildtype animals. This supposition is consistent with the observation that *let-7* family microRNA levels are reduced in wild type L2d larvae (Hammell, Karp and Ambros, 2009), and that the *let-7*-fam microRNAs collectively appear to be less critical for HBL-1 downregulation during L2d development compared to L2 (Ilbay and Ambros, 2019a)

To test for roles of the individual *let-7*-family microRNAs during L2d we generated a series of doubly-mutant strains containing the *daf-12(rh61)* allele and a mutant allele of each *let-7*-family microRNA. We observed that the removal of any single *let-7*-family member from the *daf-12(rh61)* background did not completely abrogate ascaroside-mediated suppression of *daf-12(rh61)* retarded phenotypes (Figure 5.2A). Nonetheless, the absence of each member had a different impact on the degree of suppression of the heterochronic phenotypes of *daf-12(rh61)* in the presence of ascaroside (Figure 5.2A and 5.2B), suggesting that each member contribute to downregulation of HBL-1 during L2d-to-L3 transition, and they contribute at varying degrees.

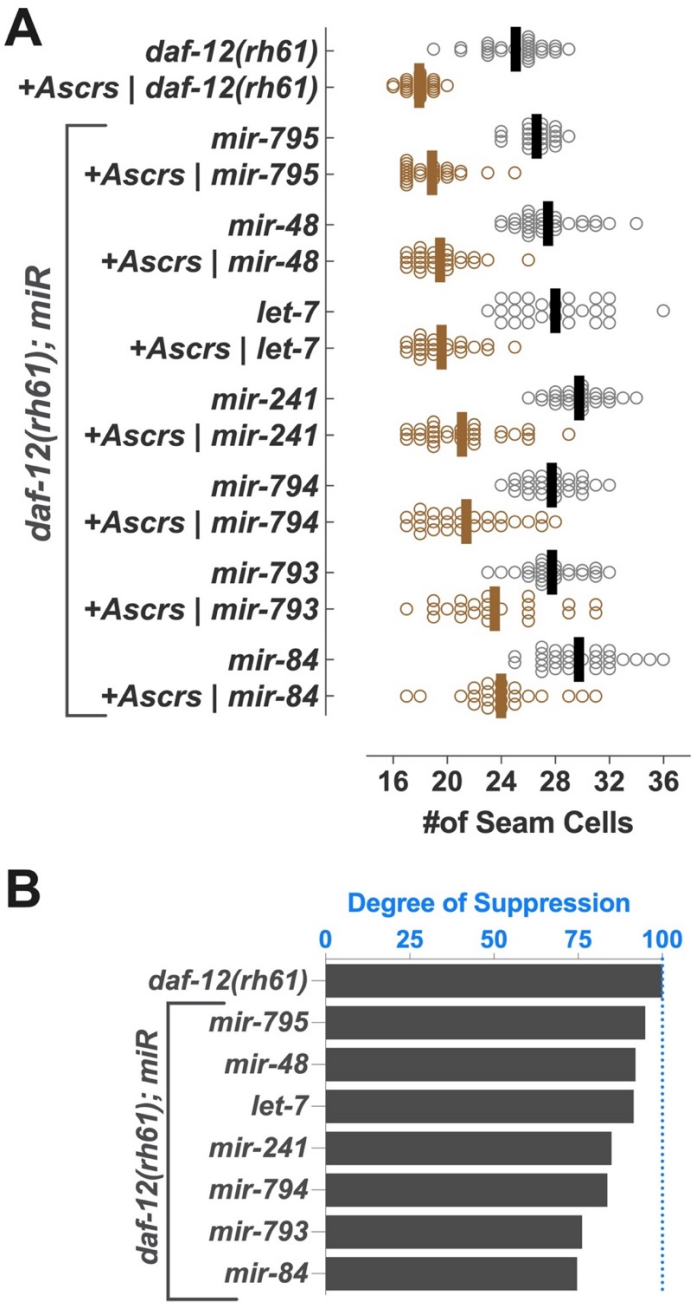

A
daf-12(rh61)
+Ascrs | daf-12(rh61)
mir-795
+Ascrs | mir-795
mir-48
+Ascrs | mir-48
let-7
+Ascrs | let-7
mir-241
+Ascrs | mir-241
mir-794
+Ascrs | mir-794
mir-793
+Ascrs | mir-793
mir-84
+Ascrs | mir-84
daf-12(rh61); miR
16 20 24 28 32 36
#of Seam Cells
B
Degree of Suppression
0 25 50 75 100
daf-12(rh61)
mir-795
mir-48
let-7
mir-241
mir-794
mir-793
mir-84
daf-12(rh61); miR

Figure 5.2. Redistribution of roles among *let-7*-family microRNAs during L2d rewiring

(A) Number of seam cells observed in young adult worms in *daf-12(rh61)* and mutants of *let-7*-family in the *daf-12(rh61)* background in the absence (black dots and vertical bars) and presence (brown dots and vertical bars) of ascarosides. Each dot represents the number of seam cells in a single animal and vertical bars indicate the average number of seam cells. (B) Degree of suppression [relative to *daf-12(rh61)*] for each double mutant are shown. Degree of suppression is calculated as the percent distance of the average number of seam cells of each mutant strain to the average number of seam cells of *daf-12(rh61)* in the presence of ascarosides.

Importantly, *mir-48*, which is the major *let-7*-family member that regulates L2-to-L3 cell-fate transitions -- all other members act redundantly with *mir-48* (Abbott *et al.*, 2005)(Figure 5.1) -- was much less critical for the L2d suppression or L2-to-L3 cell-fate transitions when animals develop through L2d instead of L2. Overall, these results suggest that the prominent role of *mir-48* in the context of HBL-1 downregulation or L2-to-L3 cell-fate transitions during rapid development is lost during L2d rewiring whereas certain other members, such as *mir-84, mir-793,* and *mir-794* -- functions of which appear to be only to support *mir-48* during rapid development -- become more important during L2d.

Factors regulating *hbl-1* both independently of and through *let-7/lin-4* complementary sites in the *hbl-1* 3'UTR are at play in the L2d alternative heterochronic pathway; and LCS-independent factors regulating *hbl-1* are potentiated by L2d-inducing ascarosides

Let-7-family microRNAs become less important but are still at play during L2d (Ilbay and Ambros, 2019a), suggesting that the *let-7*-complementary sites (LCSs) in the *hbl-1* 3'UTR should also be important for the L2d alternative heterochronic pathway. Furthermore, these LCSs could be essential for the compensation against reduced levels of *mir-48/84/241* microRNAs by the rest of the *let-7*-family members. Alternatively, these LCSs might not be essential in the L2d alternative heterochronic pathway, for example, *let-7*-family microRNAs -- at least some of them -- could be acting independently of the *let-7*-complementary sites in the *hbl-1* 3'UTR, perhaps by regulating *lin-46* or genes that act upstream

of *lin-46*, such as *lin-14* or *lin-28*. If this was the case, the extra seam cell phenotypes of the *hbl-1* 3'UTR mutant allele that removes all LCSs (*ma354*) *(*Figure 5.3A) would be suppressed by L2d-inducing conditions.

In order to test these hypotheses, we determined whether the extra seam cell phenotypes of *hbl-1(ma354) (*Figure 5.3A) were suppressed by inducing L2d ascarosides. We found that the extra seam cell phenotypes of *hbl-1(ma354)* were partially suppressed by ascarosides (Figure 5.3B). We also combined *hbl-1(ma354)* with the *daf-12(rh61)* allele (which has reduced levels of *let-7*-family microRNAs) and found that the extra seam cell phenotype of this double mutant was also partially suppressed by ascarosides. These results indicate that factors that regulate *hbl-1* through the LCSs and factors acting independently of the LCSs in the *hbl-1* 3'UTR are (almost equally) important in the L2d alternative configuration of the heterochronic pathway. Additionally, these results indicate that factors that regulate *hbl-1* independently of the LCSs in the *hbl-1* 3'UTR are activated or potentiated by L2d-inducing ascarosides.

Stress-response genes are integrated into the heterochronic pathway during L2d

The L2d rewiring involves an increased reliance on the regulation of HBL-1 by *lin-46,* which suggests that posttranslational inhibition of HBL-1 activity helps compensate for reduced translational repression of *hbl-1* by *let-7*-family microRNAs.

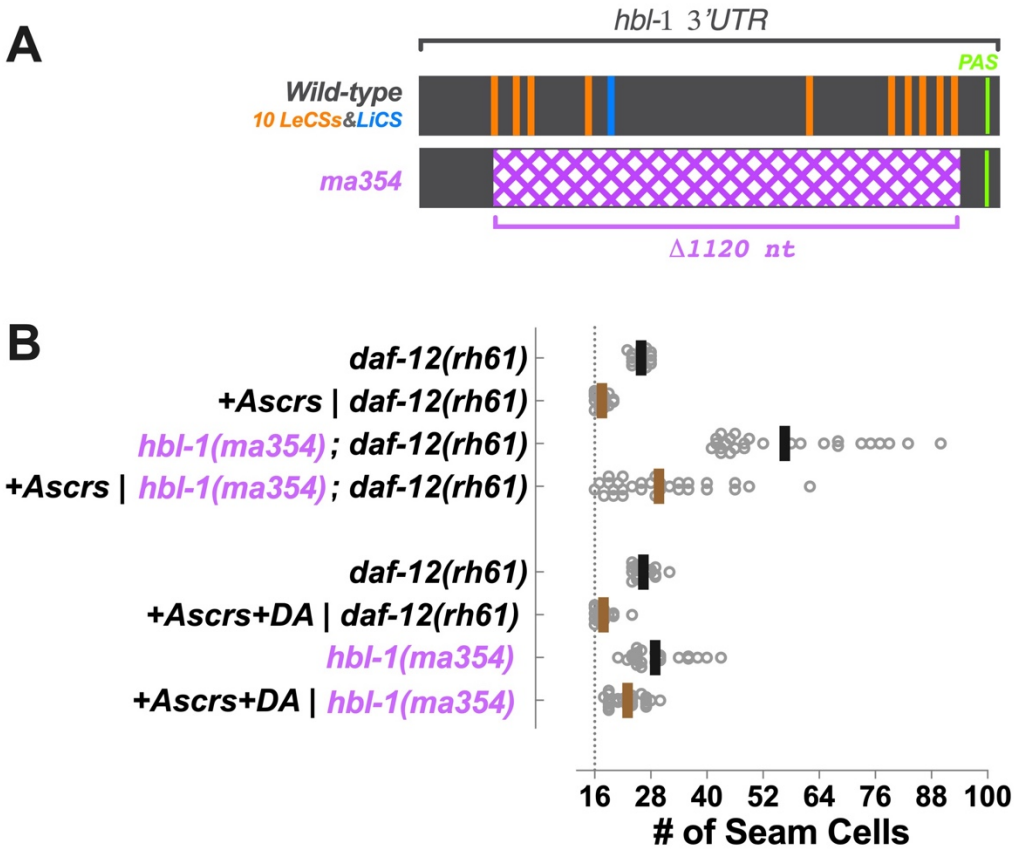

A
hbl-1 3'UTR
PAS
Wild-type
10 LeCSs&LiCS
ma354
Δ1120 nt
B
daf-12(rh61)
+Ascrs | daf-12(rh61)
hbl-1(ma354); daf-12(rh61)
+Ascrs | hbl-1(ma354); daf-12(rh61)
daf-12(rh61)
+Ascrs+DA | daf-12(rh61)
hbl-1(ma354)
+Ascrs+DA | hbl-1(ma354)
16 28 40 52 64 76 88 100
# of Seam Cells

Figure 5.3. Factors regulating *hbl-1* both independently of and through *let-7/lin-4* complementary sites in the *hbl-1* 3'UTR collaborate in the L2d alternative heterochronic pathway; and, LCS-independent factors regulating *hbl-1* are potentiated by L2d-inducing ascarosides

(A) The 3'UTR of *hbl-1* (horizontal gray bar) and ten *let-7*-(LeCS, vertical orange bars) and one *lin-4*-(LiCS, vertical blue bar) complementary sites as well as the polyadenylation signal (PAS) are shown. In the *ma354* allele, a 1120 base pair genomic region containing all LeCSs and the LiCS is deleted. (B) Number of seam cells in the absence (blue) and presence (brown) of ascarosides are plotted. *Ma354* allele enhances the extra seam cell phenotype of *daf-12(rh61).* However, this enhanced phenotype or the extra seam cell phenotype observed in *hbl-1(ma354)* are still suppressed in the presence of ascarosides.

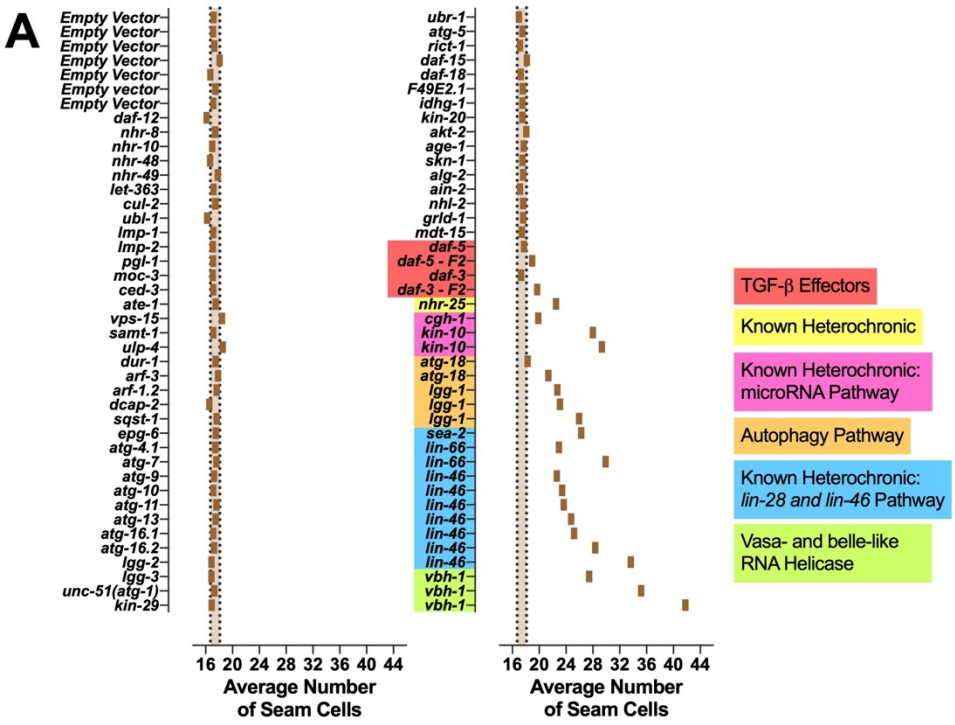
We hypothesized that factors related to the L2d trajectory, but perhaps not previously known to be developmental timing regulators, may be involved in the L2d alternative configuration of the heterochronic pathway. For example, certain L2d- or stress-activated factors could boost the activities of *let-7*-family microRNAs or LIN-46; or regulate HBL-1 independently of microRNA and/or LIN-46 pathways. Because in *daf-12(rh61); daf-7(e1372)* double mutants *let-7*-family microRNAs are downregulated and the compensatory factors of the L2d alternative heterochronic pathway are activated [which presumably suppresses the extra seam cell phenotypes of observed in *daf-12(rh61)* mutants], we reasoned that knocking down these potential compensatory factors of the L2d alternative heterochronic pathway would results in loss of L2d suppression of *daf-12(rh61)* and hence extra seam cell phenotypes in *daf-12(rh61); daf-7(e1372)* animals.

We performed a small-scale pilot RNAi screen to identify genes whose loss of function would abrogate suppression of *daf-12(rh61)* retarded phenotypes by *daf-7(e1372),* and hence result in an extra seam cell phenotype for *daf-12(rh61); daf-7(e1372)* double mutants (Figure 5.4A). As positive controls, we included RNAi clones against genes knockdown of which are predicted to compromise rewiring. These genes included *daf-3* (the downstream effector of *daf-7*), *lin-46* and known regulators of the *lin-28-lin-46* pathway (*lin-66* and *sea-2*), *cgh-1, kin-10* (required for the microRNA pathway). We also tested potential genes in the signaling pathways activated during L2d, such as *daf-5,*

daf-15, age-1, and L2d-activated stress response genes, such as the autophagy genes.

We found that, as expected, knockdown of *daf-3*, *lin-46, lin-66, sea-2, cgh-1, kin-10 (*as well as *daf-5* and *nhr-25)* resulted in the loss of L2d suppression (an increase in the number of seam cells) in *daf-12(rh61); daf-7(e1372)* double mutants (Figure 5.4A). Interestingly, knockdown of the autophagy genes, *lgg-1 and atg-18,* and the vasa- and belle-like DEAD box RNA helicase, *vbh-1* -- which is shown to be protective against heat shock and oxidative stress(Paz-Gomez *et al.*, 2014) -- also resulted in the loss of L2d suppression, indicating that these genes/pathways are involved in compensation response of the L2d alternative heterochronic pathway.

Lastly, we asked if *lgg-1* or *vbh-1* was also required for controlling L2-to-L3 cell-fate transitions during rapid development in wild-type animals or L2d development, which is induced by the *daf-7(e1372)* mutation (Figure 5.4B). We used RNAi to knockdown *lgg-1* or *vbh-1* in wild-type versus *daf-7(e1372)* animals. We found that *lgg-1* or *vhb-1* knockdown in *daf-7(e1372)* mutants resulted in extra seam cell phenotypes but not in wild-type worms, suggesting that *lgg-1* and *vbh-1* are integrated into the heterochronic pathway under L2d-inducing conditions and these genes become important for controlling L2-to-L3 cell-fate transitions, and presumably for proper temporal downregulation of HBL-1 during L2d.


A
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Empty Vector
Empty Vector
Empty Vector
Empty vector
Empty Vector
daf-12
nhr-8
nhr-10
nhr-48
nhr-49
let-363
cul-2
ubl-1
lmp-1
lmp-2
pgl-1
moc-3
ced-3
ate-1
vps-15
samt-1
ulp-4
dur-1
arf-3
arf-1.2
dcap-2
sqst-1
epg-6
atg-4.1
atg-7
atg-9
atg-10
atg-11
atg-13
atg-16.1
atg-16.2
lgg-2
lgg-3
unc-51(atg-1)
kin-29
ubr-1
atg-5
rict-1
daf-15
daf-18
F49E2.1
idhg-1
kin-20
akt-2
age-1
skn-1
alg-2
ain-2
nhl-2
grld-1
mdt-15
daf-5
daf-5 - F2
daf-3
daf-3 - F2
nhr-25
cgh-1
kin-10
kin-10
atg-18
atg-18
lgg-1
lgg-1
lgg-1
sea-2
lin-66
lin-66
lin-46
lin-46
lin-46
lin-46
lin-46
lin-46
lin-46
lin-46
vbh-1
vbh-1
vbh-1
16 20 24 28 32 36 40 44
Average Number of Seam Cells
16 20 24 28 32 36 40 44
Average Number of Seam Cells
TGF-β Effectors
Known Heterochronic
Known Heterochronic: microRNA Pathway
Autophagy Pathway
Known Heterochronic: lin-28 and lin-46 Pathway
Vasa- and belle-like RNA Helicase

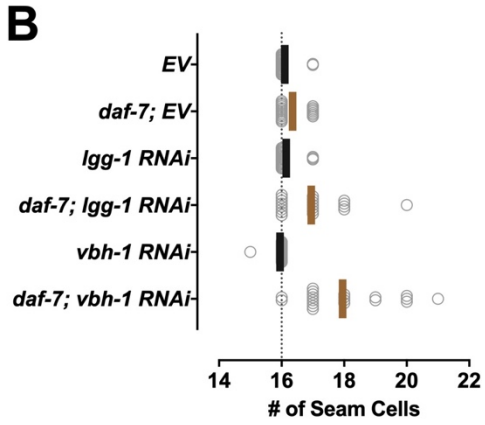
B
EV
daf-7; EV
lgg-1 RNAi
daf-7; lgg-1 RNAi
vbh-1 RNAi
daf-7; vbh-1 RNAi
14 16 18 20 22
# of Seam Cells

Figure 5.4. Stress-response genes are integrated into the heterochronic pathway during L2d.

(A) A small-scale RNAi screen to identify genes required for the suppression of extra seam cell phenotypes of *daf-12(rh61)* by L2d, induced by *daf-7(e1372)* (VT3705). Vertical dotted lines and the area between them represent the variability of the number of seam cells observed in VT3705 on empty vector. RNAi clones that resulted in an increase in the average seam cell number (of 20 animals) are listed on the right bottom and the gene names are highlighted with colors matching their descriptions in the boxes on the right. (B) Number of seam cells are shown for wildtype versus *daf-7(e1372),* VT3704, on empty vector (EV) RNAi versus *lgg-1* or *vbh-1* RNAi.

Discussion

In this study, we addressed questions related to the L2d compensation mechanism, which complements the reduction in the *let-7*-family microRNA levels under L2d-inducing stresses with the activation an alternative configuration of the heterochronic pathway to downregulate HBL-1 -- a *let-7*-family target.

We analyze the roles of the extended family of *let-7*-family microRNAs in regulating L2-to-L3 cell-fate progression under normal versus L2d-inducing conditions and show that all six *let-7*-sisters contribute to proper larval cell-fate progression both under normal and L2d-inducing conditions. We show that the L2d rewiring, which consists of the reduction in *let-7*-family levels and the activation of the (compensatory) L2d alternative heterochronic pathway, also involves a redistribution of roles among *let-7*-family microRNAs to control L2-to-L3 cell-fate progression.

Moreover, we show that the *let-7-/lin-4*-complementary sites (LCSs) in the *hbl-1* 3'UTR as well as factors acting independently of these LCSs are at play to ensure robust HBL-1 downregulation during L2d (Figure 5.5). Our previous findings suggest that *lin-46* is one of these LCS-independent factors (Ilbay and Ambros, 2019b) that is important for the L2d compensation (Ilbay and Ambros, 2019a).

Lastly, by investigating genes that might be required for the L2d compensations, we identified two genes: a key autophagy gene *lgg-1* (also

known as LC3 or ATG8), and the DEAD box RNA helicase *vbh-1* that are integrated into the heterochronic pathway only under L2d-inducing conditions. L2d-specific roles of *lgg-1* and *vbh-1* in temporal cell-fate regulation illustrate that stress-response pathways can be integrated into the heterochronic pathway to maintain stage-matched cell-fate progression despite delays and uncertainties in stage progression.

Under favorable growth conditions, *let-7*-family microRNAs (primarily *mir-48)* are expressed highly and downregulate *hbl-1* effectively enough to minimize the need for additional negative regulators of HBL-1 activity, such as LIN-46. In the presence of crowding or starvation conditions that are diapause-inducing but below the commitment threshold, animals develop continuously through L2d. During L2d, *let-7*-family microRNA levels are reduced (Hammell, Karp and Ambros, 2009). However, although expressed at substantially lower levels when compared to favorable or L2 conditions, *let-7*-family microRNAs are still needed for proper L2-to-L3 cell-fate transitions during L2d. Interestingly, *mir-48* become less important, whereas other members, such as *mir-84, mir-793,* and *mir-794* become more important for L2-to-L3 cell-fate transitions during L2d (Figure 5.2A).

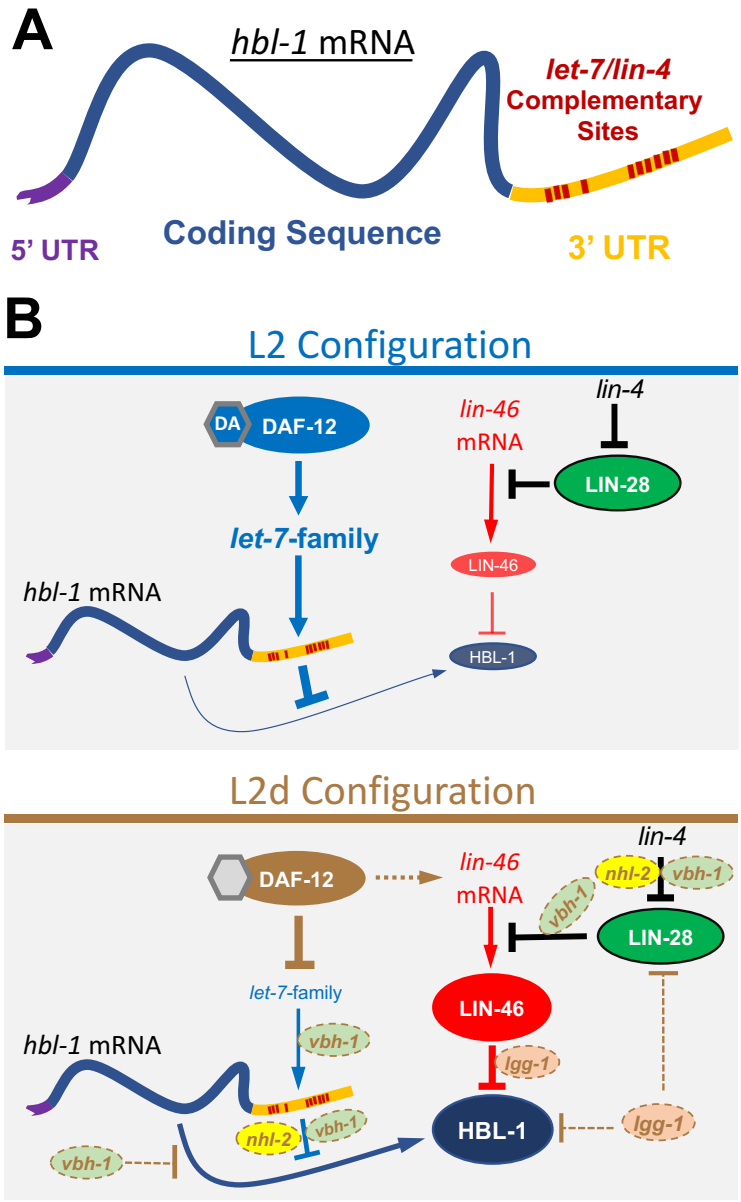
A
hbl-1 mRNA
let-7/lin-4
Complementary
Sites
5' UTR
Coding Sequence
3' UTR
B
L2 Configuration
DA
DAF-12
let-7-family
hbl-1 mRNA
lin-46
mRNA
lin-4
LIN-28
LIN-46
HBL-1
L2d Configuration
DAF-12
let-7-family
hbl-1 mRNA
vbh-1
nhl-2
vbh-1
lin-46
mRNA
lin-4
nhl-2
vbh-1
vbh-1
LIN-28
LIN-46
lgg-1
HBL-1
lgg-1

Figure 5. 5. The L2d rewiring: the shift from the L2 configuration to the L2d configuration of the heterochronic circuit regulating *hbl-1* activity.
(A) A depiction of the *hbl-1* mRNA showing the 5'UTR, protein coding region, and the 3'UTR harboring *let-7-/lin-4*-complementary sites. (B) L2 versus L2d configuration of the heterochronic circuit controlling *hbl-1* activity. Thickness of lines showing regulatory interactions indicate predicted or inferred strengths of these interactions. Potential locations where *nhl-2, vbh-1,* or *lgg-1* can be integrated into the heterochronic pathway under L2d conditions are shown in the L2d configuration panel using dotted lines.

These differences among *let-7*-family members in terms of their effects on ascaroside sensitivity could be a reflection of differences among *let-7*-family members in their capacities 1) to target and downregulate *hbl-1* in the presence of ascarosides, 2) to interact with genes in the signaling pathways downstream of ascarosides, which would affect the strength of L2d rewiring, including the activation of the L2d alternative heterochronic pathway, 3) to regulate the *lin-28-lin-46* pathway, which controls the nuclear accumulation of HBL-1 and is a critical component of the L2d alternative heterochronic pathway.

In the context of inducing L2d and dauer arrest, ascarosides act by repressing *daf-7* (a TGF-β ligand) expression, resulting in the activation of the downstream effector *daf-3* (a SMAD*).* Both *daf-7* and *daf-3* are predicted targets of *let-7*-family microRNAs. Similarly, both *lin-28* and *lin-46,* which are important for the L2d alternative heterochronic pathway, are predicted targets of *let-7*-family microRNAs. Therefore, it is possible that 1) all *let-7*-family members might interact with all of these targets *(hbl-1, daf-7, daf-3, lin-28*, and *lin-46*) and these interactions are altered by L2d-inducing conditions, or 2) certain *let-7*-family members interacts more strongly with a subset of these potential targets; and some of these interactions become more important to control L2-to-L3 cell-fate progression under L2d-inducing conditions, leading to the observation that the roles among *let-7*-family members are redistributed during L2d.

Accordingly, if a member of the *let-7*-family mainly functions to repress *daf-7* or *lin-28* expression, loss of this microRNA would result in higher

levels of DAF-7 or LIN-28, which might lead to a reduction in the degree of suppression (Figure 5.2B) -- due to reduced DAF-3 or LIN-46 activity. Thus, the stronger reductions in the degree of suppression in the *daf-12(rh61)* mutants lacking *mir-84, mir-793,* or *mir-794* might be due to regulation of *daf-7* or *lin-28* specifically by these *let-7*-family members. Other genes in the TGF-β signaling pathway, which include *daf-1, daf-4, daf-5, and daf-12,* are also predicted *let-7*-family targets and could potentially be involved in regulatory interactions with specific *let-7*-family members leading to differences in their roles in regulating cell-fate progression during L2 versus L2d trajectory. Investigating how different *let-7*-family members interact with different targets and how these interactions might be modulated by environmental factors would help better understand the role of *let-7*-family in coordinating temporal cell-fates with developmental trajectory.

During L2d, *let-7-/lin-4*-complementary sites in the *hbl-1* 3'UTR and factors that are acting independently of these LCSs collaborate to control L2-to-L3 cell fates. *lin-46* -- the importance of which increases during L2d -- is a posttranslational regulator acting independently of the LCSs in the *hbl-1* 3'UTR (Ilbay and Ambros, 2019b). Similar to *lin-46*, we found that *lgg-1and vbh-1* become important for temporal cell-fate control during L2d. However, we do not know if these factors act on the LCSs or they act independently of the LCSs in the *hbl-1* 3'UTR (Figure 5.5).

Autophagy is increased in the presence of dauer-inductive signals, which probably serves to repurpose the cellular components of the larva for remodeling of the tissues during dauer arrest (Melendez *et al.*, 2003). *lgg-1* and many other autophagy genes are required for dauer larva morphogenesis (Melendez *et al.*, 2003). The DEAD-box RNA helicase, *vbh-1*, is a paralog of *cgh-1*, which is an miRISC (microRNA-induced silencing complex) cofactor (Chu and Rana, 2006; Hammell *et al.*, 2009), and is protective against heat-shock and oxidative stress (Paz-Gomez *et al.*, 2014). Like *lin-46*, *lgg-1* and *vbh-1* could be posttranslational regulators of HBL-1, although *vbh-1* might more likely act post-transcriptionally, perhaps by promoting microRNA (*let-7*-family and or *lin-4*) activity or regulating the translation of genes involved in the L2d rewiring. *lgg-1* and *vbh-1* could be involved in the *lin-28-lin-46* pathway, regulating either *lin-28* or *lin-46* specifically under L2d-inducing conditions. Further genetic analysis is required to identify how *lgg-1* and *vbh-1* interact with the heterochronic pathway in response to L2d-inducing conditions.

Our RNAi screen was limited to certain select genes and was not comprehensive in terms of potential L2d-responsive genes. Therefore, it is possible that there are other genes or pathways that are integrated into the heterochronic pathway under L2d-inducing conditions. Furthermore, there are other known environmental or nutritional stress conditions that can alter the rate of development or developmental trajectory. These stresses might activate

different response pathways, which could also become important to maintain stage-matched temporal cell-fate progression under such conditions.

In brief, our analysis of the extended *let-7*-family microRNAs and the requirements for the L2d alternative configuration of the heterochronic pathway mechanism reveal the differences in the roles of *let-7*-family microRNAs during normal and under L2d conditions, the collaboration between LCS-dependent and independent factors that regulate *hbl-1*, and the integration of stress-response genes into the heterochronic pathway under L2d conditions (Figure 5.5).

Materials and Methods

C. elegans culture conditions

C. elegans strains used in this study and corresponding figures in the paper are listed in Table 5.1. *C. elegans* strains were maintained at 20°C on nematode growth media (NGM) and fed with the Escherichia coli HB101 strain.

Assaying extra seam cell phenotypes

As described before, in Chapter 3, the worms were scored at the young adult stage (determined by the gonad development) for the number of seam cells using fluorescence microscopy with the help of the *maIs105 [pCol-19::gfp]* transgene, which marks the lateral hypodermal cell nuclei, or the *wIs51[pScm::gfp]* transgene, which marks the seam cell nuclei.

Each circle on the genotype versus number of seam cells plots shows the observed number of seam cells on one side of a single young adult worm. More

than ten worms for each genotype were analyzed and the average number of seam cells are denoted by vertical bars in the genotype versus number of seam cell plots. GraphPad Prism 8 software was used to plot the graphs and for statistical analysis.

Generation of new alleles using CRISPR/Cas9

CRISPR/Cas9 genome editing tools were used to generate null alleles of mir-793 and mir-795.

A mixture of plasmids encoding SpCas9 (pOI90), and a pair of single guide RNAs [sgRNAs, expressed from pOI83; (Ilbay and Ambros, 2019a)] targeting both sites of interest (for primers, see Table 5.2) and the *unc-22* gene (pOI91) as co-CRISPR marker (Kim *et al.*, 2014), and a *rol-6(su1006)*-containing plasmid (pOI124) as co-injection marker was injected into the gonads of young adult worms. F1 roller and/or twitcher animals (~20 or more worms until the desired allele was detected) were cloned and screened by PCR amplification (for primers, see Table 5.2) for the presence of the expected size PCR product consistent with deletion of the genomic region spanning between the sites targeted by the pair of guides.

In all new CRISPR alleles, genomic regions spanning the deletion site were sequenced using Sanger sequencing. For each allele, a single worm with a precise (HR) edited locus was cloned and backcrossed twice before being used in the experiments.

L2d-inducing ascaroside plates

L2d-inducing plates that were prepared as described previously (Ilbay and Ambros, 2019a)**.**

RNAi Screening for genes functioning in the L2d alternative heterochronic pathway

RNAi by feeding was used to knockdown genes of interest. RNAi clones were obtained from Ahringer RNAi feeding library, except for the *nhl-2*, which was cloned by Katherine McJunkin. RNAi clones used in the screen are listed in Table 5.3. The identity of each RNAi clone was sequence confirmed by Sanger Sequencing. Eggs were placed on the RNAi plates and the worms (20 worms per RNAi clone) that reach young adult stage were scored for their extra seam cell phenotypes. Positive hits were tested at least twice.

Strain Number	Genotype	Related to
VT1307	*mir-48(n4097) maIs105*	1A
VT3488	*mir-48(n4097) maIs105 V; mir-84(n4027) X.*	1A
VT1296	*mir-48 mir-241(nDf51) maIs105 V*	1A, B, C
VT3551	*mir-48(n4097) maIs105 V; mir-793(ma292) X*	1A
VT3492	*mir-794(tm4915) I; mir-48(n4097) maIs105 V*	1A
VT3552	*mir-795(ma278) I; mir-48(n4097) maIs105 V*	1A
VT1367	*maIs105 V*	1B, C
VT3579	*mir-48 mir-241(nDf51) maIs105 V; mir-84(n4037) X*	1B, C
VT3345	*mir-48mir-241(nDF51) maIs105 V; mir-793(ma292) X*	1B, C
VT3139	*mir-794(tm4915) I; mir-48 mir-241(nDf51) maIs105 V*	1B, C
VT3346	*mir-795(ma278) I; mir-48mir-241(nDf51) maIs105 V*	1B, C
VT791	*maIs105 V; daf-12(rh61) X*	2
VT3335	*mir-795(ma278) I; maIs105 V; daf-12(rh61) X*	2
VT2966	*mir-48(n4097) maIs105 V; daf-12(rh61) X*	2
VT2950	*maIs105 V; daf-12(rh61) let-7(n2853ts) X*	2
VT3225	*mir-241(n4315) maIs105 V; daf-12(rh61) X*	2
VT3130	*mir-794(tm4915) I; maIs105 V; daf-12(rh61) X*	2
VT3320	*maIs105 V; mir-793(ma292) daf-12(rh61) X*	2
VT2971	*wIs51 V; mir-84(n4937) daf-12(rh61) X*	2
VT3553	*wIs51 V; hbl-1(ma354) daf-12(rh61) X*	3B
VT3500	*wIs51 V; hbl-1(ma354) X*	3B
VT3705	*daf-7(e1372) III: wIs51 V; daf-12(rh61) X*	4A
VT3704	*daf-7(e1372) III: wIs51 V*	4B

Table 5.1. *C. elegans* strains used in Chapter V.

Cloning/ PCR primers	Primer Name	Primer Sequence	Plasmid name and/or Purpose
Cloning: Annealed primer-pairs were cloned into pOI83 to express sgRNAs	priOI280F	tcttgaggaaaaacattcatactga	pOI77 and pOI101 sgRNA expressing plasmids were used to delete a 220 base-pair genomic region, containing *mir-793* locus.
	priOI281R	aaactcagtatgaatgtttttcctc	
	priOI331F	tcttgcaatcaataggaaataaagg	
	priOI332R	aaacccttatttcctattgattgc	
	priOI288F	tcttggctgatcaatctacctcagc	pOI81 sgRNA expressing plasmid was used to mutate the *mir-795* locus.
	priOI289R	aaacgctgaggtagattgatcagcc	
PCR primers	priOI284F	acaaaatcgcatctgaaaacca	To PCR amplify and Sanger Sequence a genomic region flanking *mir-793.*
	priOI285R	tcaacaacgtttaatttcaccacg	
	priOI286F	ctttcgcccaatctcaccct	To PCR amplify and Sanger Sequence a genomic region flanking *mir-795.*
	priOI287R	atcggtgtgccttacgtgtt	

Table 5.2. Cloning and PCR primer used in Chapter V.

#	Gene Name	Library Location	Lethality/ Larval Arrest	#	Gene Name	Library Location	Lethality/ Larval Arrest
1	***age-1***	**II-7J02**		34	***laf-1***	**III-8O23**	**Y**
2	***ain-1***	**X-7O20**	**Y**	35	***let-363***	**I-9A17**	
3	***ain-2***	**I-2E07**		36	***lgg-1***	**II-4P03**	
4	***akt-1***	**V-7I17**		37	***lgg-2***	**IV-5F02**	
5	***akt-2***	**X-8A04**		38	***lin-46***	**V-14E10**	
6	***alg-1***	**X-6D15**	**Y**	39	***lin-66***	**IV-9A08**	
7	***alg-2***	**II-1F05**		40	***lmp-1***	**X-3M14**	
8	***arf-1.2***	**III-3A13**		41	***lmp-2***	**X-1C20**	
9	***arf-3***	**IV-4E13**		42	***mdt-15***	**III-3E17**	
10	***atg-10***	**II-6E18**		43	***moc-3***	**IV-4I05**	
11	***atg-18***	**V-14D09**		44	***nhl-2***	**McJunkin Lab**	
12	***atg-5***	**I-9P22**		45	***nhr-10***	**III-3D10**	
13	***bec-1***	**IV-2N20**		46	***nhr-23***	**I-3F11**	
14	***cbp-1***	**III-5A06**	**Y**	47	***nhr-25***	**X-6I19**	
15	***cgh-1***	**III-4A17**		48	***nhr-48***	**X-8A19**	
16	***cul-2***	**III-8P20**		49	***nhr-49***	**I-4N14**	
17	***daf-12***	**X-5M11**		50	***nhr-8***	**IV-3J14**	
18	***daf-15***	**IV-4H20**		51	***pgl-1***	**IV-3M20**	
19	***daf-18***	**IV-1I23**		52	***rict-1***	**II-8J07**	
20	***daf-3***	**X-1M03**		53	***samt-1***	**IV-9M16**	
21	***daf-5***	**II-9A19**		54	***skn-1***	**IV-2N18**	
22	***dcap-2***	**IV-7M21**		55	***sop-2***	**II-8A20**	
23	***dre-1***	**V-14L07**	**Y**	56	***sqst-1***	**IV-6K12**	
24	***dur-1***	**IV-5M09**		57	***uba-1***	**IV-5M23**	**Y**
25	***epg-7/atg-11***	**X-3F20**		58	***ubl-1***	**III-1N05**	
26	***F49E2.1***	**X-4B24**		59	***ubq-1***	**III-3K09**	**Y**
27	***grld-1***	**IV-1C19**		60	***ubr-1***	**I-1J13**	
28	***hsp-1***	**IV-8O17**	**Y**	61	***ulp-4***	**II-6A07**	
29	***idhg-1***	**III-8C02**		62	***unc-51(atg1)***	**V-14G22**	
30	***kin-10***	**I-4G07**		63	***vbh-1***	**I-9B10**	
31	***kin-19***	**III-2C13**		64	***vhp-1***	**II-4G06**	

Table 5.3. RNAi clones used in Chapter V.

CHAPTER VI -- General Discussion

Findings presented in this thesis illustrate how a key *C. elegans* developmental gene product, the transcription factor Hunchback-like-1 (HBL-1), is regulated by two parallel pathways via two distinct mechanisms. One arm of HBL-1 regulation involves *let-7*-family (*mir-48, mir-84*, and *mir-241*) microRNAs, which post-transcriptionally regulate HBL-1 abundance through *let-7* complementary sites in the *hbl-1* 3'UTR (Abbott *et al.*, 2005), whereas the second arm, which consists of the *lin-28-lin-46* pathway (Moss, Lee and Ambros, 1997; Pepper *et al.*, 2004), regulates the nuclear accumulation of HBL-1, independently of the *hbl-1* 3'UTR, and most likely, via a post-translational mechanism.

Additionally, we show that *let-7*-family microRNAs and LIN-46 that control HBL-1 expression act downstream of signaling pathways that control larval stage progression, including TGF-β and insulin signaling pathways and the nuclear hormone receptor DAF-12, forming a robustness network that coordinate cell-fate transitions with larval stage progression (Figure 6.1). This robustness network ensures that temporal cell-fates remain anchored to specific larval stages, while at the same time, controlling the developmental trajectory (larval stage progression) in accordance with the environmental or physiological conditions.

This robust coordination mechanism of cell-fate transitions and larval stage progression ensures that the L2 stage cell-fate determinant transcription factor HBL-1 is first kept ON throughout the rapid and deterministic L2 stage or

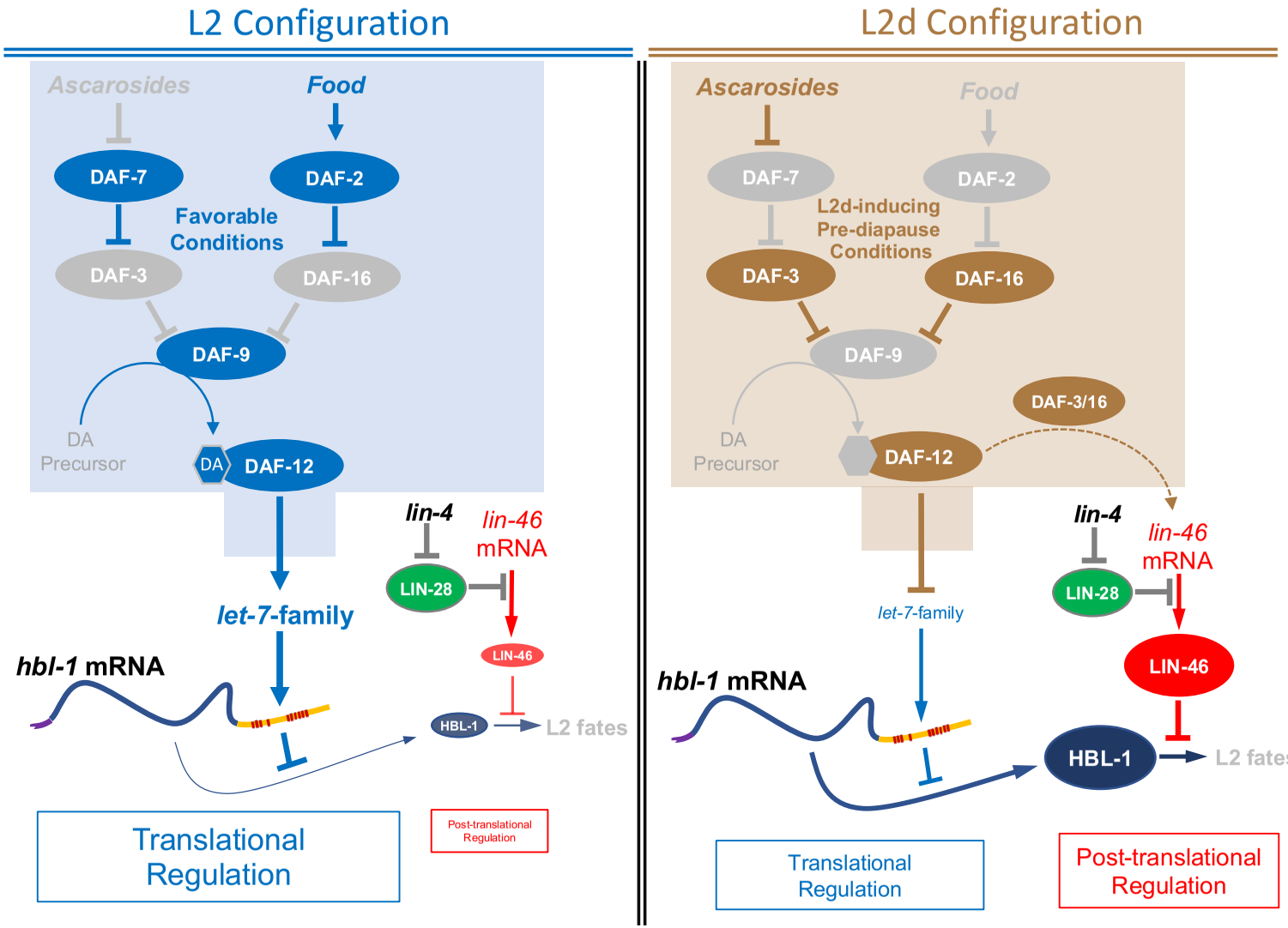
L2 Configuration
L2d Configuration
Ascarosides
Food
DAF-7
DAF-2
Favorable Conditions
DAF-3
DAF-16
DAF-9
DA Precursor
DA
DAF-12
lin-4
lin-46 mRNA
LIN-28
let-7-family
LIN-46
hbl-1 mRNA
HBL-1
L2 fates
Translational Regulation
Post-translational Regulation
L2d-inducing Pre-diapause Conditions
DAF-3/16

Figure 6.1. Signaling events controlling the larval stage progression and the L2d rewiring and corresponding L2 to L2d configurations of the heterochronic circuit controlling *hbl-1* activity.

Regulation of the L2d rewiring and the changes in the heterochronic circuit controlling *hbl-1* activity during the L2 rewiring are illustrated. The L2d rewiring is regulated by DA-dependent and DA-independent activities of DAF-12, which are controlled by the TGF-β (DAF-7 and DAF-3) and insulin (DAF-2 and Daf-16) signaling pathways. Thickness of lines showing regulatory interactions or the size of the fonts indicate inferred (or predicted) strengths of the interactions or genes. Gray boxes or fonts indicate genes or factors that are not active under L2 or L2d conditions. Different regions of the *hbl-1* mRNA are marked with different colors as indicated in Figure 5.5A.

during the lengthened and bipotential L2d stage, which may be followed by the L3 stage of larval development or by dauer diapause. Then, regardless of the developmental trajectory (whether it is from L2 to L3, L2d to L3, or L2d to dauer), concomitant with the progression to the L3 stage HBL-1 is robustly turned OFF -- which is necessary to permit progression to L3 cell-fates.

Previous studies showed that *let-7*-family microRNAs are developmentally and environmentally regulated in *C. elegans*: *let-7*-family microRNAs are expressed at the L2 and later stages under favorable conditions (Abbott *et al.*, 2005), however, they are transcriptionally repressed at the L2 (more precisely, L2d) stage in response to dauer-inducing signals (Hammell, Karp and Ambros, 2009). LIN-46 acts in parallel with *let-7*-famiy microRNAs in controlling L2 cell-fates (Pepper *et al.*, 2004). We found that under dauer-inducing conditions (e.g. the presence of population density pheromones) *lin-46* compensates for reduced *let-7*-family microRNA levels and post-translationally prevents ectopic HBL-1 activity, which otherwise results in retarded heterochronic phenotypes, including extra seam cell and gapped alae phenotypes (Figure 6.1).

In addition to *lin-46*, heterochronic genes *lin-4* and *nhl-2* are also involved in this compensatory arm of the L2d alternative heterochronic pathway. Together with previous findings, our results and observations favor a model wherein *lin-4* and *nhl-2* function in the L2d alternative heterochronic pathway not by directly

affecting HBL-1 activity but rather indirectly by modulating the activities of *let-7*-family microRNAs, or LIN-46, or both (Figure 5.5).

Lin-4 is a microRNA and can potentially regulate HBL-1 directly through a conserved complementary site in the *hbl-1* 3'UTR (Lin *et al.*, 2003; Karp and Ambros, 2012), however, the compensation pathway is still functional in *hbl-1* 3'UTR deletion mutants that no longer possess the *lin-4* complementary site (Figure 5.3). Therefore, our results do not support the model wherein *lin-4*-mediated repression of *hbl-1* mRNA compensates for reduced levels of *let-7*-family microRNAs. On the other hand, *lin-4* is known to regulate LIN-28 (Moss, Lee and Ambros, 1997), therefore it can indirectly regulate LIN-46 abundance and hence the compensation response. NHL-2 functions as a cofactor for the microRNA-induced silencing complex (miRISC) that modulates microRNA activity (Hammell *et al.*, 2009), indicating that NHL-2 can act by supporting or boosting the activities of *let-7*-family microRNAs as well as *lin-4* in the context of compensating against reduced levels of *let-7*-family microRNAs (Figure 5.5).

We call the overall response of the heterochronic pathway to dauer-inducing signals, which include the reduction in *let-7*-family microRNA levels and reliance, and the engagement of the compensatory *lin-46* pathway, as a "rewiring" response or *the L2d rewiring*. This rewiring is similar to previously described altered heterochronic pathway in post-dauer animals (Karp and Ambros, 2012), but importantly, here we show that the rewiring response that ultimately lead to the observation of an altered heterochronic pathway in post-dauer animals is initiated

before dauer commitment and by dauer-inductive signals. Of note, we observe that the strength of this rewiring correlates with the strength of the dauer/L2d-inducing signals, suggesting that environmental stress signals modulate the rewiring response. This ability of dauer-inductive signals, and conserved signaling pathways, TGF-β and insulin, to modulate temporal cell-fate programs in *C. elegans* raises the exciting possibility of similar roles for these signaling pathways in engaging similar cell-fate programs in humans (Antebi, 2019), potentially affecting cell identities in various settings by regulating *let-7*-family microRNAs or their targets via controlling alternative gene regulatory pathways, which may or may not involve a homolog of *lin-46* (Gephyrin), *lin-4*, or *nhl-2* (Trim-NHL family proteins)

After dauer commitment, the rewiring of the heterochronic pathway is augmented or completed: *let-7*-family microRNAs are no longer required (they become decoupled from HBL-1 regulation) whereas the reliance on *lin-46* is further increased. To distinguish the rewiring event --both already known and yet unknown -- occurring before and after dauer commitment, we call the totality of the changes to the heterochronic pathway occurring before and after dauer commitment as the L2d rewiring, and the post-dauer rewiring, respectively. Importantly, although the effects of the post-dauer rewiring on heterochronic phenotypes are stronger than that of the L2d rewiring, the L2d rewiring and post-dauer rewiring mechanisms involve the same set of genes -- at least so far -- and similar changes in their roles in controlling L2 to L3 cell-fate

transitions, suggesting that the post-dauer rewiring might merely be an enhancement of the L2d rewiring, which presumably invariably precedes dauer commitment and dauer formation.

Because going through L2d is much faster and it involves fewer steps than dauer formation and recovery, elucidating genetic programs involved in coordinating cell-fate progression with developmental trajectory is easier by investigating the L2d rewiring rather than the post-dauer rewiring. For example, the L2d rewiring is more compatible with functional tests using RNAi. This allowed us to perform a small-scale (~60 genes), pilot RNAi screen to determine whether potential L2d-activated genes were required for the L2d rewiring (Figure 5.4). We found that two autophagy related genes, *lgg-1* and *atg-18*, and a DEAD-box helicase gene, *vbh-1,* were required for the L2d rewiring, indicated by the loss of suppression of heterochronic phenotypes of *daf-12(rh61)* in the L2d-inducing *daf-7(e1372)* background. And, importantly, RNAi knock-down of *lgg-1* and *vbh-1* resulted in retarded heterochronic defects under L2d-inducing conditions but not under normal conditions, suggesting that *lgg-1* and *vbh-1* are integrated into the heterochronic pathway only under L2d-inducing conditions.

In this pilot screen, for each RNAi clone, 20 worms were analyzed under an inverted microscope for the number of seam cells at young adult stage. Although labor-intensive and time-consuming, expanding this screen to several hundred *C. elegans* genes (for example, certain classes of genes such as nuclear hormone receptors, F-box proteins, or RNA helicases) seems feasible and would potentially

reveal new factors and pathways regulating temporal cell-fates under crowding or starvation stress.

A comprehensive screen of all *C. elegans* genes (perhaps a forward genetic screen), however, would require a different, more high-throughput, approach in terms of determining changes in the number of seam cell -- which appears to be the most direct and sensitive way to assess (abnormally prolonged) HBL-1 activity. Developing brighter fluorescent reporters that mark the seam cells would allow or facilitate screening of worms for extra seam cells under a dissecting microscope. Also, in order to be able to readily distinguish between genes regulating HBL-1 under normal conditions versus genes regulating HBL-1 specifically under L2d conditions (such as *lgg-1* and *vbh-1*), it would be ideal to perform the genetic screens using worm strains that are maintained heterozygous for L2d-inducing *daf-7* or *daf-2* partial loss of function alleles.

The nuclear hormone receptor, DAF-12, is central to the coordination of temporal cell-fates with larval stage progression. Dauer-promoting, unliganded, DAF-12 represses the transcription of *let-7*-family microRNAs, which is an important aspect of the L2d rewiring response. During L2d, reduced *let-7*-family levels are thought to permit proper DAF-12 accumulation for optimal dauer decision (Hammell, Karp and Ambros, 2009) as well as prolonging HBL-1 expression (Ilbay and Ambros, 2019a), perhaps to prevent precocious cell-fate transitions during L2d. Under dauer/L2d-inducing conditions, d*af-12* is also required for the activation of the compensatory *lin-46* pathway that is responsible

for suppressing the retarded heterochronic phenotypes caused by insufficient expression of *let-7*-family microRNAs (Figure 2.6G). Therefore, I hypothesize that DAF-12 directly or indirectly regulates *lin-46*, coordinating the reduction of *let-7*-family levels with a compensatory increase in LIN-46 level and/or activity.

A model wherein DAF-12 coordinates *let-7*-family microRNAs with LIN-46-mediated compensatory pathway is compatible with all our finding and observations and may include the following steps. Because DAF-12 is a transcription factor, it could regulate *lin-46* transcriptionally (Figure 6.1). Namely, at the L2 stage while repressing the promoters of *let-7*-family microRNAs, DAF-12 could also activate the promoter of *lin-46*, increasing the rate of *lin-46* mRNA accumulation. The *lin-46* mRNA is repressed until the L3 stage (by LIN-28) therefore it can accumulate to varying levels at the L2/L2d stage without any danger of being translate -- which, otherwise, would precociously inhibit HBL-1 activity. At the L3 stage, however, the amount of the L2/L2d stage accumulated *lin-46* mRNA would determine the rapidity of LIN-46 protein accumulation. Accordingly, if *the lin-46* mRNA accumulates at a high enough level at the L2/L2d stage, then LIN-46 protein can accumulate fast enough at the L3 stage to reach the critical level to inhibit HBL-1 activity and hence the inappropriate execution of L2 cell-fates due to ectopic HBL-1 activity at the early L3 stage (Figure 6.2).

Our preliminary observations suggest that the L2d rewiring may indeed include a potential regulation of the rate of LIN-46 accumulation at the early L3

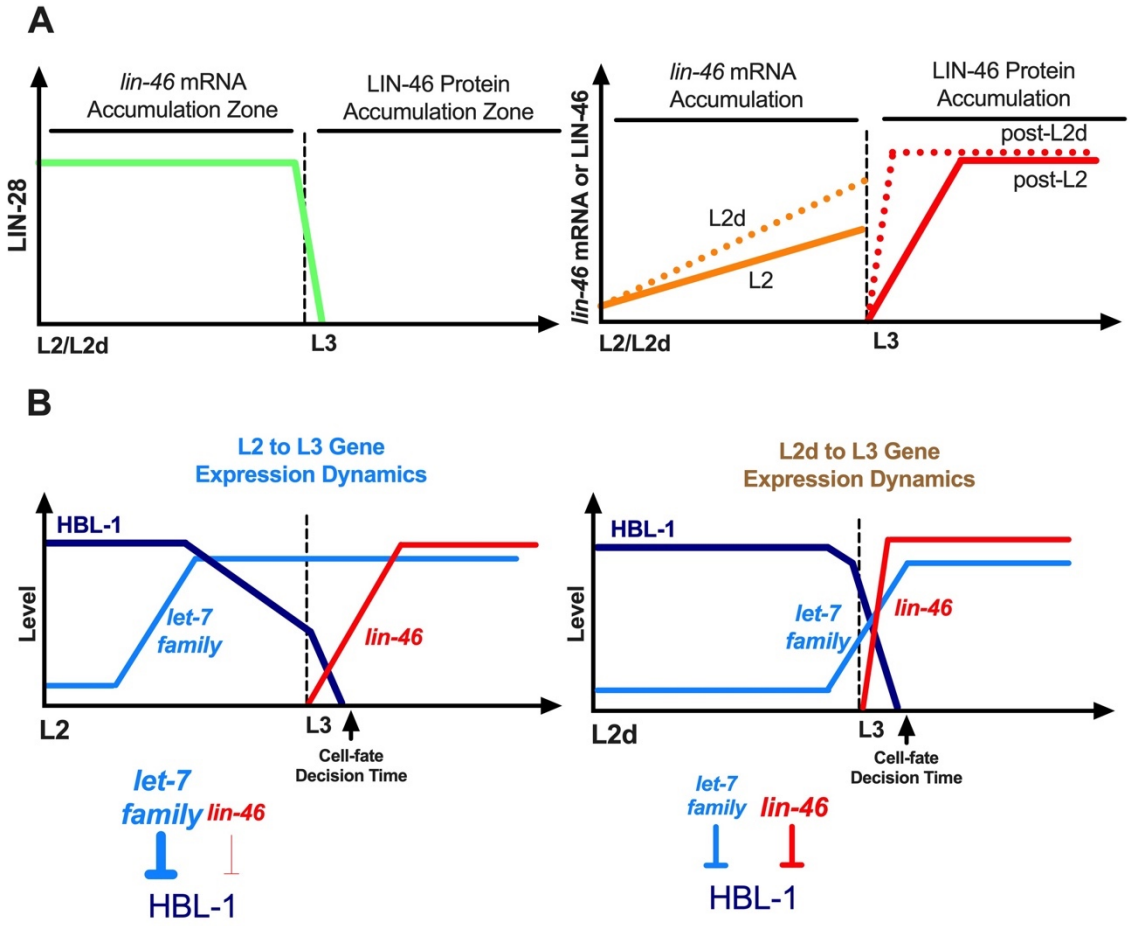
A
lin-46 mRNA Accumulation Zone
LIN-46 Protein Accumulation Zone
LIN-28
L2/L2d
L3
lin-46 mRNA Accumulation
LIN-46 Protein Accumulation
lin-46 mRNA or LIN-46
post-L2d
post-L2
L2d
L2
L2/L2d
L3
B
L2 to L3 Gene Expression Dynamics
HBL-1
Level
let-7 family
lin-46
L2
L3
Cell-fate Decision Time
let-7 family
lin-46
HBL-1
L2d to L3 Gene Expression Dynamics
HBL-1
Level
let-7 family
lin-46
L2d
L3
Cell-fate Decision Time
let-7 family
lin-46
HBL-1

Figure 6.2. Models of gene expression dynamics at the L2/L2d and L3 stages illustrating the potential regulation of LIN-46 accumulation and hence HBL-1 activity in early L3 animals.

(A) LIN-28 (protein) and *lin-46* mRNA are expressed at the L2/L2d stage and the LIN-28 represses *lin-46* mRNA. We hypothesize that *lin-46* mRNA accumulation may be regulated during L2/L2d to control the rate of LIN-46 accumulation at the L3 stage. (B) LIN-46 could accumulate faster in the post-L2d L3 animals compared to post-L2 L3 animals, which would inhibit HBL-1 before the cell-fates are executed thereby preventing reiteration of L2 cell-fates.

stage. When LIN-46 protein levels in early post-L2 L3 versus post-L2d L3 larvae are compared, LIN-46 appears to be higher in post-L2d L3 animals. These experiments were, however, designed to gain preliminary insights, so, they should be repeated in a more thorough manner, using more sensitive tools, and perhaps several time points spanning early to mid-L3 stage. *lin-46* tagged with mCherry at its endogenous locus produces visible but dim fluorescent signal, therefore, brighter fluorescent tags, such as mScarlet-I or tagging *lin-46* with multiple copies of a fluorescent protein (e.g. two mScarlet-I tags in tandem) should help increase the sensitivity of the assays.

In addition to LIN-46 protein levels, because of the uncoupling of *lin-46* mRNA expression and LIN-46 protein expression by LIN-28, investigating the *lin-46* mRNA accumulation pattern in the L2 versus L2d larvae would help determine if the differences in the *lin-46* mRNA levels at the end of L2 versus L2d stage could underlie the differences in LIN-46 protein levels at the early L3 stage in post-L2 versus post-L2d animals. Finally, if the *lin-46* mRNA accumulation is different under L2d-inducing conditions, then the potential role of DAF-12 in regulating *lin-46* expression during L2d should be investigated.

Unliganded DAF-12, which promotes L2d and dauer formation, could directly promote *lin-46* mRNA accumulation, or alternatively, indirectly affect LIN-46 protein levels by positively regulating *lin-4* or negatively regulating the expression of *lin-28*. However, *lin-4* levels in L2 versus L2d larvae [albeit assessed in a *daf-12(rh61)* background] does not seem to change (Figure 2.4); and

preliminary analysis of LIN-28 expression suggests that LIN-28 levels are comparable in L2 versus L2d larvae. Although, it should be note that the assays used to quantify LIN-28 levels weren't sensitive enough to detect subtle (less then 2-fold) or dynamic (less than 4 hours) changes, which appear to matter: half-dose of the *lin-28* gene is observed to suppress the heterochronic defects of *daf-12(rh61)*. Lastly, we cannot rule out that DAF-12 could regulate LIN-46 indirectly, provided that this indirect regulation also does not involve changes in the levels of *lin-4* and LIN-28.

We found that new *let-7*-family members, *mir-793, mir-794,* and *mir-795*, act redundantly with *mir-48* and *mir-241* to regulate L2-to-L3 cell-fates. Interestingly, when all seven members were investigated in the context of L2 versus L2d trajectory in the *daf-12(rh61)* background, we found that the reliance on different members of the *let-7*-family for proper progression to L3 cell-fates during the L2d trajectory is different than during the L2 trajectory. For example, *mir-48,* which is the major regulator of the L2-to-L3 cell-fate transitions under normal (L2) conditions, is not as important as *mir-84* or *mir-793* under L2d conditions in *daf-12(rh61)* background. To be sure, the already altered levels of *let-7*-family members in the *daf-12(rh61)* background (Hammell, Karp and Ambros, 2009) makes it harder to interpret these results, but one reason for the increased importance for *mir-84* and *mir-793* under L2d-inducing conditions might be member-specific regulation of certain mRNA targets that are related to L2d rewiring, such as the TGF-β or insulin signaling factors, *daf-7* or *daf-2,* or the

heterochronic genes *lin-28* or *lin-14.* These factors might be upregulated specifically in the mutants of *mir-84* or *mir-793* opposing the effects of L2d-inducing conditions. Similarly, *lin-46* might be regulated specifically by *mir-48* but not by other family members, and higher levels of LIN-46 in *mir-48*-null animals may potentiate the effects of L2d-inducing signals, resulting in efficient suppression of retarded heterochronic phenotypes under L2d-inducing conditions.

The widely conserved member of the *let-7*-family, the *let-7* microRNA, is expressed late during larval development and it controls larval to adult cell-fate transitions. *Caenorhabditis*-specific *let-7* paralogs, *mir-48/84/241,* are expressed earlier, by the L2 stage, and those paralogs redundantly regulate HBL-1, a key regulator of L2 cell-fates. The evolutionarily younger, and, also more recently identified paralogs, *mir-793/794/795* are expressed at various stages and at lower levels (Kato *et al.*, 2009). However, we found that *mir-793/794/795* also, similar to *mir-48/84/241*, contribute to the regulation of the L2-to-L3 cell-fate transitions, which suggest that they presumably regulate *hbl-1*. In *C. elegans*, where (in which cells) each *let-7*-family member is expressed during larval development is not clear: tissue-specificity of microRNA expression is inferred from GFP expression driven by “microRNA promoters”, which are usually defined as up to 2 kb upstream DNA sequences. To understand the overlapping and distinct functions of the members of the *let-7*-family -- as well as other microRNAs in other families -- it is

critical to develop techniques to reliably identify spatiotemporal expression patterns of accumulation of mature microRNAs.

In *C. elegans,* there are more than 100 transcripts whose 3'UTRs contain at least one conserved *let-7*-complementary site. Only a few of these targets are experimentally validated, however, complementary site conservation appears to be a good predictor of microRNA targeting. Therefore, it would be safe to assume that many of these 100-plus targets would prove to be *bona fide* targets and be regulated by *let-7* and its paralogs. However, it is not known if all seven members of the *let-7* family regulate all target transcripts to a certain degree, or whether different *let-7* family members can regulate distinct sets of targets. It is likely that there are overlapping spatiotemporal expression domains where two or more *let-7* family microRNAs regulate co-expressed targets. It is also likely that there are distinct spatiotemporal domains where only one *let-7* family member regulates co-expressed target transcripts. Determining where, when, and by which members of the *let-7* family microRNAs, target transcripts are regulated would help better understand how temporal cell-fate and larval stage progression are coordinated and what roles *let-7* family microRNAs play in this coordination during *C. elegans* development.

Of special interest to the coordination of cell-fate progression and developmental trajectory in *C. elegans*, many "*daf*" (abnormal DAuer Formation) genes that play key roles in gene regulatory pathways controlling developmental trajectory, including *daf-12, daf-16, daf-9, daf-5, daf-4, daf-7, daf-1, daf-3, daf-*

2, are predicted *let-7* targets. Aside from *daf-12* (Hammell, Karp and Ambros, 2009), it is not known whether these *daf* genes are regulated by *let-7* family microRNAs, and, if so, it is not known how regulation of these genes by *let-7* family microRNAs might affect temporal cell-fates and/or developmental trajectory.

HBL-1 activity is required for executing L2 cell-fates, which occurs soon after the L1 molt at the early L2 or L2d stage. Therefore, one expectation could be that once L2 cell-fates are executed by the early L2/L2d stage, HBL-1 would be downregulated. However, we found that HBL-1 is present throughout the L2 or L2d stage and is robustly downregulated in early L3 larvae (Figure 2.9). The reason for a continuing HBL-1 expression during L2/L2d stage until the L2-to-L3 molt could be to prevent precocious cell-fate progression. For example, if HBL-1 was downregulated by mid L2 stage soon after the proper execution of L2 cell-fates, L3 or L4 larvae could execute cell-fates precociously (e.g. seam cell of L4 larva might terminally differentiate as they normally do at the adult stage).

The L2d rewiring provides insights into how the worms maintain HBL-1 expression throughout the L2 stage or the lengthened L2d stag -- the length of which correlates with the severity of the conditions -- and then turn off HBL-1 expression before the L3 stage to permit proper progression to L3 cell-fates. In terms of temporal downregulation of HBL-1, the role of *let-7*-family microRNAs is more prominent during L2, when the developmental progression is deterministic (unlike the L2d larva L2 larva does not have the option to arrest as dauer larva). L2 versus L2d trajectory decision is made at the end of the L1 stage; and if L2 is

elected (or L2d option is eliminated) rapid progression to L3 stage is anticipated. Therefore, in accordance with this anticipation of rapid development and the deterministic nature of the L2-to-L3 stage progression, the transcription of *let-7*-family microRNAs is activated by early L2 stage to initiate the *hbl-1* downregulation program of L2 stage (Figure 6.1).

MicroRNAs can inhibit the translation of target mRNAs, and depending on the configuration of the regulatory circuit, inhibition of translational can function to facilitate the downregulation of the target (Figure 1.1). However, the rate of downregulation will also be influenced by the rate of target protein degradation or the stability of the target protein. If the target protein is relatively stable, inhibition of translation by microRNAs may only marginally alter the temporal downregulation pattern. Thus, in such a case, target downregulation may be too slow: the concentration of the target protein may abnormally remain above a certain threshold when a critical developmental time point is reached.

let-7-family microRNAs, which are the major factors required for proper downregulation of HBL-1 during the L2 (duration of which is 7-8 hours at 25ºC), must be activated at an early enough (in accordance with the stability of HBL-1) time point to allow enough time for the degradation of the already synthesized HBL-1 before the L3 stage. This L2 mode of *hbl-1* regulation, which is heavily dependent on *let-7*-family microRNA, or translational repression, appear to be consistent with the rapid and deterministic nature of the L2 to L3 stage progression.

On the other hand, if, at the end of the L1 stage, L2d is elected, *let-7*-family activation and hence HBL-1 downregulation are postponed until the next trajectory decision (L3 versus dauer arrest). This decision is made at the end of the L2d stage, and if the larva elects reproductive development, there is less -- and perhaps not enough -- time to accumulate *let-7*-family microRNAs to effectively repress *hbl-1* translation and degrade the already synthesized HBL-1 before the new cell-fates are executed at the early L3 stage. Therefore, a post-translational regulator of HBL-1, LIN-46, comes into play to support the delayed microRNA-mediated repression of *hbl-1* translation by inhibiting the nuclear accumulation of the residual, already synthesized, HBL-1. Consequently, this residual HBL-1 at the L3 stage accumulates in the cytoplasm -- where it cannot run the transcriptional program of L2 cell-fates -- until it is degraded. Thus, this mode of *hbl-1* regulation, which relies on the cooperation between translational regulation exerted by microRNAs and posttranslational regulation exerted by LIN-46, is more suited to the dynamics of the bipotential L2d trajectory (Figure 6.1).

MicroRNAs have been associated with developmental robustness as well as developmental canalization (the evolutionary process of acquiring and/or increasing robustness of developmental traits). Several examples indicate that microRNAs confer robustness to gene regulatory networks and phenotypic outcomes (Ebert and Sharp, 2012; Pelaez and Carthew, 2012). It was also argued that microRNAs -- merely because they function to repress translation and reduce noise in gene expression in certain network circuits -- can increase the robustness

of some but not necessarily all network circuits (Bartel, 2018). In addition to robustness-conferring microRNAs, the core conserved biological processes have deconstraining properties that confer robustness to living systems (Kirschner and Gerhart, 1998).

In this study, we found that microRNAs collaborate with an ancient and widely conserved gene, *lin-46*, to regulate the developmental transcription factor, HBL-1. Together with the conserved signaling pathways (TGF-β and insulin), the nuclear hormone receptor DAF-12, accessory developmental timing genes *lgg-1* and *vbh-1*, the heterochronic circuit of L2 cell-fates that is comprised of *let-7*-family microRNAs and *lin-46* constitute a gene regulatory network that confers the necessary precision and robustness to *hbl-1* regulation (Figure 6.1).

In addition to *lgg-1* and *vbh-1*, other factors and/or pathways might also be involved in this robustness network, perhaps in order to integrate other environmental, physiological, or developmental signals into the heterochronic pathway. The current view of the gene regulatory network conferring robustness to *hbl-1* expression illustrates that robustness of key developmental genes can require the integration of various signals and utilization of diverse gene regulatory pathways controlling gene activities at different levels such as translational and post-translational. Therefore, our results indicate that developmental robustness mechanisms can be more complex than previous examples or models wherein robustness is conferred by a single gene, such as HSP90 (Queitsch, Sangster and Lindquist, 2002) or *mir-7* (Li *et al.*, 2009), or a gene class, such as microRNAs

(Ebert and Sharp, 2012; Pelaez and Carthew, 2012; Posadas and Carthew, 2014).

Heterochronic defects evidently reduce the fitness of the worms, in some cases substantially (Choi and Ambros, 2019). In other words, optimal fitness is linked to -- among other things -- executing temporal cell-fates properly. The expression of heterochronic transcription factors (TFs), LIN-14, HBL-1, and LIN-29, are necessary and sufficient for the execution of stage-specific cell-fates, therefore, failure to express or ectopically express these TFs results in skipping or reiterating stage-specific fates, respectively. On the other hand, unlike the cell-fate progression, stage progression is plastic or flexible: various environmental or nutritional signals accelerate/decelerate stage progression or induce programmed developmental arrests. Our results show that gene regulatory networks controlling temporal cell-fates are capable of robustly adapting to changes in the rate of stage progression, anchoring the expression of heterochronic TFs and hence specific cell-fates to specific stages.

Heterochronic and other developmental genes that affect organismal fitness are delicately regulated to ensure the correct dosing and spatiotemporal precision of gene products and hence to prevent deviations from optimal phenotypic outcomes. Gene regulatory networks controlling these critical developmental genes have been evolving (inventing and optimizing) mechanisms to buffer gene expression against environmental and physiological perturbations. These buffering or robustness mechanisms presumably promote the consistency

of organismal success in a broader range of conditions, potentially allowing evolving species to spread into new territories. Therefore, it may not be surprising to encounter complex robustness mechanisms especially in species that are geographically widespread (e.g. *C. elegans*) and are capable of living under diverse conditions.

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