11-15-2023

# Evolutionary conservation and times of action of heterochronic genes

Maria Ivanova
*Rowan University*

# EVOLUTIONARY CONSERVATION AND TIMES OF ACTION OF HETEROCHRONIC GENES

by

Maria Ivanova

A Dissertation

Submitted to the
Department of Molecular Cell Biology and Neuroscience
School of Translation Biomedical Engineering and Science
In partial fulfillment of the requirement
For the degree of
Doctor of Philosophy
at
Rowan University
September 15, 2023

Dissertation Chair: Sergei Borukhov, Ph.D., Associate Professor, Department of Cell Biology and Neuroscience

Committee Members:
Ronald E. Ellis, Ph.D., Professor, Department of Molecular Biology
Eric Moss, Ph.D., Associate Professor, Department of Molecular Biology
Michael Anikin, Ph.D., Assistant Professor, Department of Cell Biology & Neuroscience
Sal Caradonna, Ph. D., Chair, Department of Molecular Biology

**Acknowledgments**

I would like to thank all the wonderful people I worked with during the 6 years of my PhD research:

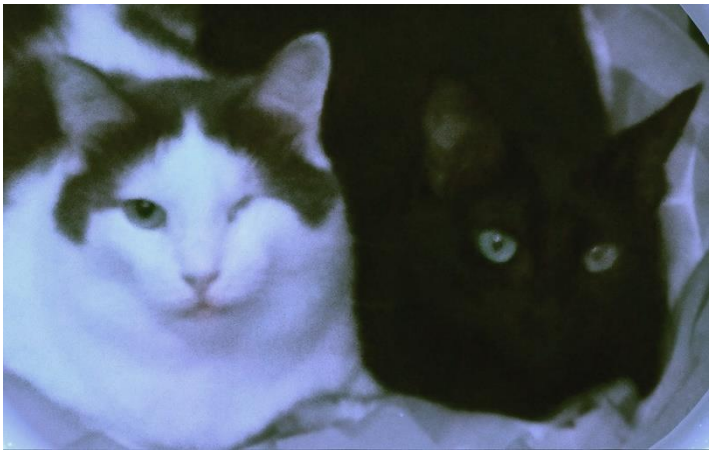
Dr. Moss for being a great mentor and giving me the opportunity to grow as a scientist.

Dr. Ellis for help, support, and thorough review of my texts.

My colleagues Madeleine Minutillo, Kevin Kemper, Jon Harbin, Satheeja Santhi Velayudhan and Youngquan Shen for answering all my questions and being amazing lab mates.

I would like to thank my parents and grandparents for believing in me and always being there for me even from far away.

I would like to thank my cats Bohr and Eris for filling my days with purrs and cheering me up at times of troubles. Sadly, Bohr left me in the middle of my PhD journey. I will always miss you, my best friend, and dedicate my work to you.



## Abstract

Maria Ivanova
EVOLUTIONARY CONSERVATION AND TIMES OF ACTION OF HETEROCHRONIC GENES
2023-2024
Sergei Borukhov, Ph.D.
Doctor of Philosophy

The heterochronic pathway of *C. elegans* is the most well-characterized system to date for controlling the sequence and timing of developmental events. However, we still have critical unanswered questions to address. First, little is known about the evolution of the heterochronic pathway, and of developmental timing in general. To determine if the roles of major heterochronic genes are conserved, I made mutants in orthologs of these genes in *C. briggsae*, using CRISPR/Cas9 revealing a significant drift in the roles of some of the genes. Although all of them are still involved in the developmental timing regulation, and several important interactions between the genes are conserved. Second, previous studies showed that *C. elegans lin-14*, a transcription factor controlling L1 and L2 developmental events, has two activities separated in time during development. To learn if other heterochronic genes also had separate activities that worked at different points in development, I applied a system of targeted protein degradation – the auxin-inducible degron system – to study the times of action for the core heterochronic genes *lin-28, hbl-1*, and *lin-41*. My results revealed that each *lin-28* and *hbl-1* had two activities separated in time as well, and that all these genes act in a temporal sequence that reflects their positions in the epistasis pathway. Thus, I deepened our understanding of when the *C. elegans* heterochronic genes act. This will improve our understanding of how developmental timing mechanisms work.

## Table of Contents

**Table of Contents (Continued)**

**Table of Contents (Continued)**

## List of Figures

## List of Figures (Continued)

Figure Page

## List of Figures (Continued)

Figure Page

## List of Tables

# Chapter 1

# Introduction

## Development

Development is one of the fundamental processes that allows the transformation of one cell into a complex multicellular organism. Understanding how development works is one of the major goals of modern science. Ultimately, we would like to model all the stages of development and the molecular interactions that underlie it, and be able to manipulate them when needed.

To achieve this goal, we need to understand developmental regulatory mechanisms. My work is focused on developmental timing, as it is regulated by the heterochronic pathway in nematodes (Rougvie and Moss, 2013). Our understanding of this process grew in a stepwise manner. First, a forward genetics approach was used to identify the genes that controlled this process. This approach involves inducing mutations with radiation, chemicals, or insertional mutagenesis, or using naturally occurring mutations. Then mutants with phenotypes of interest were identified, and the affected genes mapped, cloned sequenced. This step is largely complete for the heterochronic pathway in *C. elegans*.

Next, the interactions between genes were analyzed using epistasis, suppression, and enhancement. To do this, mutants were crossed to generate lines that carry mutations in two or more genes. Such lines can have phenotypes resembling one of the single mutants (epistasis), the phenotype can return to the wild type (suppression) or become more

severe (enhancement). This process helped determine the order in which heterochronic genes act in *C. elegans.*

We have now reached the stage where comparative (or evolutionary) analysis is possible. To do this, we need to study mutations similar to those generated in the original organism in related species, to understand how the structure of this regulatory pathway might have changed during evolution. This had not yet been done for the heterochronic pathway of *C. elegans*.

***Caenorhabditis elegans***

*Caenorhabditis elegans* is well suited for studying developmental timing. It is easy to maintain in the laboratory, has a short life cycle (developing from a single cell embryo to an adult in less than 70 hours), and is transparent, so all its cells can be observed, and their fates traced.

The *C. elegans* life cycle consists of embryogenesis, four larval stages, and adulthood (Fig. 1.1). The larval stages are separated by periods of lethargus and subsequent molts. In certain environmental conditions (lack of food, overcrowding, or high temperature) L1 and L2 larvae of *C. elegans* reprogram their development to enter the dauer pathway. Dauers do not eat, are extremely resistant to stress and can survive for months. When growing conditions return to optimal, dauers continue development through the L4 stage to adult.

There is a fixed number of somatic cells in *C. elegans*; hermaphrodites have exactly 959 cells and males have 1033. Post-embryonic cell lineages were described by Sulston and

Horvitz in 1977 and the entire embryonic cell lineage was described by Sulston et al., 1983.

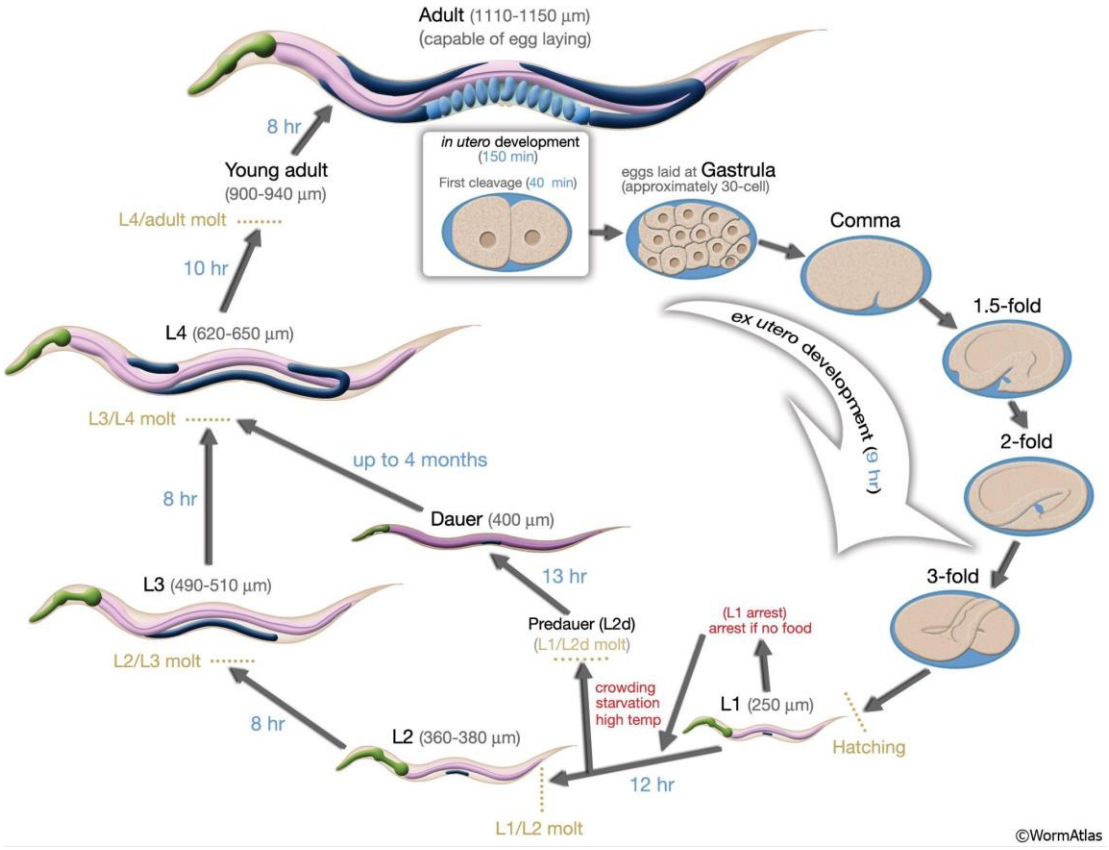



*Figure 1.1.* The life cycle of *C. elegans* at 22ºC. Blue numbers along the arrows indicate the length of each larval stage. The size of worms in micrometers (µm) at each stage is indicated in parentheses above the stage. Unfavorable conditions lead to an alternative developmental stage known as the dauer larvae, which specializes in dispersal and can survive without food. (Figure courtesy of WormAtlas).

## Developmental Pathways in *C. elegans*

Proper development must be orchestrated in space and time. For species that have different sexes, it also requires systems that determine the sex of an individual and modify development accordingly. Some well-studied developmental pathways of *C. elegans* include homeobox-regulated neurogenesis and body plan patterning (Aboobaker and Blaxter, 2003; Reilly et al., 2020), sex determination (Ellis, 2022), and the heterochronic pathway (Rougvie and Moss, 2013).

The sex-determination pathway attracted scientists' attention because it could provide insights into the genetic regulation of cell fate. It was one of the first developmental processes of *C. elegans* that was dissected with genetic analysis. The first mutants that caused incorrect sex determination were discovered in the 1970s in ethyl methanesulfonate mutagenesis screens (Hodgkin and Brenner, 1977; Klass et al., 1976). Then, epistatic relationships between those genes were studied and the structure of the sex determination pathway was deciphered (Hodgkin, 1987, 1980). Comparative analysis of sex determination pathways in *C. elegans* and its close relatives *C. briggsae* and *C. remanei* revealed additional features of the pathway and evolutionary trends. For example, pleiotropic proteins like pre-mRNA splicing factors and GLD-1 tend to be more conserved in sequence, and rapidly changing proteins, nevertheless, preserve interactions with their partners, like TRA-1/TRA-2ic and FEM-3/TRA-2ic (Haag, 2005). Finally, some genes appear *de novo* in different species, like *fog-2* in *C. elegans.* Comparative studies can reveal crucial and dispensable interactions within the pathway that improve our understanding of the system's function.

**Heterochronic Pathway**

The heterochronic pathway is one of the best studied systems for controlling developmental timing. Heterochronic genes act in a sequence to regulate patterns of development specific to each of the four larval stages in *C. elegans*. They are best known for their control of lateral hypodermal seam cell fates, although some of them have other, pleiotropic effects.

Lateral hypodermal seam cells are blast cells located on each side of the worm. They undergo reiterative divisions at each larval stage, during which one of the daughter cells joins the hypodermal syncytium and another remains at the seam and divides at the next stage. These divisions are all asymmetric. However, some seam cells (H1, V1-V4, and V6) undergo additional symmetric divisions at the beginning of the L2 stage, in which both daughter cells stay at the seam and retain blast cell fates (Fig. 1.2). The pattern of H1 divisions is slightly different—one of the daughter cells that stay at the seam after the L2 divisions does not undergo further divisions. The pattern of seam cell divisions resembles that of stem cells, where one daughter cell is directed to differentiation and another retains a stem cell fate. Thus, seam cells provide a model for learning how a cell knows what developmental program to use.

The number of seam cells increases because of the L2 symmetric divisions. L1 larvae have 10 seam cells on each side, and adult worms have 16. At the end of development, seam cells differentiate and form the cuticle structures known as adult alae (Sulston and Horvitz, 1977).

Mutations in heterochronic genes can cause developmental events associated with certain larval stages to be skipped or reiterated in some tissues, although other tissues retain normal developmental timing. When developmental events are skipped, later developmental programs are executed during earlier larval stages, and the phenotype is called precocious. But when early developmental events are reiterated during later stages, the phenotype is called retarded (Abbott et al., 2005; Ambros and Horvitz, 1987, 1984; Chalfie et al., 1981).

Some heterochronic genes encode proteins and others make miRNAs. The proteins are usually expressed at the beginning of development, and each of their levels drops at a specific larval stage. By contrast, the expression of the miRNAs increases as development proceeds (Fig. 1.3). These miRNAs silence the expression of mRNAs needed to produce heterochronic proteins (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Olsen and Ambros, 1999). They do this by targeting the 3'-UTRs of the transcripts (Ha et al., 1996).

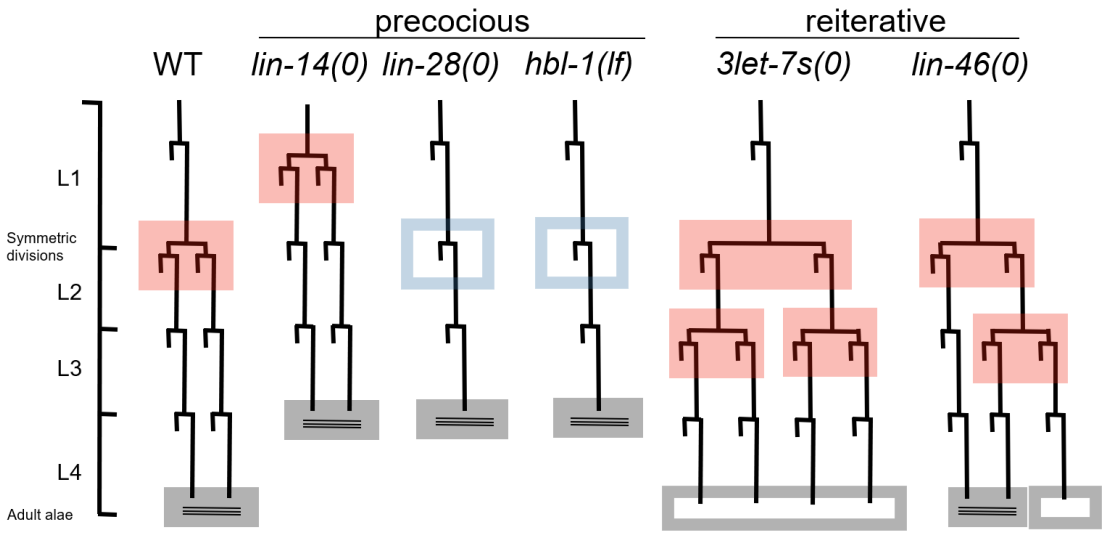



*Figure 1.2.* Heterochronic lateral hypodermal seam cells lineages in *C. elegans***.** The first diagram (WT) shows the wild-type cell lineage pattern of the V1-V4 and V6 seam cells in a *C. elegans* hermaphrodite. Vertical lines indicate the passage of time, and horizontal lines indicate a cell division. At the L1, L3, and L4 stages the seam cells divide asymmetrically, so that one daughter cell remains at the seam and keeps dividing and the other joins the hypodermal syncytium. A red box marks the symmetric division that normally occurs in the L2 stage, in which both daughter cells remain at the seam and later divide. A gray box indicates the final differentiation of seam cells and formation of adult alae. To the right, seam cell lineages for three precocious mutants and two reiterative mutants are shown. Precocious mutants skip certain stages and the seam cell differentiation occurs one stage earlier; those mutants that skip the symmetric divisions (indicated by a blue open box) generate a reduced number of seam cells. Reiterative mutants repeat certain larval stages. Their seam cells might fail to differentiate at the end of development and not form adult alae (indicated by an open gray box). If some seam

cells differentiate and some do not, the alae appear gapped. Reiteration of symmetric divisions causes an increase in seam cell numbers.

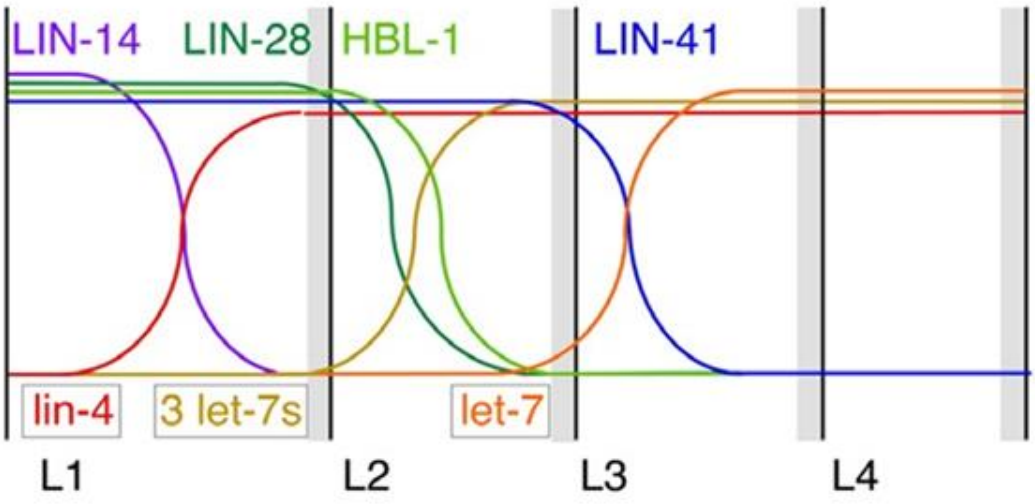



*Figure 1.3.* The pattern of heterochronic gene expression. The diagram shows relative expression levels of heterochronic genes during development. The genes encoding for proteins (top) are expressed in the beginning of the development and become silenced at different stages. The expression of miRNA genes is low in the beginning, and each increases at a specific larval stage. (Rougvie and Moss, 2013).

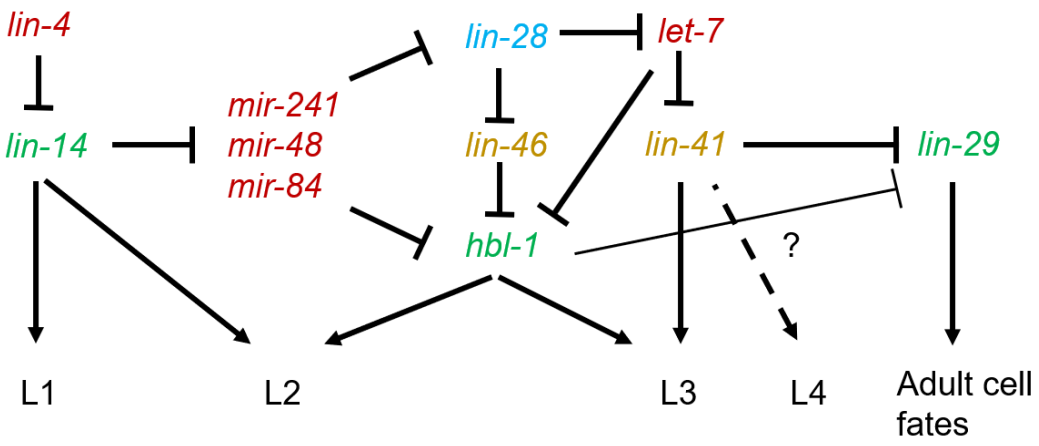



*Figure 1.4.* The *C. elegans* heterochronic pathway. The scheme shows the regulatory relationships between heterochronic genes and the stages that they regulate. Dark red indicates miRNAs, green - transcription factors, yellow - proteins that interact with other proteins, blue - an RNA-binding protein. Arrows indicate positive regulation, and flats indicate negative regulation.

**Genes that Act at the L1 Stage**

The *lin-4* gene was the first miRNA and heterochronic gene to be identified. Mutations in *lin-4* cause reiterations of L1 developmental events at later stages, and also cause extra molts. At adulthood, *lin-4* mutants lack adult alae and are vulvaless (Chalfie et al., 1981). The *lin-4* miRNA works by negatively regulating *lin-14* expression, but there are also binding sites in the 3'-UTR of *lin-28* (Wightman et al., 1991, 1993; Moss et al., 1997). It is highly conserved in *Caenorhabditis* and *lin-4* sequences from *C. briggsae* and some other species can rescue the *C. elegans lin-4(0)* phenotype (Lee et al., 1993).

LIN-14 is a transcription factor localized in the nucleus. It has no orthologs in animals other than nematodes. However, recent studies of the predicted folded structure of LIN-14 showed its similarity to the crystal structure of BEN domain proteins that are present in other animals, including fruit flies and mammals (Greene et al., 2023). *lin-14* encodes 3 isoforms that all share the only known functional domain of LIN-14–an amphipathic helix near its carboxyl terminus (Hong et al., 2000). Its expression peaks during the L1 stage in various tissues and completely fades after the L2 (Ruvkun and Giusto, 1989). *lin-14* controls a variety of developmental processes that include lateral hypodermal seam cell divisions and differentiation, and vulval development (Ambros and Horvitz, 1984; Euling and Ambros, 1996). The mutations in *lin-14* that remove the protein activity cause the skipping of L1 seam cell fates, the formation of precocious alae, and a protruding vulva. By contrast, deletions in the gene's 3'-UTR can cause reiterations of cell division patterns characteristic of the L1 and L2 stages. Experiments with temperature-sensitive alleles of *lin-14* showed that it had two activities separated in time, named *lin-14a* and *lin-14b*. The absence of only *lin-14a* activity caused an execution of L2 developmental programs at the L1 stage, whereas further development remained normal. The absence of only *lin-14b* activity caused a skipping of L2 developmental events rather than those of the L1. So far, the molecular mechanisms that distinguish these two activities remain unknown (Ambros and Horvitz, 1987).

**Genes that Act at the L2 Stage**

LIN-14 inhibits the expression of three *let-7* family miRNAs: *mir-241*, *mir-48*, and *mir-84*. These miRNAs, also called the *3-let-7s*, regulate L2 to L3 developmental transitions by downregulating the heterochronic genes *lin-28* and *hbl-1* (Abbott et al., 2005;

Tsialikas et al., 2017). The *3-let-7s* act redundantly, because removing only one of them does not cause significant defects. However, removing all three causes reiterations of L2 and L3 cell fates at later stages, and the absence of adult alae.

LIN-28 is a highly conserved RNA-binding protein that is present in the majority of clades in the animal kingdom, including humans (Moss and Tang, 2003). Its function is provided by a cold-shock domain and retroviral-type zinc-fingers. LIN-28 is localized in the cytoplasm and its expression peaks at the L1 stage, but then decreases and is not detectable after the L2 stage (Moss et al., 1997). LIN-28 has two mechanisms of action. First, it binds to the 5'-UTR of *lin-46*, inhibiting its expression, and second, it binds the *let-7* precursor preventing its maturation (Van Wynsberghe et al., 2011; Vadla et al., 2012; Ilbay et al., 2021). Mutations in *lin-28* cause the skipping of L2 developmental events and the precocious execution of L3 and L4 events. Sometimes *lin-28* mutants skip two stages and the precocious differentiation of seam cells occurs at the L2 molt (Ambros and Horvitz, 1984). Finally, *lin-28* positively regulates *lin-14* via an unclear mechanism while LIN-14 inhibits the transcription of *lin-28* suppressors (Arasu et al., 1991, Moss et al., 1997, Tsialikas et al., 2017). Thus, there is a positive feedback loop regulation between *lin-14* and *lin-28*.

LIN-46 is an unusual dimeric protein with homology to C-terminal domains of mammalian scaffolding protein gephryn. It was discovered as a suppressor of the heterochronic defects in *lin-28* mutants. LIN-46 is localized in the nuclei and cytoplasm of hypodermal cells, and its levels peak around each molt. LIN-46 is also constitutively expressed in AVB neurons. Although the function of LIN-46 during molts and in neurons remains unclear. The loss of LIN-46 function causes gaps in adult alae in around 32% of

mutant worms at 20ºC. Furthermore, an increased number of seam cells was observed in *lin-46(lf)* mutants at 15ºC. Experiments with temperature sensitive alleles showed that LIN-46 acts during the L2 stage to promote earlier and later defects (Pepper et al., 2004). It is likely that LIN-46 acts by preventing the nuclear accumulation of HBL-1 (Ilbay and Ambros, 2019).

HBL-1 is a zinc-finger transcription factor that is expressed in ectodermal cells during embryonic and larval development. HBL-1 plays an important role in embryonic development and also promotes L2 to L3 cell-fate transitions. Depletion of HBL-1 causes embryonic lethality and precocious development (Fay et al., 1999; Abrahante et al., 2003; Abbott et al., 2005).

**Genes that Act at the L3 and L4 Stages and the Transition to Adult Stage**

*let-7* is a highly conserved miRNA. Lack of *let-7* prevents the formation of adult alae and causes extra molts in worms, while its overexpression causes the precocious differentiation of seam cells (Reinhart et al., 2000). Similar to *lin-28*, it is found in the majority of animal species (Pasquinelli et al., 2000). The relationship between these two genes is also conserved: the downregulation of *lin-28* by *let-7* is associated with stem cell differentiation and cancer cell proliferation in mammals (Newman et al., 2008; Rybak et al., 2008; Pan et al., 2011). In *C. elegans*, the expression of *let-7* is robust at the L4 and adult stages (Abbott et al., 2005; Johnson et al., 2003). *let-7* promotes larval to adult cell fate transitions by downregulating *lin-41* and *hbl-1* (Reinhart et al., 2000; Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003), and is itself negatively regulated by HBL-1 in a feedback loop (Roush and Slack, 2009).

LIN-41 is a member of the RBCC family of proteins, which are believed to participate in protein-protein interactions. This gene is also found in other animals and is associated with stem cells (Rybak et al., 2009; Cuevas et al., 2015). In *C. elegans*, LIN-41 acts downstream of *let-7* and prevents the transition to adult cell fates. It is localized in the cytoplasm and constitutively expressed in many tissues; however, in the hypodermis its expression ceases during the L4 stage. Mutations that remove LIN-41 activity result in a precocious differentiation of seam cells and some other defects unrelated to the developmental timing, including a dumpy phenotype and sterility (Slack et al., 2000).

At the end of the development, seam cells undergo a final transition to the adult state. This transition is regulated by a zinc-finger transcription factor LIN-29. The heterochronic pathway prevents LIN-29 from premature accumulation until the late L4 stage (Rougvie and Ambros, 1995; Bettinger et al., 1996). *lin-29* is negatively regulated by LIN-41 and HBL-1 (Slack et al., 2000; Abrahante et al., 2003). Moreover, LIN-29 exists in two isoforms, LIN-29a and LIN-29b with slightly different functions, and HBL-1 and LIN-41 respectively negatively regulate these different isoforms (Azzi et al., 2020).

**Times of Actions of Heterochronic Genes**

Heterochronic genes act in a sequence to specify each of the four larval stages. Multiple methods including GFP fusions, Northern blots, Immunofluorescence, and RT-PCR were used to determine when heterochronic genes are expressed (Abbott et al., 2005; Bettinger et al., 1996; Johnson et al., 2003; Moss et al., 1997; Tsialikas et al., 2017). It was found that some genes are expressed early in larval development and then down-regulated, whereas others are initially upregulated at later stages. However, the time of expression

alone does not indicate when genes are active nor how many separate activities they have.

Experiments with a temperature-sensitive allele of *lin-14* showed that it has two activities whose functions are separated in time (Ambros and Horvitz, 1987). Determining if other heterochronic genes—*lin-28, hbl-1,* and *lin-41*—also have dual functions would improve our understanding of developmental timing control. These times of actions can be found using a method for turning genes on or off at certain times in development and then observing the resulting phenotypes. Auxin-inducible degrons are ideal for this purpose.

**Evolution of Development**

Developmental systems evolve even if the resulting phenotype does not change. This happens because mutations that do not significantly alter fitness slowly accumulate over time, and their effects can be balanced by those of other random mutations. Thus, molecular factors might join or leave the pathway, change the way they interact with other molecules, or change the time or place they act. This process is called developmental systems drift (True and Haag, 2001).

Developmental systems drift causes an incompatibility between developmental programs of close species, so the hybrids might be inviable, sterile, or have a defect in traits that are identical in both parent species. For example, both *D. melanogaster* and *D. simulans* have thoracic bristles, but this trait is lost in hybrids (Takano, 1998, True and Haag, 2001).

Because of developmental systems drift, mutations in some genes can cause similar phenotypes but the mechanisms underlying those phenotypes turn out to be different. For

example, *lin-39* mutants of *C. elegans* and *P. pacificus* are both vulvaless, although in *C. elegans* vulval precursor cells fuse with the epidermis instead of forming vulval structures, whereas in *P. pacificus* the same cells undergo apoptosis (Sommer, 1997, True and Haag, 2001).

Thus, developmental systems drift causes diversity in conserved regulatory processes, and the structure of regulatory pathways cannot be inferred from the resulting phenotype. Sometimes studying the same pathway in two closely related species reveals conserved aspects of it that had not yet been discovered. I will consider two examples of how developmental pathways change during evolution.

First, the sex determination genes are known to rapidly change in sequence in nematodes, flies, and mammals (O'Neil and Belote, 1992; Tucker and Lundrigan, 1993; Whitfield et al., 1993; McAllister and McVean, 2000). The functions of some sex-determination genes differ in close species. For example, Sxl regulates sex determination in *D. melanogaster* but lacks this function in *M. domestica* even though the sequences of orthologs are highly conserved (Meise et al., 1998). In worms, *fem* genes are required for spermatogenesis in *C. elegans* but not in *C. briggsae* (Hill et al., 2006). Sometimes the interactions between proteins are conserved, but not their sequences. For example, TRA-1 and TRA-2 interact with each other in both *C. elegans* and *C. briggsae* but there is no cross-species interaction (Wang and Kimble, 2001).

Thus, neither the inability to rescue null mutants by homologs from a close species nor a high divergence of sequences proves that the structure of a developmental pathway or the interactions between genes of that pathway have changed. To understand the evolution of

developmental systems, it is necessary to study the functions of proteins and pathway structures within each species first.

Gene sequences might change rapidly due to positive selection or because genes are functionally dispensable. The latter seems unlikely because sex-determination genes also influence reproduction rates and success. At the same time, there are several reasons why positive selection might be active in the sex-determination pathway. The presence of males changes the optimal timing of sperm-to-oocyte transition in hermaphrodites, intracellular factors like organelles and parasites that try to shift the sex ratio, or conflict between maternal-effect genes and zygotically expressing genes (Eberhard, 1980; Cosmides and Tooby, 1981; Hodgkin and Barnes, 1991; Werren and Hatcher, 2000; Stothard and Pilgrim, 2003).

Second, the Homeotic (Hox) genes represent another developmental system - the one that regulates the formation of the body plan in bilaterians. Clusters of homeobox genes are located along chromosomes in a specific order that is conserved across different animal species. Moreover, this order reflects the location of Hox gene activities along the anterior-posterior axis of the embryo. The addition, duplication or loss of Hox genes is tightly related to differences in the body morphology between species (Holland, 2013). The evolution of Hox genes in nematodes appears to be more rapid compared to other animals. Multiple cases of Hox gene loss were observed in different nematode groups, although canonical orthologous groups are preserved. Close relatives of nematodes, nematomorphs, have a similar body plan, however, they possess a full set of Hox genes (Aboobaker and Blaxter, 2003). The reason for the rapid evolution and frequent loss of Hox genes in nematodes remains unclear.

Overall, our understanding of the evolution of developmental pathways is in its early stages. There are few studies that compare multiple null alleles of orthologous regulatory genes from developmental pathways in related species. Furthermore, the evolution of the heterochronic pathway in nematodes had not been studied previously. Thus, discovering the functions of heterochronic genes in another *Caenorhabditis* species would build a base for understanding how developmental timing changes during evolution and would broaden our knowledge of developmental systems evolution in general.

### *Caenorhabditis briggsae*

*C. elegans* and *C. briggsae* are remarkably similar nematodes despite being separated evolutionarily by 5 to 30 million years (Cutter et al., 2008). They both occupy similar ecological niches and have nearly identical patterns of development, as revealed by cell lineage analyses (Zhao et al., 2008; Félix and Duveau, 2012). Although they are nearly indistinguishable anatomically, only 60% of their loci are clearly orthologous to each other (Stein et al., 2003). That divergence fits nicely with the fact that the rate of genomic rearrangements in Caenorhabditis is four times faster than in *Drosophila* (Coghlan and Wolfe, 2002), which suggests that developmental pathways might also evolve more rapidly.

A previous comparison of *C. elegans* and *C. briggsae* orthologs by RNA-interference (RNAi) showed that only a small fraction (91 of 1333 orthologs) have significantly different loss-of-function phenotypes in the two species (Verster et al., 2014). Some heterochronic gene orthologs were included in that study, although the analysis was not detailed, and more thorough studies focused specifically on heterochronic genes are required to understand the extent of their conservation in *C. elegans* and *C. briggsae.*

There are several valuable approaches for generating mutant phenotypes in *C. elegans* and other nematodes.

**CRISPR/Cas9**

CRISPR/Cas9 is a revolutionary gene-editing technology that allows scientists to precisely modify DNA sequences. CRISPRs (clustered regularly interspaced short palindromic repeats) were discovered in *E. coli* by Ishino et al (1987) and were initially used for genotyping different bacterial strains such as *M. tuberculosis* and *S. pyogenes* (Groenen et al., 1993; Hoe et al., 1999). In the middle of the 2000s, it was shown that CRISPR sequences were similar to bacteriophages and other infectious agents; a function of CRISPR as a bacterial immune system was suggested (Makarova et al., 2006; Mojica et al., 2005). Then it was experimentally shown that CRISPR sequences provided resistance against phages that had similar sequences in their genome (Barrangou et al., 2007). The CRISPR system works by taking a short length of DNA from the phage and inserting it into the bacterial genome as a spacer. If the bacterial cell becomes re-infected, the spacer is transcribed into a small RNA called a crRNA that forms a complex with CRISPR-associated (Cas) proteins, which then recognize the phage genome and stop it from replicating by cutting and destroying its DNA.

Bacteria and archaea have a variety of CRISPR-Cas systems with slightly different structures and mechanisms of action (Koonin and Makarova, 2022). The most widely used system nowadays is the CRISPR/Cas9 class 2 system. The first applications of CRISPR/Cas9 to genome editing in human and mouse cells were documented in 2013. Several modifications were introduced into the system: codons were optimized to efficiently express Cas9 in mammalian cells, nuclear localization signal was added to

Cas9, and the guide RNA (gRNA) was extended (Cho et al., 2013; Jinek et al., 2013). The group of George Church was the first to utilize homologous repair in mammalian cells following double-stranded DNA breaks introduced by CRISPR through the addition of repair templates to the CRISPR mix (Cong et al., 2013; Mali et al., 2013). Hwang et al (2013) were the first to use CRISPR in zebrafish, and they also demonstrated that CRISPR could be used to edit the genome in the germ line to generate mutant lines (Hwang et al., 2013). Around the same time, CRISPR/Cas9 was also adapted for use with *C. elegans* (Chen et al., 2013; Friedland et al., 2013; Waaijers et al., 2013).

CRISPR/Cas9 is more efficient than conventional methods such as transgenesis, microparticle bombardment, and Mos1 transposition that were used previously in *C. elegans* (Mello et al., 1991; Praitis et al., 2001; Robert and Bessereau, 2007; Frøkjær-Jensen et al., 2008). It targets the genome directly, allows for precise editing of sequences with low number of off-target effects, and eliminates the need for negative selection. It is sufficient to inject around 20 animals to isolate multiple mutant alleles (Paix et al., 2015).

**RNAi**

Another method for producing loss-of-function phenotypes is RNAi. Double-stranded RNA (dsRNA) introduced in the *C. elegans* cells is converted by endogenous systems into short interfering RNAs (siRNAs) that can silence gene transcription or cause the degradation of mRNAs that have complementary sequences (Meister and Tuschl, 2004; Grishok, 2005). The effect of RNAi can also be inherited and persist for several generations. *C. elegans* can be injected with dsRNA, fed, or soaked in it (Fire et al., 1998; Tabara et al., 1998; Timmons and Fire, 1998). Although RNAi usually does not produce null phenotypes, it does not require screening for mutations. This method is still valuable

if there is a need to analyze a range of loss-of-function phenotypes. It is especially useful if null alleles are lethal.

### Auxin-Inducible Degron System

In recent years, a system of targeted protein degradation—the auxin-inducible degron (AID) system has been adapted for use in *C. elegans* (Zhang et al., 2015). It requires the expression of modified *Arabidopsis thaliana* protein TIR-1 and the fusion of a target protein with an AID tag. Then, upon the addition of the plant hormone indole-3-acetic acid (IAA, auxin), TIR-1 binds the AID tag and recruits a ubiquitin-ligase complex that attaches ubiquitins to the tagged protein, targeting it for degradation. This system allows for conditional generation of loss-of-function phenotypes and is reversible. The major advantage of this system compared to RNAi is that the degradation occurs within an hour after the addition of auxin and the levels of constitutively expressed proteins can be restored in 24 hours after removal from auxin. However, some background degradation might occur even in the absence of auxin. Thus, a modified AID system was developed where phenylalanine 79 is replaced with glycine in the TIR-1 enzyme (Hills-Muckey et al., 2022). This modification eliminates background degradation and makes the system 10 times more sensitive to the auxin analog 5-Ph-IAA. This system can be used to inactivate proteins at specific times during development and identify their times of action.

### Rationale

The goal of my first project was to study the conservation of the roles of heterochronic genes. I chose *C. briggsae* as a close relative of *C. elegans*. It is also hermaphroditic and easy to work with, its genome has been sequenced, and it has orthologs of all the heterochronic genes. I chose CRISPR/Cas9 as the most effective approach to make

targeted mutations. In the case of *hbl-1,* I used RNAi and AID to study the loss-of-function phenotype because *hbl-1(0)* is embryonic lethal in *C. elegans*. I also built double mutants to study the conservation of interactions between heterochronic genes in *C. elegans* and *C. briggsae*. Based on the data, I was able to identify parts of the heterochronic pathway that are highly conserved, and parts that are undergoing evolutionary change.

The goal of my second project was to find the times of actions for several heterochronic genes in *C. elegans* - *lin-28, hbl-1,* and *lin-41*. I chose an auxin-inducible degron system that allows for rapid degradation of target proteins within an hour but also allows for reactivation of proteins in certain cases, when their expression is not down-regulated after the degradation. Using this system, I was able to identify time frames when the activities of these heterochronic genes are required for the regulation of developmental events specific to certain larval stages.

# Chapter 2

# Materials and Methods

## Sequence Analysis

*C. briggsae* orthologs of *C. elegans* heterochronic genes were identified previously in Wormbase (wormbase.org) and miRBase (mirbase.org) and were confirmed by reciprocal BLAST on each genome. Links to the database entry for each gene are given in Chapter 3, Table 3.1.

## Strains and Culture Conditions

Nematodes were grown at 20°C on standard NGM plates seeded with *E. coli* AMA1004 unless otherwise indicated.

## Strains Used

### *C. elegans Strains*

RG733 (*wIs78* [pDP#MM016B (*unc-119*) + pJS191 (*ajm-1::gfp* + pMF1(*scm::gfp*) + F58E10]) (wild type for this study),

HML1029 *cshIs140[rps-28pro::TIR1(F79G)_P2A mCherry-His-11; Cbr-unc-119(+)] LGII,*

ME502 *cshIs140[rps-28pro::TIR1(F79G)_P2A mCherry-His-11; Cbr-unc-119(+)] LGII; hbl-1(aeIs8[hbl-1::aid]); wIs78,*

ME504 *cshIs140[rps-28pro::TIR1(F79G)_P2A mCherry-His-11; Cbr-unc-119(+)] LGII; lin-41(aeIs10[lin-41::aid]); wIs78,*

ME506 *cshIs140[rps-28pro::TIR1(F79G)_P2A mCherry-His-11; Cbr-unc-119(+)] LGII; lin-28(aeIs10[lin-41::aid]); wIs78,*

ME507 *cshIs140[rps-28pro::TIR1(F79G)_P2A mCherry-His-11; Cbr-unc-119(+)] LGII; lin-14(aeIs5[lin-14::aid]); wIs78.*

***C. briggsae Strains***

AF16 (wild type),

ME421 *Cbr-lin-28(ae25),*

ME444 *Cbr-dpy-5(v234) +/+ Cbr-lin-28(ae35),*

ME449 *Cbr-lin-46(ae38),*

ME450 *Cbr-lin-28(ae39),*

ME451 *Cbr-lin-46(ae38); Cbr-lin-28(ae39),*

ME454 *Cbr-dpy-5 (v234) +/+ Cbr-lin-28(ae39),*

ME480 *Cbr-lin-46(ae43),*

ME482 *Cbr-lin-46(ae44),*

ME486 *Cbr-let-7(ae47),*

ME487 *Cbr-let-7(ae48),*

ME489 *Cbr-lin-14(ae51) Cbr-let-7(ae50),*

ME493 *Cel-lin-4(ae53),*

ME494 *Cbr-lin-28(ae39); Cbr-let-7(ae47),*

ME497 *Cbr-lin-4(ae54),*

ME500 *Cbr-lin-4(ae55),*

ME511 *Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]),*

ME514 *Cbr-lin-14(ae58),*

ME515 *Cbr-lin-14(ae59),*

ME519 *Cbr-dpy-8(v262) + Cbr-unc-7(v271)/+ Cbr-lin-14(ae62) Cbr-unc-7(v271),*

ME520 *Cbr-dpy-8(v262) + Cbr-unc-7(v271)/+ Cbr-lin-14(ae63) Cbr-unc-7(v271),*

ME526 *Cbr-mir-241(ae64),*

ME527 *Cbr-mir-48(ae65),*

ME529 *Cbr-mir-84(ae68),*

ME530 *Cbr-mir-84(ae69),*

ME531 *Cbr-mir-241(ae64); Cbr-mir-84(ae70),*

ME533 *Cbr-mir-241(ae64); Cbr-mir-84(ae69),*

ME534 *Cbr-lin-4(ae71); Cbr-dpy-8(v262) + Cbr-unc-7(v271)/+ Cbr-lin-14(ae62) Cbr-unc-7(v271),*

ME535 *Cbr-lin-4(ae72); Cbr-dpy-8(v262) + Cbr-unc-7(v271)/+ Cbr-lin-14(ae62) Cbr-unc-7(v271),*

ME538 *Cbr-mir-241 Cbr-mir-48(ae73),*

ME541 *Cbr-mir-241 Cbr-mir-48(ae73)/+; Cbr-mir-84(ae70),*

ME544 *Cbr-lin-41(ae76),*

ME545 *Cbr-spe-8 (v142) +/+ Cbr-lin-41(ae77),*

ME547 *Cbr-lin-41(aeIs14[Cbr-lin-41::aid]),*

ME548 *Cbr-trr-1(v76) +/+ Cbr-lin-29(ae75),*

ME549 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]; Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]),*

ME550 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)],*

ME552 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]; Cbr-lin-41(aeIs14[Cbr-lin-41::aid]),*

ME553 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]; Cbr-mir-241 Cbr-mir-48(ae73); Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]) Cbr-mir-84(ae70),*

ME554 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]; Cbr-lin-28(aeIs13[Cbr-lin-28::aid]); Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]),*

ME555 *aeEx44[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)],*

ME556 *aeEx45[Cbr-lin-28::gfp],*

ME558 *Cbr-lin-41(ae76); Cbr-let-7(ae48),*

ME559 *aeEx46[Cbr-lin-28::gfp(Y35R F37A C127R C137A)],*

ME561 *aeEx48[Cbr-lin-28::gfp(Y35R F37A C127R C137A) 3'-UTR deletion],*

ME562 *Cbr-lin-41(ae76); Cbr-lin-29(ae75)/+,*

ME563 *Cbr-lin-28(ae39); Cbr-lin-29(ae75)/+,*

ME564 *aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)] Cbr-lin-4(ae79); Cbr-mir-241 Cbr-mir-48(ae73); Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]) Cbr-mir-84 (ae70),*

ME565 *Cbr-lin-41(ae76); aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]; Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]),*

ME566 *Cbr-hbl-1(aeIs12[Cbr-hbl-1::aid]) Cbr-let-7(ae48); aeIs15[rps-28pro>atTIR1(F79G)::P2A::gfp::His-11; Cbr-unc-119(+)]*

ME567 *Cbr-lin-28(ae39); Cbr-mir-48(ae65)*

ME568 *Cbr-dpy-5(v234) +/+ Cbr-lin-28(ae80)*

**Development Synchronization**

To generate developmentally synchronized populations of worms, adults filled with eggs were washed from crowded plates into 15ml tubes, spinned down, the excess liquid then was removed leaving a worm pellet. 500-1000µl of household bleach solution was added to the tube and vortexed every 2 minutes until cuticles of worms were dissolved enough to release eggs. Tubes then were filled with sterilized distilled water to slow the bleach activity and centrifuged at 400g for 3 minutes, then the supernatant was discarded and

eggs were washed twice with sterilized distilled water and then twice with M9. Then eggs were transferred to plates with M9 and left on a shaker for 20-48 hours at room temperature. The liquid with larvae was centrifuged to concentrate larvae and they were transferred to plates with food in a small amount of M9.

**Microscopy**

Animals were examined using DIC and fluorescence microscopy on a Zeiss Axioplan2 microscope with Zeiss objectives: Plan-NEOFLUAR 5x, Plan-NEOFLUAR 16x, Plan-NEOFLUAR 63x, alpha Plan-FLUAR 100x. Images were acquired using AxioCam with AxioVision software.

**Seam Cell and Gonad Phenotype Scoring**

Fluorescent seam cell markers to facilitate the counting of seam cells are not yet available for *C. briggsae* as for *C. elegans*. Therefore, there was some variability in seam cell counts for animals of apparently identical age due to occasional difficulty distinguishing seam cells from lateral hypodermal nuclei by DIC microscopy. Seam cells were confidently identified when located along the lateral midline or slightly off and having a visible oval-shaped outline. Errors in counting may occur when seam cell nuclei or hypodermal syncytial nuclei lay away from or close to the apparent midline, respectively.

A disorganized gonad was scored if gonad or oocyte contents leaked into the pseudocoelom or oocytes or spermatozoa were found outside the gonad.

**CRISPR/Cas9**

We followed general protocols described in Paix *et al.* 2017. gRNA was synthesized from PCR-amplified templates using Invitrogen™ MEGAshortscript™ T7 Transcription

Kit (Catalog# AM1354) and purified with Invitrogen™ MEGAclear™ Transcription Clean-Up Kit (Catalog# AM1908). Purified gRNAs were mixed with Cas9 (EnGen® Spy Cas9 NLS, Catalog# M0646T) and used in microinjections. Typical concentrations of the components in the injection mix: gRNA (up to 200ng/µl), Cas9 (250 ng/µl), co-injection *Cbr-myo::GFP* plasmid (35 ng/µl).

When making insertions, a hybrid dsDNA repair template was used as described in (Dokshin *et al.* 2018). Repair templates were melted and cooled before injections (Ghanta and Mello 2020). The repair template then was added to the CRISPR mix to the final concentration of 100-500 ng/µl of DNA.

**RNAi**

RNA interference used to knockdown *Cbr-hbl-1* expression was performed as described (Hammell and Hannon 2016). Part of the *Cbr-hbl-1* ORF flanked by T7 promoters was amplified using these primers: 5'-GCGCGCTAATACGACTCACTATAGGTCCCAGCACCCCTACCACCAC-3', 5'-GCGCGCTAATACGACTCACTATAGTGGTGACGCCGGCTCTCCTTT-3'

RNA was synthesized and purified with the in-vitro transcription kit mentioned above. Purified RNA was diluted with sterile distilled water to 200 ng/µl concentration and injected into gonads.

**Auxin-Inducible Degron System**

We used a modified auxin-inducible system with *TIR1(F79G)*, using 5-Ph-IAA as the auxin analog (Zhang *et al.* 2015; Hills-Muckey *et al.* 2021). To express *TIR1*, wildtype *C. briggsae* were injected with a modified pCMH2074 plasmid (C. Hammell, pers. comm.)

containing *TIR1(F79G)* mutation and GFP in place of mCherry. Bright fluorescing animals carried a stable array with a high inheritance rate (50-75%) were selected and a strain with stable extrachromosomal expression was established (*aeEx44*).

To integrate *TIR1(F79G)* into the genome, fluorescent L4 and young adult animals were transferred to a plate without bacteria and irradiated with UV in an UV-crosslinker set to an energy level of 13 kJ/cmˆ2 to generate chromosomal breaks and attach the array to a chromosome. Fluorescent F1 animals were isolated and then plates were screened for 100 or 75% transmission rates or for animals displaying uniform (vs. mosaic) fluorescence. Stable integrants were identified as animals with uniform fluorescence with 100% penetrance. This strain then was outcrossed at least 3 times.

To assess phenotypes using AID system, animals carrying alleles with fused degron tag and expressing *TIR1(F79G)* were grown on plates containing 50µmol of 5-Ph-IAA that was spread on standard NGM plates to approximately 0.005 µM concentration in the agar.

### ***Cbr-lin-28*::GFP Plasmid**

The *Cbr-lin-28::GFP* expression plasmid was produced by PCR and restriction digestion and ligation techniques. Q5® High-Fidelity DNA Polymerase (NEB #M0491S) and Phusion® High-Fidelity DNA Polymerase (NEB #M0530S) were used for amplification. *Cbr-lin-28* with promoter region and 3'UTR were amplified from the *C. briggsae* (AF16) genome, and the *C. elegans*-optimized GFP sequence with introns was amplified from pVT221 (Moss *et al.*, 1997). To mutate the plasmid, a Q5® Site-Directed Mutagenesis Kit

(NEB #E0552S) was used. The plasmid scheme and mutation sequences are shown in Chapter 3, Fig. 3.13.

**Plots and Statistics**

Data was analyzed using Prism software. *P*-values were calculated using unpaired Welch's t-tests for absolute values and Fisher's exact tests for fractions. Error bars in plots indicate 95% CI, asterisks indicate the following: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, ns - not significant ($p > 0.05$).

# Chapter 3

## Orthologs of the *C. Elegans* Heterochronic Genes Have Divergent Functions in *C. Briggsae*


### Abstract

The heterochronic genes of *C. elegans* comprise the best-studied pathway controlling the timing of tissue and organ formation in an animal. To begin to understand the evolution of this pathway and the significance of the relationships among its components, we characterized 11 *C. briggsae* orthologs of *C. elegans* heterochronic genes. Using CRISPR/Cas9, we made a variety of alleles and found that several mutant phenotypes differ in significant ways from those of *C. elegans*. Although most mutant orthologs displayed defects in developmental timing, their phenotypes could differ in which stages were affected, the penetrance and expressivity of the phenotypes, or by having additional pleiotropies that were not obviously connected to developmental timing. However, when examining pairwise epistasis and synergistic relationships, we found those paralleled the known relationships between their *C. elegans* orthologs, suggesting that the arrangements of these genes in functional modules is conserved, but the modules' relationships to each other and/or to their targets has drifted since the time of the species' last common ancestor. Furthermore, our investigation has revealed a relationship to this pathway to other aspects of the animal's growth and development, including gonad development, that is relevant to both species.

## Introduction

A developmental regulatory system performs its function in part due to the specific activities of its components and in part due to the manner in which these components interact. It has been found through comparative analysis that as these systems evolve, components may be replaced, or their relationships may change. Such investigations can illuminate important features of a developmental regulatory system and how it performs its function (True and Haag 2001; Hill *et al.* 2006; Sommer 2012; Ellis 2022).

The heterochronic pathway of the nematode *Caenorahbditis elegans* is the most thoroughly characterized developmental regulatory system controlling timing of tissue and organ development in an animal (Rougvie and Moss 2013). The components of the core pathway include transcription factors, RNA-binding proteins, and several microRNAs (miRNAs). This is the pathway in which miRNAs were discovered, and they play an important role in how it works: several regulatory factors are down-regulated at specific times during larval development by the miRNAs. Furthermore, the transcription and processing of miRNAs are temporally regulated and, in some cases, under the control of other heterochronic regulators.

Mutations in heterochronic genes alter the relative timing of diverse developmental events independent of spatial or cell type specific regulation. Similar animal-wide timing pathways have not been characterized in other species. The core heterochronic pathway includes the protein-coding genes *lin-14, lin-28, lin-29, lin-41, lin-46* and *hbl-1*, and the miRNA-encoding *lin-4*, *let-7*, *mir-241*, *mir-28,* and *mir-84* (Rougvie and Moss 2013). (Several other genes with heterochronic effects are not considered here.)

Most of the proteins encoded by heterochronic genes are expressed in the beginning of postembryonic development whereas the miRNAs are not. The levels of the miRNAs rise during the larval stages and block the expression of proteins whose activities promote stage-specific developmental events. In general, when the miRNAs are missing or defective, developing mutant animals repeat some stage-specific events and postpone later events, which is called a reiterative phenotype. Mutations that delete miRNA binding sites from the 3'UTRs of their heterochronic gene targets also cause reiterative phenotypes. By contrast, when target genes are defective due to loss-of-function mutations, stage-specific events are skipped, which is called a precocious phenotype.

The core heterochronic genes of *C. elegans* have one-to-one orthologs in *C. briggsae*. Some have orthologs in other phyla, such as the miRNAs and *lin-28*, and others belong to conserved gene families, such as *hbl-1*, *lin-29*, and *lin-41* (Rougvie and Moss 2013). *lin-14* and *lin-46* are found only in the *Caenorhabditis* genus of nematodes. The degree of conservation does not correlate with how important a gene is in the regulation of development: *lin-14* has a key role in controlling L1 and L2 cell fates, and in many ways it is the paradigmatic heterochronic gene (Ambros and Horvitz 1987).

*C. briggsae* is a close relative of *C. elegans* that is often used as a model for studies of evolution within one genus. These species are remarkably similar—they occupy similar ecological niches, anatomically indistinguishable and have nearly identical development and cell lineages (Zhao *et al.* 2008; Félix and Duveau 2012). Despite the similarities, clear orthologs constitute only 60% of their genomes (Stein *et al.* 2003).

Comparative developmental studies—especially of the sex determination pathway in *C. elegans*, *C. briggsae*, and other *Caenorhabditis* species—have revealed that many alterations, shifts, and substitutions of components and their relationships are possible while preserving morphology, life history, and behavior (Ellis 2017, 2022). Such evolution in developmental pathways while the resulting morphology remains unchanged is a phenomenon called developmental systems drift (True and Haag 2001). Random mutations that do not dramatically decrease fitness may linger for several generations and become suppressed by other mutations. Over time, many genetic differences can accumulate, causing the roles of individual genes to change and components of developmental pathways to be replaced or change their relationships to their targets.

Our goal was to investigate the functional organization of the heterochronic pathway by seeing how much of its composition and arrangement are the same across a short evolutionary time—short enough so that orthologs are identifiable for all components, but where sufficient time has passed for developmental systems drift to have occurred. We began by mutating each *C. briggsae* ortholog of the 11 core heterochronic genes and characterizing their phenotypes, as well as comparing some well-characterized epistasis relationships. To study genes expected to have early embryonic lethal, pleiotropic, or infertile null phenotypes we used the auxin-inducible degron system in *C. briggsae* (Zhang *et al.* 2015; Hills-Muckey *et al.* 2021).

## Results

### *Wild-Type Seam and Intestinal Cell Fates are Similar in* C. briggsae *and* C. elegans

Heterochronic phenotypes of *C. elegans* can be reliably observed in the postembryonic lineages of the lateral hypodermal seam cells (Ambros and Horvitz 1984, 1987; Ambros 1989). Seam cells are located along each side of the newly hatched larva, dividing at each larval stage and differentiating at adulthood (Sulston and Horvitz 1977). We counted seam cells at each larval stage and observed their divisions in wildtype *C. briggsae* to see if the lineage patterns resembled those of *C. elegans*.

We found that *C. briggsae* L1 larvae had 10 seam cells within 3 hours of hatching (n = 10, Fig. 3.1). Seam cells were observed to divide in L1 larvae within 6 hours after hatching, with one of the daughters staying at the midline while the other moved dorsal or ventral to join the hypodermal syncytium. As a result, we saw that molting L1 larvae still had 10 seam cells (n = 10). Seam cells H1, V1-V4, and V6 divided symmetrically in L2 larvae, and late L2 or early L3 larvae had 15.5+/-0.5 seam cells (n = 16). Both L3 and L4 larvae still had asymmetrical divisions like those in the L1. L4 larvae and adult worms had 15-16 seam cells (Fig. 3.4A). All seam cells aligned along the midline and produced cuticular alae at adulthood. These observations indicate that the numbers and division patterns of seam cells in *C. briggsae* are like those of *C. elegans* at each larval stage.

Also like *C. elegans*, newly hatched *C. briggsae* had 20 intestinal nuclei. In *C. elegans*, 10 to 14 of 20 intestinal nuclei divide at the beginning of L1 lethargus (Sulston and Horvitz 1977; Hedgecock and White 1985). In *C. briggsae,* some intestinal cell nuclei also divided during the L1 lethargus and molt, with some divisions coinciding with the

first round of the L2 seam cell divisions. In addition, we observed a slight but statistically significant increase in the average number of intestinal nuclei between the L3 and L4 stage. It is possible that some intestinal nuclei in *C. briggsae* divide during stages after the L1, which does not occur in *C. elegans* (Fig. 3.2).

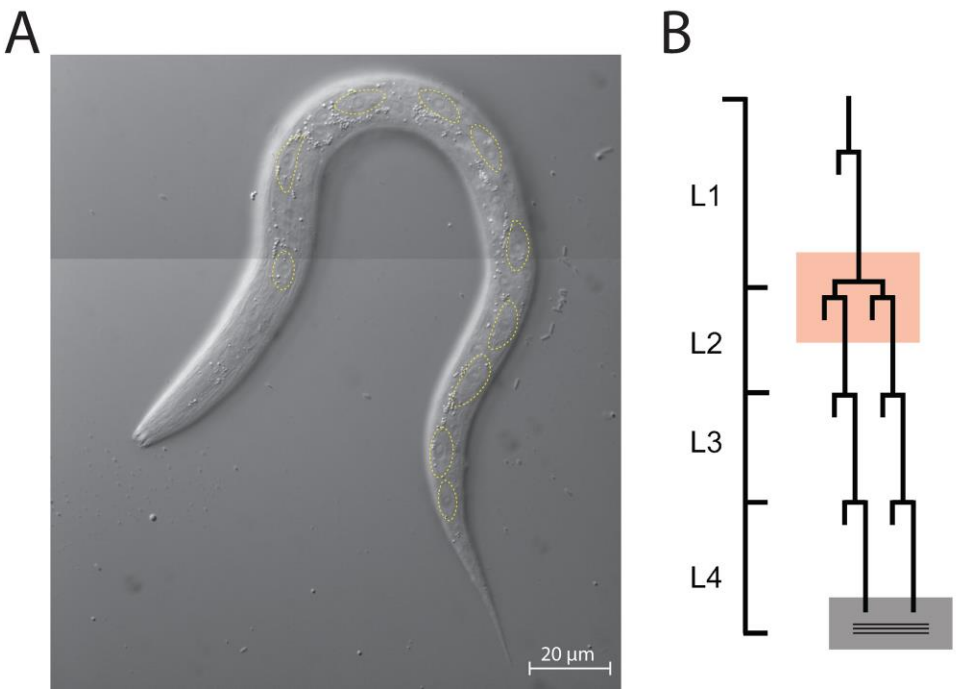



*Figure 3.1.* Seam cell lineages of *C. briggsae* are identical to those of *C. elegans*. (A) DIC microphotograph of an L1 worm less than 3 hours after hatching. 10 seam cells are outlined with the dotted line. (B) Cell lineage diagram summarizing the divisions of V1-V4 and V6 seam cells during the four larval stages. Vertical lines represent a passage of time and horizontal lines indicate cell divisions. The lines that stop indicate terminal differentiation, either by joining the hypodermal syncytium, or by differentiation into adult seam cells that produce alae (gray square with horizontal lines). The red square

highlights symmetric cell divisions that each produce two daughters that remain at the seam and continue dividing. This cell lineage is identical for *C. elegans* and *C. briggsae*.

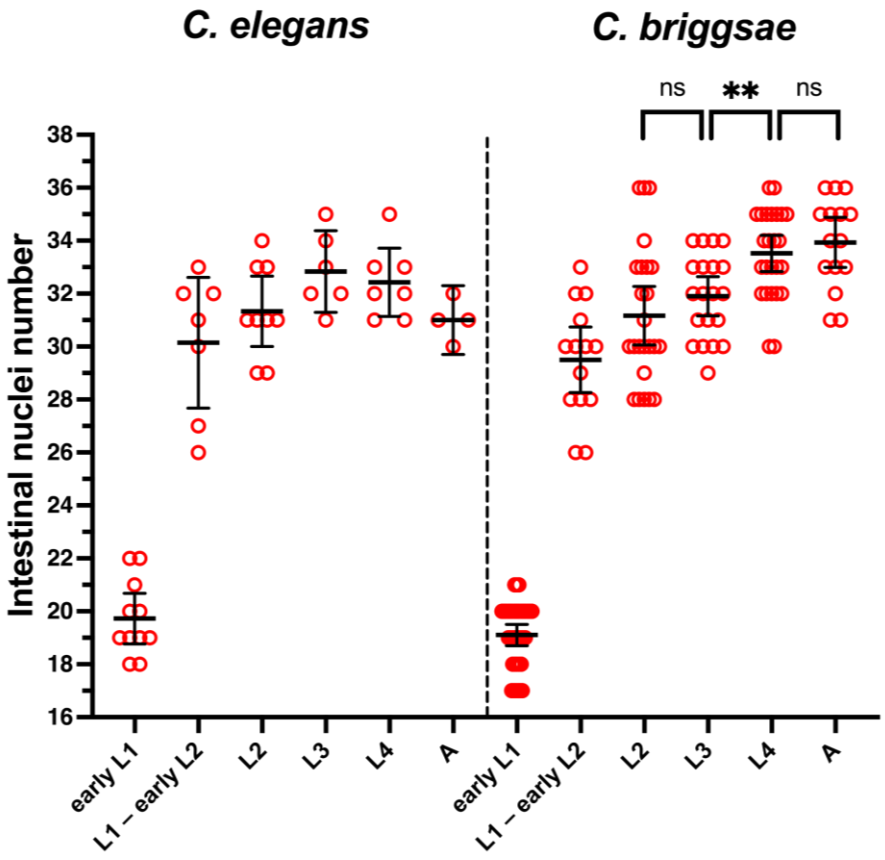



*Figure 3.2.* Intestinal nuclei divide after the L1 stage in *C. elegans* and *C. briggsae*. Division in *C. briggsae* occurs after the L2 stage, but not in *C. elegans*. "Early L1" refers to animals before the L1 seam cell divisions. "L1 – early L2" refers to animals during and after the L1 seam cells divisions but before the L2 (symmetric) seam cell divisions. "L2" refers to animals both during and after the L2 seam cell divisions. Statistical analysis is described in Materials and Methods.

We used seam cell and intestinal nuclei, as well as vulval lineages, which have been previously documented (Brown 2001), to characterize developmental timing phenotypes in *C. briggsae* mutants. However, we also observed phenotypic changes in other tissues, as described below.

**Table 3.1**

*Genetic Lesions*

| ***Cbr-lin-4*** https://wormbase.org/species/c_briggsae/gene/WBGene00255721#g--10 | | ***Allele type*** |
|---|---|---|
| WT | GCCTGTTCCCTGAGACCTCAAGTGTGAGCGTTCTGAACAT | wt |
| *ae54* | GCCTGTT------GACCTCAAGTGTGAGCGTTCTGAACAT | 0 |
| *ae55* | GCCTGTT--------**T**CTCAAGTGTGAGCGTTCTGAACAT | 0 |
| *ae71* | GCCTGT-------GACCTCAAGTGTGAGCGTTCTGAACAT | 0 |
| *ae72* | GCCTG-------AGACCTCAAGTGTGAGCGTTCTGAACAT | 0 |
| *ae79* | GCC------CTGAGACCTCAAGTGTGAGCGTTCTGAACAT | 0 |
| ***Cel-lin-4*** https://wormbase.org/species/c_elegans/gene/WBGene00002993#g--10 | | |
| WT | GCCTGTTCCCTGAGACCTCAAGTGTGAGTGTACTATTGAT | wt |
| *ae53* | GCCTGT-------GACCTCAAGTGTGAGTGTACTATTGAT | 0 |
| ***Cbr-lin-14*** https://wormbase.org/species/c_briggsae/gene/WBGene00036988#g--10 | | |
| WT | E V H D L R T A V N<br>GAGGTTCACGATCTACGGACGGCAGTAAAT | wt |
| *ae62* | E V H G R Q *<br>GAGGTTCACG-------GACGGCAGTAAAT | 0 |
| *ae63* | E V H Y G R Q *<br>GAGGTTCAC----TACGGACGGCAGTAAAT | 0 |
| *ae51* | E V H D H D G R Q *<br>GAGGTTCACGATC**ACG**ACGGACGGCAGTAAAT | 0 |
| *ae58* | ATTCCAAAAAAAAATTCGCCCT< 1381 bp del.>CCTCGGAA | gf |
| *ae59* | ATTCCAAAAAAAAATTCGCCCTTTCTATCCCATCC< 1368 bp del.>CCTCGGAA | gf |
| ***Cbr-lin-28*** https://wormbase.org/species/c_briggsae/gene/WBGene00033626#g--10 | | |
| WT | D D N T G E D L F V H Q S N L N M Q G F R<br>GATGATAACACCGGGGAAGATCTTTTTGTGCATCAATCCAATTTGAATATGCAAGGATTTAGAA | wt |
| *ae25* | D D N R G R S F C A S I Q F E Y A R I *<br>GATGATA--ACCGGGGAAGATCTTTTTGTGCATCAATCCAATTTGAATATGCAAGGATTTAGAA | (0) |
| *ae35* | D D M E R V K V M Q H T E K I F L C I N P I *<br>GATGATA**TGGAGAGGGTGAAGGTGATGCAACATACGGAA**AAGATCTTTTTGTGCATCAATCCAATTTGAAT | (0) |

| | | |
|---|---|---|
| ***Cbr-lin-28*** https://wormbase.org/species/c_briggsae/gene/WBGene00033626#g--10 | | |
| *ae39* | D R G R S F C A S I Q F E Y A R I *<br>GAT--------CG**T**GGAAGATCTTTTTGTGCATCAATCCAATTTGAATATGCAAGGATTTAGAA | (0) |
| *ae80* | D D N <160 aa del.> K A G K *<br>GATGATAAC< 1025 bp del.>AAGGCTGGAAAATAGgcgtcgagaccatggagaac | 0 |
| ***Cbr-lin-29*** https://wormbase.org/species/c_briggsae/gene/WBGene00025748#g--10 | | |
| WT | Y L S Q H M R I H L G I K P F G P C N Y C G K<br>TACCTCTCCCAACACATGCGAATCCATTTGGGAATCAAACCGTTTGGGCCATGCAATTATTGTGGAAAG<br>K F T Q L S H L Q Q H I R T H T G E K P Y K<br>AAGTTCACACAGCTCTCACATCTTCAACAACACATTCGGACGCACACGGGAGAGAAACCGTATAAA | wt |
| *ae75* | Y L S H A N P F G N Q T V W A M Q L L W K<br>TACCTCTC-----ACATGCGAATCCATTTGGGAATCAAACCGTTTGGGCCATGCAATTATTGTGGAAAG<br>E V H T A L T S S T T H S D A H G R E T V *<br>AAGTTCACACAGCTCTCACATCTTCAACAACACATTCGGACGCACACGGGAGAGAAACCGTATAAA | 0 |
| ***Cbr-lin-41*** https://wormbase.org/species/c_briggsae/gene/WBGene00040670#g--10 | | |
| WT | M T T T T S T A T L T L E T T D G G E Q H<br>ATGACCACCACCACGAGTACGGCAACGCTGACACTGGAAACCACCGACGGCGGTGAGCAGCAC | wt |
| *ae76* | M T T K P P P R V R Q R *<br>ATGACCACCA**AACCACCA**CCACGAGTACGGCAACGCTGACACTGGAAACCACCGACGGCGGTGAGCAGCA<br>C | lf |
| *ae77* | M T T M P K D P A K P P A K A Q V V G W P P V<br>R<br>ATGACCACCATGCCTAAAGATCCAGCCAAACCTCCGGCCAAGGCACAAGTTGTGGGATGGCCACCGGTGA<br>GA<br>S Y R K N V M V S C Q K S S G G P E A A A F V<br>K<br>TCATACCGGAAGAACGTGATGGTTTCCTGCCAAAAATCAAGCGGTGGCCCGGAGGCGGCGGCGTTCGTGA<br>AG<br>T T S T A T L R R R K E D D H H A *<br>ACCACGAGTACGGCAACGCTGAGAAGACGTAAAGAGGATGACCACCATGCCTAAAGATCCAGCCAAACCT<br>CC<br>GGCCAAGGCACAAGTTGTGGGATGGCCACCGGTGAGATCATACCGGAAGAACGTGATGGTTTCCTGCCAA<br>AA<br>ATCAAGCGGTGGCCCGGAGGCGGCGGCGTCGACACTGGAAACCACCGACGGCGGTGAGCAGCAC | 0 |
| ***Cbr-lin-46*** https://wormbase.org/species/c_briggsae/gene/WBGene00031058#g--10 | | |
| WT | R Y P P Q S K V V E<br>CGATATCCACCTCAATCCAAAGTTGTAGAA | wt |
| *ae38* | R Y P P W Y H P S Y P N Q T D L P S L I R G L G<br>CGATATCCACC**ATGGTATCACCCATCTTATCCGAATCAGACTGATCTTCCCAGCCTTATCCGCGGTCTCGG<br>T**<br>L R Y S S C Q K R K N C I *<br>**CTCAGATACAGTTCTTGCCAAAAGCGCAAAAACTGTATCTGACGCATATCGACGCAGAAGTGGAAGGCGAA<br>G**<br>TCCAAAGTTGTAGAA | 0 |
| WT | M S S V G<br>F<br>TAAACATTTACATAA*TTACAG*GTAAAACCAAGAATTGTATCAGTGGGAGTCAATCCAATGAGTTCTGTAGG<br>ATTTC | wt |
| *ae43* | TAAACATTTACATAA*TTACAG*GTAAAACCAAGAATTGT------<br>GGGAGTCAATCCAATGAGTTCTGTAGGATTTC | gf |
| *ae44* | TAAACATTTACATAATTAC----------<br>AGTTCTGTAGGATTTC | lf |

| | | |
|---|---|---|
| ***Cbr-let-7*** https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=Cbr-let-7 | | |
| WT | ATTTTTCAGGGGATTGCAGGATGATGGCTCTACACTGGGGTACGGTGAGGTAGTAGGTTGTATAGTTTAG | wt |
| *ae47* | ATTTTTCAGGGGATTGCAGGATGATGGCTCTACACTGGGGTGAGGT**GGTTGTATAGTTTAGAATATTACTC TCGGT AGTTTAGAATATTACTCTCGG**AGTAGGTTGTATAGTTTAG | 0 |
| *ae48* | ATTTTTCAGGG----------TATAGTTTAG | 0 |
| *ae50* | ATTTTTCAGGGGATTGCAGGATG----------TATAGTTTA | 0 |
| ***Cbr-mir-48*** https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=Cbr-mir-48 | | |
| WT | CATTCGGGATGTTGAGGTAGGCTCAGTAGATGCGAGGAGATCCACCATTCCTCACATCGTCTGTCCTAACT CG | wt |
| *ae65* | GTTTTTCGATATCTCACATAGAAATAGAG< 2180 bp del. >ATTCCTCACATCGTCTGTCCTAACTCG | 0 |
| ***Cbr-mir-84*** https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=Cbr-mir-84 | | |
| WT | TTTTGAACAGCCGAGGAAGTTGGAATGATGTTGACTTTTCAGTTACGTCGACAGCATTGCAAACTACCTCA GA | wt |
| *ae68* | TTTTGAACAGCCGAG---- TTGGAATGATGTTGACTTTTCAGTTACGTCGACAGCATTGCAAACTACCTCAGA | 0 |
| *ae69* | TTTTGAACAGCCGA--- AGTTGGAATGATGTTGACTTTTCAGTTACGTCGACAGCATTGCAAACTACCTCAGA | 0 |
| *ae70* | TTTTGAACAGCCGAGGAA------- TGATGTTGACTTTTCAGTTACGTCGACAGCATTGCAAACTACCTCAGA | 0 |
| ***Cbr-mir-241*** https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=Cbr-mir-241 | | |
| WT | TCTTTTGTACCTCTTCCGCAAGACGGTGTCAAAGCTGAGGTAGGTGTGAGAAATGACGAAAGGCTCTTAAT CG | wt |
| *ae64* | TCTTTTGTACCTCTTCCGCA**TCT**---------- T**CC**GTGTGAGAAATGACGAAAGGCTCTTAATCG | 0 |
| ***Cbr-mir-241 Cbr-mir-48*** | | |
| *ae73* | AAATGCACGTATAGGATGGGCTTCT< 12,897 bp del.>CGGGTTGGGACACAAACAACTCTTT | 0 |
| ***Cbr-lin-28::AID*** | | |
| *aeIs13* | E T E T A D K A G K M P K D P A K P P A K A Q V V GAGACTGAAACTGC**C**GA**T**AA**A**GCTGGAAAA**ATGCCTAAAGATCCAGCCAAACCTCCGGCCAAGGCACAAGT TGT** G W P P V R S Y R K N V M V S C Q K S S G G P E **GGGATGGCCACCGGTGAGATCATACCGGAAGAACGTGATGGTTTCCTGCCAAAAATCAAGCGGTGGCCCGG AGG** A A A F V K * **CGGCGGCGTTCGTGAAGTAG**acaaggctggaaaaTAG | AID |
| ***Cbr-hbl-1::AID*** | | |
| *aeIs12* | A L H M Y Q A K H Q M P K D P A K P P A K A Q V V GCTCTCCACATGTA**T**CA**G**GC**T**AA**G**CATCAG**ATGCCTAAAGATCCAGCCAAACCTCCGGCCAAGGCACAAGT TGT** G W P P V R S Y R K N V M V S C Q K S S G G P E **GGGATGGCCACCGGTGAGATCATACCGGAAGAACGTGATGGTTTCCTGCCAAAAATCAAGCGGTGGCCCGG AGG** A A A F V K * **CGGCGGCGTTCGTGAAGTAG**taccaagcgaaacatcagTGA | AID |

| *Cbr-lin-41::AID* | | |
|---|---|---|
| *aels14* | M T T M P K D P A K P P A K A Q V<br>V<br>aaaaaagaagacgtaaagaggATGACCACC**ATGCCTAAAGATCCAGCCAAACCTCCGGCCAAGGCACAAGT**<br>**TGT**<br>G W P P V R S Y R K N V M V S C Q K S S G G P<br>E<br>**GGGATGGCCACCGGTGAGATCATACCGGAAGAACGTGATGGTTTCCTGCCAAAAATCAAGCGGTGGCCCGG**<br>**AGG**<br>A A A F V K T T S T A T<br>**CGGCGGCGTTCGTGAAG**ACCACGAGTACGGCAACG | AID |

*Note.* Dashes, deleted sequence. Bold, insertion/substitution. Underline, mature miRNA. Italics, trans splice site. Angle brackets, large deletion. Lowercase, duplicated sequence at AID insertion site. Allele type: 0, null; (0), likely null but possibly strong loss-of-function; lf, loss-of-function (not null); gf, gain-of-function; wt, wildtype.

### *Cbr-lin-4(0) Mutants Reiterate L2 Stages*

*C. elegans lin-4* is the first heterochronic gene to be identified and encodes the first miRNA to be discovered (Chalfie *et al.* 1981; Ambros and Horvitz 1984; Lee *et al.* 1993). *Cel-lin-4(0)* mutants have a profound reiterative phenotype where L1-specific events are repeated causing adult animals to lack both vulvae and alae.

We isolated two *Cbr-lin-4(0)* mutant alleles with 6 (allele *ae54*) and 8 bp (allele *ae55*) deletions that remove most or all of the miRNA seed sequence (Table 3.1). Grossly, these animals were egg-laying defective for lacking a vulva (Fig. 3.3A and C).

To learn if *Cbr-lin-4(0)* mutants reiterated stage-specific fates like *C. elegans*, we examined adult alae, seam cells and intestinal nuclei. Based on seam cell counts, *Cbr-lin-4(0)* mutants appeared to re-iterate the symmetric divisions characteristic of the L2 stage during later larval stages (Fig. 3.4A, Fig. 3.5). Seam cells divided symmetrically at L2,

and most larvae had 15.2+/-0.7 seam cells (n = 30) after the L2 divisions. Thus, L1 stages were not reiterated by most seam cells. This effect on seam cell lineages was also cold-sensitive: L4 larvae and young adults had more seam cells at 15°C than at 20°C or 25°C (Fig. 3.4B).

In contrast, both the *C. elegans* reference allele *Cel-lin-4(e912)* and *Cel-lin-4(ae53)*, a mutant generated using the same single guide RNA used for the *Cbr-lin-4(0)* mutant alleles, reiterated mostly L1 stage seam cell fates. As a result, these animals had 10.7+/-0.5 seam cells prior to the L4 stage (n = 6) and 12.7+/-1.7 seam cells after the L4 (n = 13).

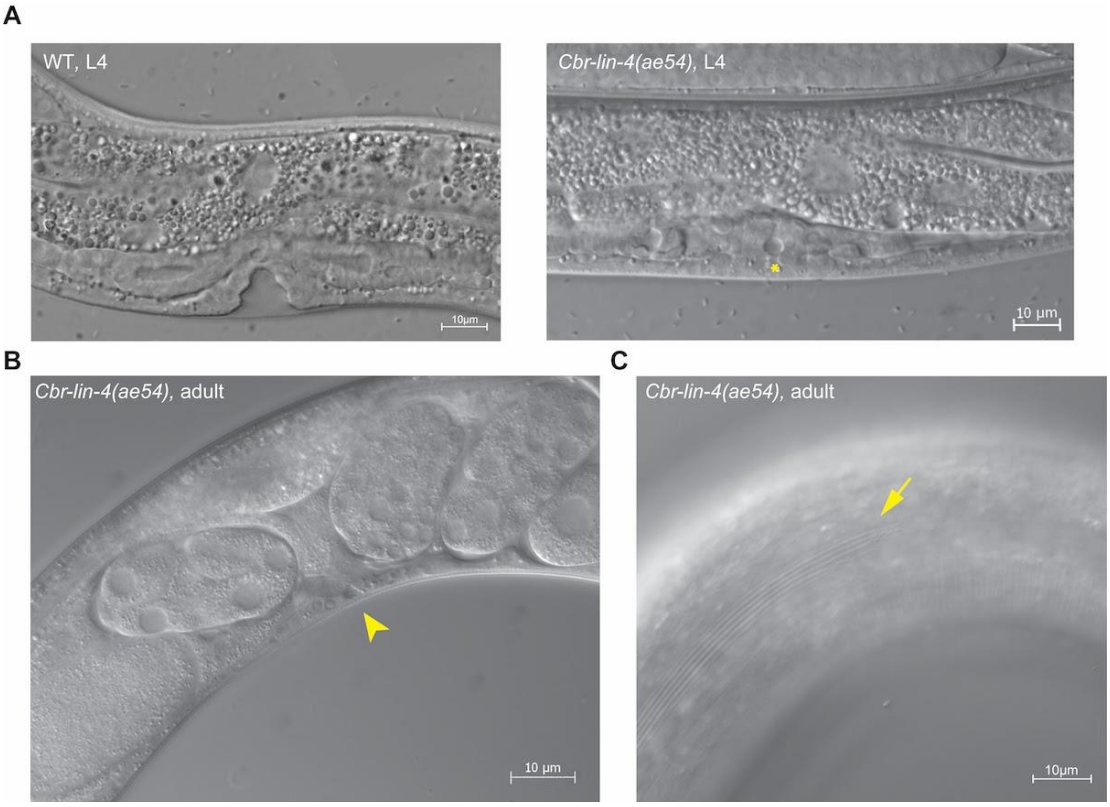



*Figure 3.3. Cbr-lin-4(0)* mutants have a reiterative phenotype. DIC micrographs showing (A) the vulval region in a *Cbr-lin-4(ae54)* L4 animal compared to a wild-type L4 animal. The vulval precursor cells failed to divide. Asterisk indicates the vulva area, and above the asterisk is the lysed anchor cell. (B) *Cbr-lin-4(ae54)* adult where the vulva failed to form resulting in the accumulation of embryos in the uterus. Arrowhead points at the vulva area. (C) Gapped alae in an adult *Cbr-lin-4(ae54)* animal; arrow points at the alae gap.

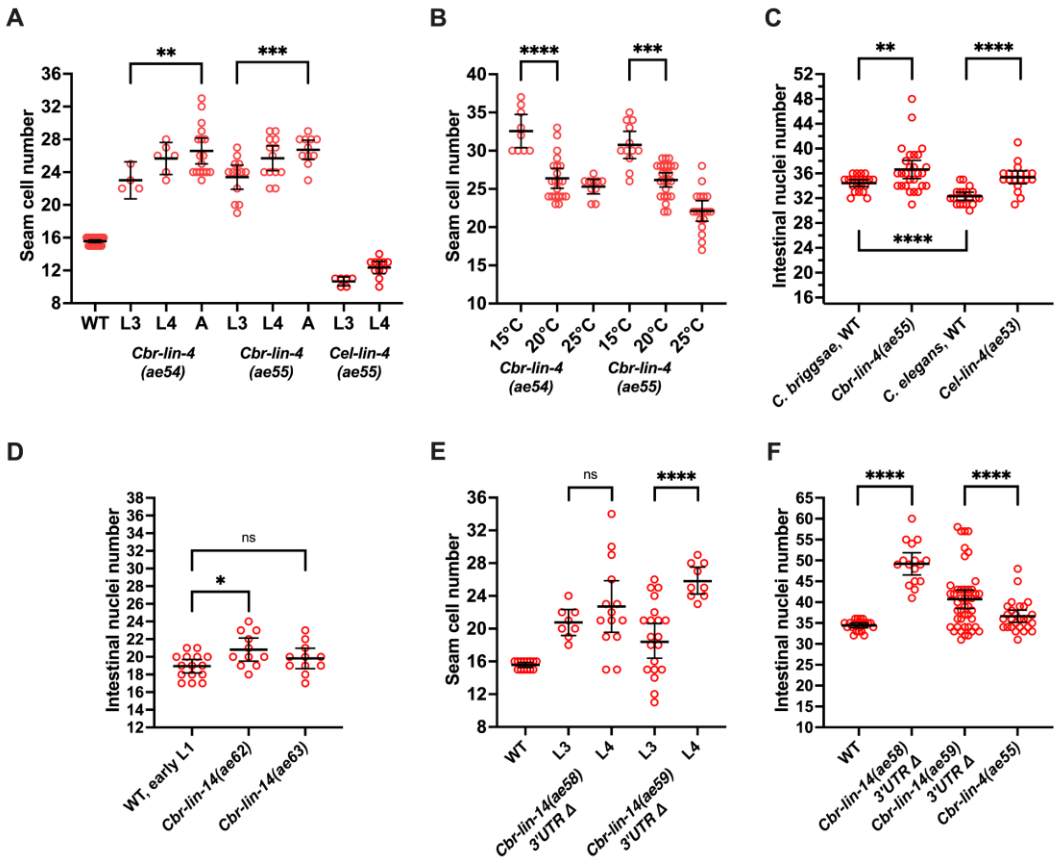



*Figure 3.4.* Seam cell and intestinal nuclei changes in *Cbr-lin-4(0)* and *Cbr-lin-14(0)* or *(gf)* mutants**.** Plots show seam cell and intestinal nuclei counts in L4 and young adults (unless otherwise specified) at 20°C (unless otherwise specified) for the strains indicated. (A) The number of seam cells is higher in *Cbr-lin-4(0)* mutants than in the wild type, and increases between the L3 and adult stages. On the contrary, *Cel-lin-4(0)* mutants have a reduced number of seam cells. (B) The number of seam cells in *Cbr-lin-4(0)* mutants increases at 15°C. (C) Intestinal nuclei numbers increase in *Cbr-lin-4(0)* mutants. (D) Intestinal nuclei numbers in the *Cbr-lin-14(0)* mutants are similar to those in early L1 larvae. (E) *Cbr-lin-14* 3'UTR deletion mutants have an increased number of seam cells. (F) *Cbr-lin-14* 3'UTR deletion mutants have even higher numbers of intestinal nuclei

than *Cbr-lin-4(0)* mutants. The data set for *Cbr-lin-4(ae55)* is the same as in panel C. The reason for the difference between *ae58* and *ae59* is unclear as both deletions had approximately the same length and location. Statistical analysis is described in Materials and Methods.

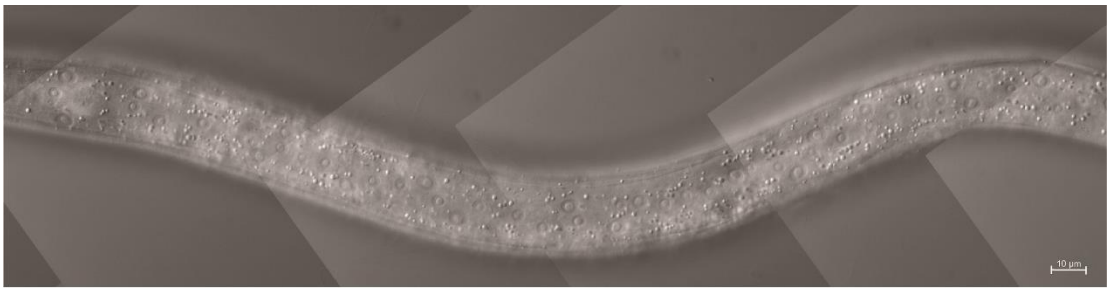



*Figure 3.5.* Cbr-lin-4(0) mutants reiterate L2 seam cell fates. DIC microphotograph of Cbr-lin-4(ae54) L3 larva with an increased number of seam cells (outlined with yellow dotted line).

Like *C. elegans lin-4(0)* mutants, *Cbr-lin-4(0)* mutant animals also have extra molts. Five L4 larvae from the *Cbr-lin-4(ae55)* strain were placed together on a plate at 20°C, and the next day, 7 shed cuticles were found in the bacterial lawn. The animals were adults (carried eggs), and 4 of them were stuck while shedding extra cuticles. Adult egg-producing wild-type *C. briggsae* were never observed shedding cuticles. Thus, *Cbr-lin-4(0)* mutants have at least one additional molt after reaching adulthood, and possibly as many as two. Unlike *Cel-lin-4(0)* mutants, whose cuticles are devoid of adult alae, *Cbr-lin-4(0)* mutants developed partial adult alae at the end of the development, most young

adults (adults without embryos) had alae "patches", meaning that more than 0% but less than 50% of their seam cells generated adult alae (50%, n = 12), while most adult worms with embryos had “gapped” alae, meaning that more than 50%, but less than 100%, of the seam cells formed alae (78%, n = 18, Fig. 3.3C). Thus, most seam cells differentiate at the end of the development in *C. briggsae lin-4* mutants, while they fail to differentiate in *C. elegans*.

In wild-type *C. elegans* and *C. briggsae*, dauer larvae represent an alternative developmental stage that forms in unfavorable environmental conditions like overcrowding or lack of nutrients. When heterochronic mutants reiterate L1 stages and do not transition to L2 they cannot form dauer larvae, which was observed for *Cel-lin-4(0)* mutants (Liu and Ambros 1989). Surprisingly, *Cbr-lin-4(0)* mutants could enter the dauer developmental pathway, reinforcing the conclusion that they enter the L2 stage. However, *Cbr-lin-4(0)* dauers had gapped dauer alae and some segments of their bodies looked expanded that was not observed in wild-type dauers (Fig. 3.6). We suspect that those worms transitioned to the dauer stage incompletely.

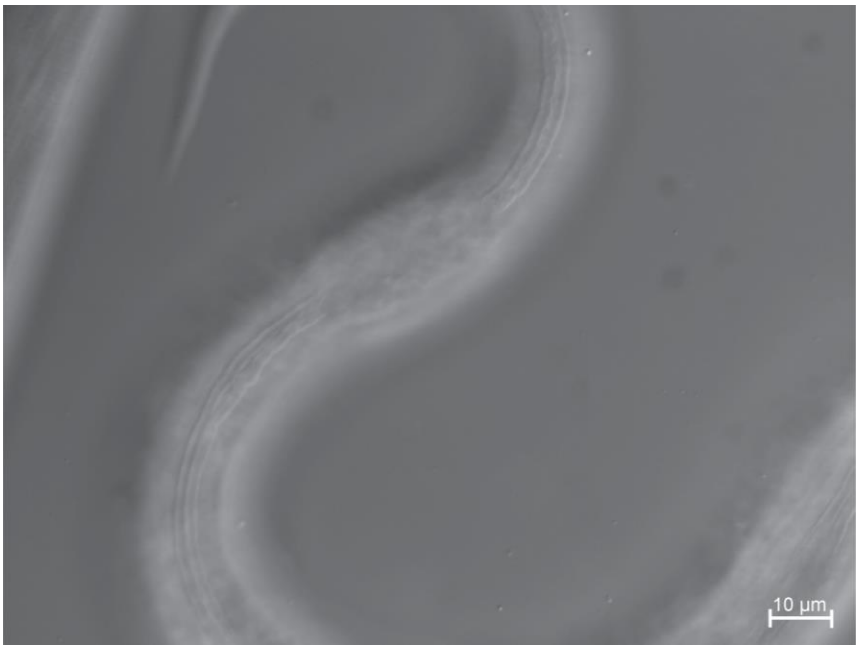



*Figure 3.6*. Cbr-lin-4(0) mutants can become dauers but incompletely. DIC microphotograph of the central body of a *Cbr-lin-4(ae54)* dauer larva. Part of its body is swollen and lacks the dauer cuticle.

Interestingly, despite these differences, both *Cbr-lin-4(0)* and *Cel-lin-4(0)* mutants had a slightly increased number of intestinal nuclei compared to wild-type (Fig. 3.4C). The number of intestinal nuclei in *Cbr-lin-4(ae55)* mutant was not significantly different ($p > 0.05$, unpaired Welch's t-test) at 15°C (36.9+/-3.1, n = 28) compared to the same mutant at 20°C. So, whereas the phenotype of *Cel-lin-4(0)* mutants is interpreted as a reiteration of L1 stage-specific fates, we find some ambiguity with *Cbr-lin-4(0)* mutants, since they appear to reiterate L1 fates in the intestine and L2 fates in the hypodermis.

### **C. briggsae lin-14(0)** ***Mutants Resemble*** **C. elegans lin-14(0*) Mutants***

*C. elegans lin-14* encodes a transcription factor unique to this genus (Ruvkun and Giusto 1989; Hristova *et al.* 2005). Alleles are of two general types: loss-of-function (lf) and null (0), which cause a precocious phenotype, and gain-of-function (gf), which lack miRNA binding sites in the 3'UTR and cause a reiterative phenotype (Ambros and Horvitz 1987; Wightman *et al.* 1991).

We made mutant alleles *ae62* and *ae63* with frameshift mutations that create premature stop codons in *Cbr-lin-14* by targeting the first exon shared by all isoforms (Table 3.1, Fig. 3.7). The mutants were often infertile, so we balanced them with a mutation that caused a visible phenotype: *Cbr-dpy-8(v262)* (Wei *et al.* 2014). *Cbr-lin-14(0)* progeny from the balanced strain resembled *Cel-lin-14(0)* mutants in several key features, including a protruding vulva, and shared a similar overall morphology (Fig. 3.8).

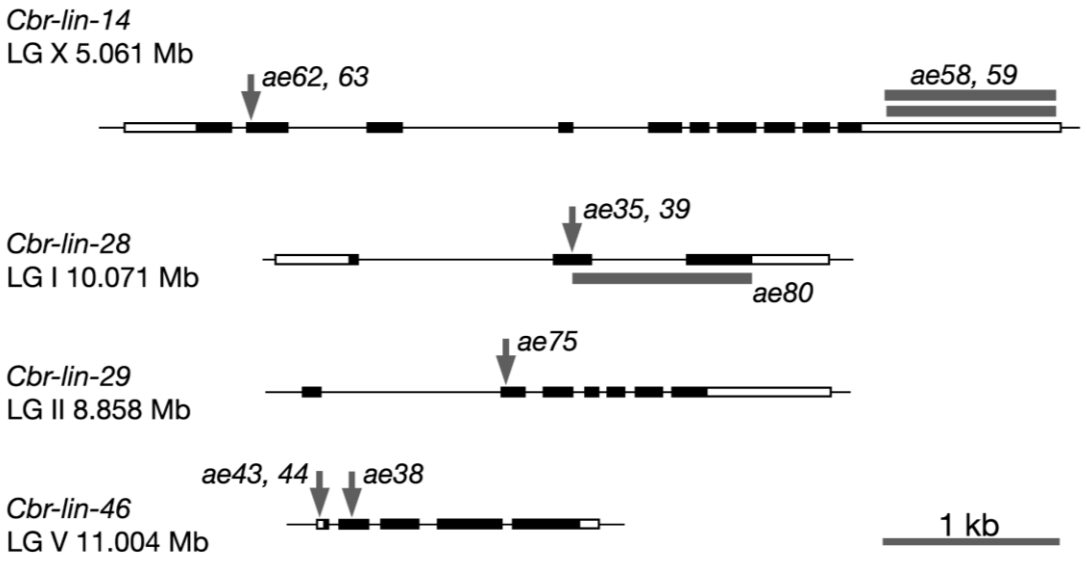



*Figure 3.7.* Genomic locations of genetic lesions listed in Table 3.1. Black rectangles, coding exons. White rectangles, non-coding regions. Arrows indicate lesions listed in Table 3.1. The bars for *Cbr-lin-14* alleles *ae58* and *ae59* show the extent of the deletions. Lesions and modifications not shown were at either the 5' or 3' end of the coding regions, as indicated in Table 3.1 and described in the text.

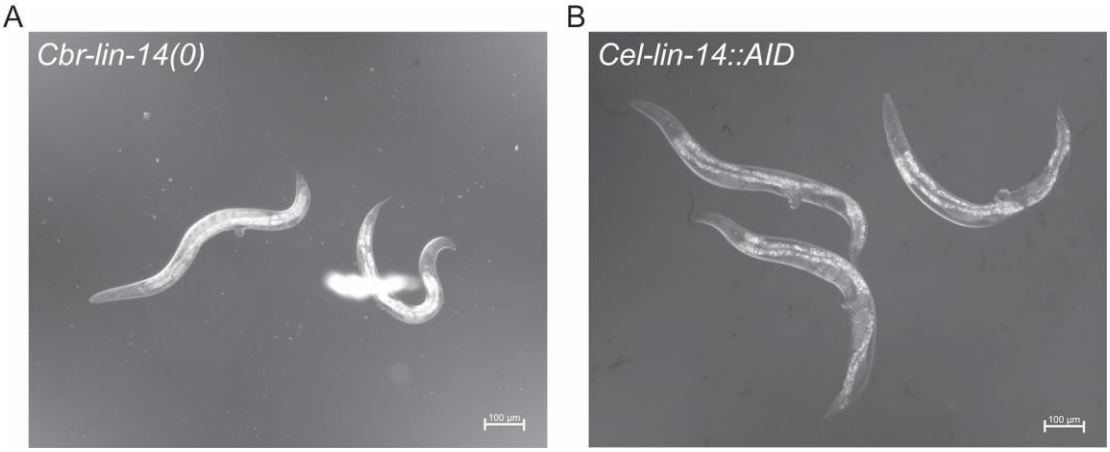



*Figure 3.8.* The body morphology of Cbr-lin-14(0) mutants is almost identical to that of Cel-lin-14(lf) mutants. DIC microphotographs of Cbr-lin-14(ae62) mutant and Cel-lin-14::AID mutants on 5-Ph-IAA (20°C).

To see if these mutants displayed a precocious phenotype, we examined the L4 cuticle, seam cell divisions, and intestinal nuclei number. *Cbr-lin-14(0)* mutants developed full adult alae by the L4 stage (100% had precocious alae, n = 20). As occurs in *C. elegans lin-14(0)* mutants, seam cell counts of *Cbr-lin-14(0)* mutants to be close to albeit slightly below the wild-type number by the L4 (Fig. 3.9). This is consistent with most seam cells of *Cbr-lin-14(0)* animals dividing symmetrically during the L1 stage, as occurs in *C. elegans lin-14(0)* mutants (Ambros and Horvitz, 1984). Also like *Cel-lin-14(0)*, the number of intestinal nuclei in later development was reduced in *Cbr-lin-14(0)* mutants, although some divisions did occur (Fig. 3.4D). Overall, our observations suggest *Cbr-lin-14* is required for the stage-appropriate expression of L1-specific fates and that the gene function is largely conserved between the two species.

### *The* Cbr-lin-4(0) *Reiterative Phenotype Requires Functional* Cbr-lin-14

In *C. elegans, lin-4* mutations that lead to a reiterative phenotype do so because they cause prolonged expression of *lin-14* (Ruvkun and Giusto 1989; Lee *et al.* 1993; Wightman *et al.* 1993). As a result, the phenotype of a loss-of-function *Cel-lin-14(0)* mutation is completely epistatic to that of *Cel-lin-4(0)* (Ambros 1989).

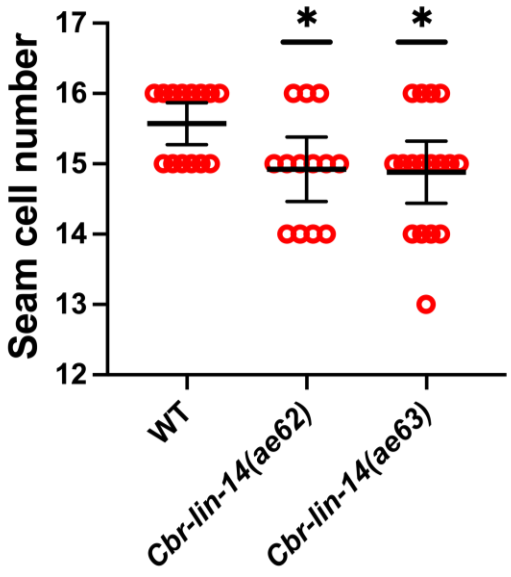



*Figure 3.9.* The number of seam cells in *Cbr-lin-14(0)* mutants is slightly reduced compared to the wild type. Seam cells were counted in L4 animals for two *Cbr-lin-14(0)* alleles, *ae62* and *ae63*. A small reduction in the seam cell number might be a result of skipping of the L2 divisions by some seam cells. Statistical analysis is described in Materials and Methods.

To test whether the temporal fate reiterations that occur in *Cbr-lin-4(0)* mutants occurred due to the elevated *Cbr-lin-14* function, we made *Cbr-lin-4(0); Cbr-lin-14(0)* double mutants by disrupting the *Cbr-lin-4* gene in a *Cbr-lin-14(ae62)* balanced strain using CRISPR/Cas9, and then isolating double homozygotes from among the progeny. We made strains with two different *Cbr-lin-4* alleles, *ae71* and *ae72* (Table 3.1).

The *Cbr-lin-4(0); Cbr-lin-14(0)* double mutant phenotype mostly resembled the *Cbr-lin-14(0)* single mutant phenotype—the number of seam cells by the L4 was 14.7+/-0.9 (n = 16, combined data from two strains) and most of the intestinal nuclei did not divide (after the L1 stage, the number of intestinal nuclei was 19.6+/-1.5, n = 16, combined data from two strains). Surprisingly, however, precocious alae were not always observed in double mutant worms: 2 of observed L4 larvae did not have precocious alae and other 2 had full precocious alae. This differs significantly from the *C. elegans* double mutant where alae appear precociously in all worms (Ambros 1989). This observation indicates a difference between the species in the relationships of *Cbr-lin-4* and *Cbr-lin-14* to downstream regulators controlling the timing of alae formation. But as in *C. elegans*, *Cbr-lin-14* appears to be required for the reiterative and vulvaless phenotypes of *Cbr-lin-4(0)*.

**Cbr-lin-14(gf) *Mutants Resemble Weak* Cel-lin-14(gf) *Alleles***

In *C. elegans*, two mutants with deletions in the 3'UTR of *lin-14* display a reiterative phenotype: an allele with nearly all miRNA sites removed reiterates L1 stages and lacks a vulva and adult alae, closely resembling *lin-4(0)*, and a weaker allele with a few intact miRNA sites reiterates both L1 and L2 stage events, also lacks a vulva, and develops some alae (Ambros and Horvitz 1987; Wightman *et al.* 1993).

*Cbr-lin-14* 3'UTR deletions (alleles *ae58* and *ae59*) were generated with CRISPR/Cas9, removing approximately 1.3 kb that includes all predicted miRNA binding sites (Table 3.1, Fig. 3.7). As adults, the mutants lacked vulvae, had extra seam cells and intestinal nuclei, and had alae patches (52%, n = 21) or lacked alae completely (48%, Table 3.2). The patches of adult alae were more transparent and thinner than wild-type alae.

**Table 3.2**

*Adult Alae in* Cbr-lin-4(0) *and* Cbr-lin-14(gf) *Strains*

| Alae completeness | *Cbr-lin-4(ae55)* young adults n = 12 | *Cbr-lin-4(ae55)* egg-producing adults n = 18 | *Cbr-lin-14(ae58) 3'UTR Δ* egg-producing adults n = 21 |
|---|---|---|---|
| Full | 8% | 17% | |
| Gaps | 8% | 78% | |
| Half | 17% | 6% | |
| Patches | 50% | | 48% |
| No alae | 17% | | 52% |

*Note.* Alae gaps mean that more than 50%, but less than 100% of seam cells generated adult alae, alae patches - more than 0% but less than 50%, half means that approximately 50% of seam cells generated adult alae.

The number of seam cells in *Cbr-lin-14(gf)* mutants was slightly lower and more variable at late stages than in *Cbr-lin-4(0)* mutants (Fig. 3.4E). Additionally, late L2 and early L3 larvae had fewer seam cells than expected if *Cbr-lin-14(gf)* phenocopied *Cbr-lin-4(0)* (mean = 11+/-1, n = 11). This difference would be explained by most seam cells

reiterating L1 cell fates before they reiterate L2 fates. Also, the number of intestinal nuclei was higher than in *Cbr-lin-4(0)* mutants—a sign of reiteration of L1 fates in this tissue—which supports the interpretation that these animals reiterate L1 stage events to some degree. Thus, *Cbr-lin-14(gf)* mutants resemble the weaker *Cel-lin-14(gf)* allele.

### **Cbr-lin-28(0)** *Mutants Have Minor Heterochronic Defects and Arrest Development at the L4 Stage*

*C. elegans lin-28* encodes an RNA-binding protein that is widely conserved in animals (Moss *et al.* 1997; Moss and Tang 2003; Vadla *et al.* 2012). *Cel-lin-28(0)* mutants display a completely penetrant precocious phenotype where they skip cell fates of the L2, and a less penetrant defect of skipping L3 fates (Ambros and Horvitz 1984; Vadla *et al.* 2012). They also show an incompletely penetrant fertility problem as a result of spermathecal defects (Choi and Ambros 2019).

We made *Cbr-lin-28* mutant alleles *ae25*, *ae35*, and *ae39* by targeting the second exon to generate frameshifts with premature stop codons (Table 3.1, Fig. 3.7). *Cbr-lin-28(0)* animals were strikingly different from *Cel-lin-28(0)* animals: many arrested their development during the late L4 stage, did not undergo the final molt and lacked adult alae. These arrested animals retained features characteristic of mid- to late- wild-type L4 animals: the reflexed gonad arms stopped developing toward each other, and the vulva ceased development during morphogenesis (Fig. 3.10A). Observing a synchronized population, we found that *Cbr-lin-28(0)* mutants developed at the same rate and produced oocytes at the same time as wild-type animals, except that wild type proceeded to the last molt and developed a vulva and alae ($n \approx 20$).

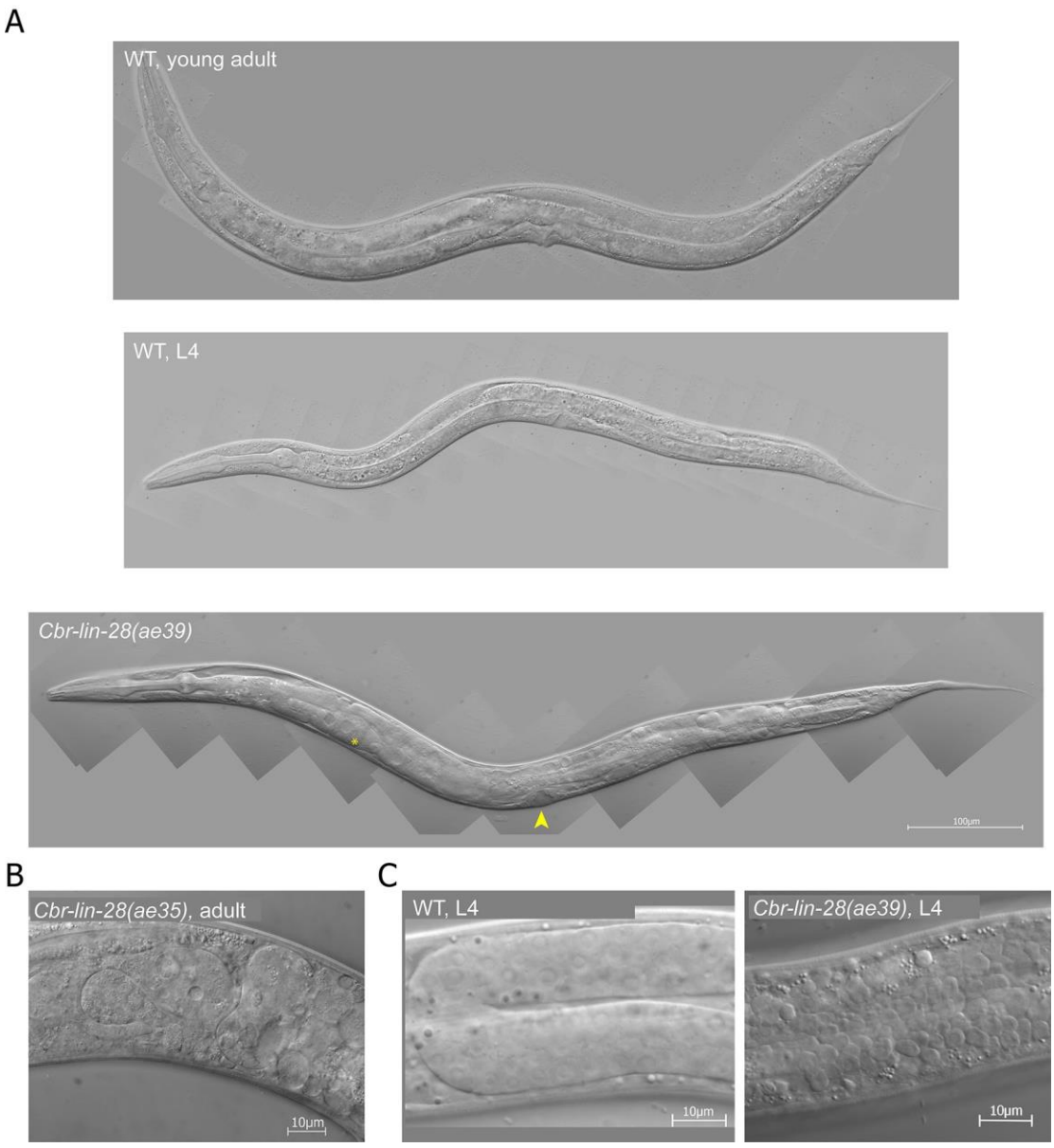



*Figure 3.10.* Developmental timing defects in *Cbr-lin-28(0)* mutants cause an L4 arrest and gonad disintegration. DIC photomicrographs of animals grown at 20°C. (A) Wild-type (AF16) L4 larva and young adult compared to *Cbr-lin-28(ae39)* with "arrested L4" phenotype. Arrowhead indicates L4-like vulva (larval trait) and asterisk indicates oocytes (adult trait). (B) A closer view at the disorganized gonad of a *Cbr-lin-28(ae35)* adult. (C) An earlier stage gonad disorganization that occurs in some *Cbr-lin-28(ae39)* mutant L4

larvae compared to a normal gonad. All animals are oriented anterior end left, dorsal side up.

The gonads of *Cbr-lin-28(0)* mutants became disorganized or disintegrated after animals arrested in L4 or reached adulthood. Gonad contents leaked into the pseudocoelom and sometimes the gonad fell apart into separate cells (Fig. 3.10B). In some cases, gonad disorganization was not visible at first but manifested later (Fig. 3.11). The underlying defect is unknown. Some mutants developed through the L4 stage and had normal vulvae and adult alae, but became stuck in the L4 cuticle during molting. Those few that completed the L4 molt ("successfully molted") were not morphologically different from the wild type, although sometimes they had disorganized gonads.

In contrast to what happens in *C. elegans*, *Cbr-lin-28(0)* mutants had only weak heterochronic defects: most animals had a small patch of precocious alae near the pharynx at the L4 stage, and the number of seam cells was similar to the wild type (Fig. 3.12A). Undergoing dauer development suppressed precocious alae, but did not suppress L4 arrest (Fig. 3.12B and C). By contrast, dauer development completely suppresses *C. elegans lin-28(0)* heterochronic defects (Liu and Ambros 1991). Some other observed phenotypes included rolling (less than 10%), protruding vulvae (10-20%), larvae stuck at L2 and L3 molts, incompletely shed cuticles ("belts"), and sometimes gonads losing their structure during the L4 stage (Fig. 3.10C).

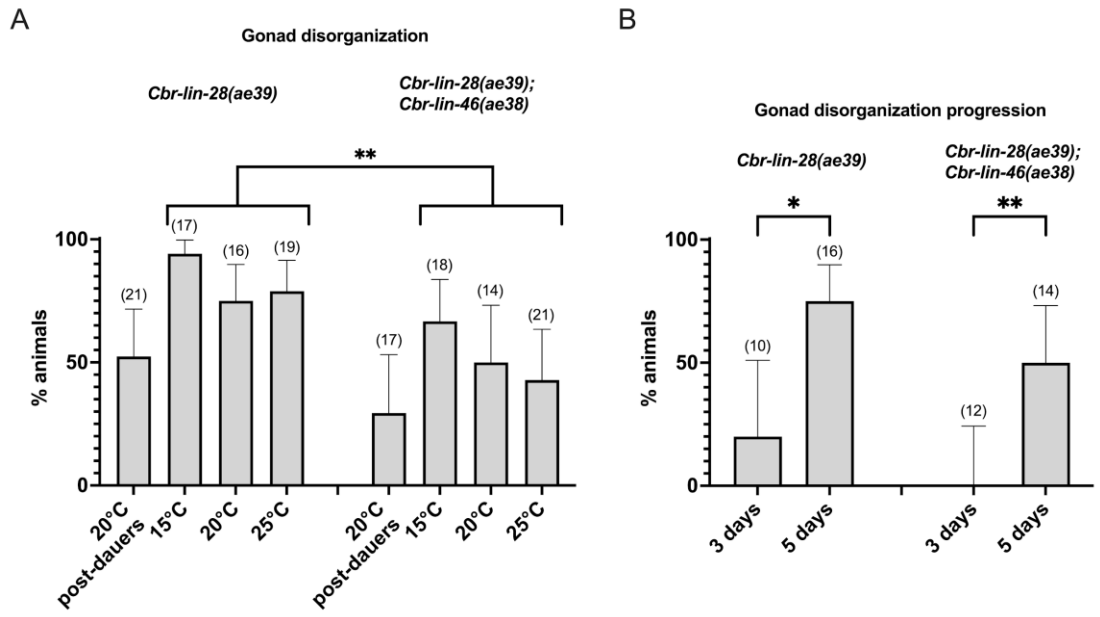



*Figure 3.11.* Gonad disorganization manifests with age and is slightly suppressed by *Cbr-lin-46(0)* in *Cbr-lin-28(0)* mutants**.** (A) Gonad disorganization in *Cbr-lin-28(0)* mutants is partially suppressed by the dauer pathway, although *p-value* was > 0.05 between post-dauers and normal animals at 20°C. *Cbr-lin-46(0)* mutation partly suppressed *Cbr-lin-28(0)* gonad disorganization, $p < 0.005$ when the data from all temperatures was combined in one data set in each strain. (B) Gonad disorganization manifests with age. In both *Cbr-lin-28(0)* and *Cbr-lin-28(0)*; *Cbr-lin-46(0)* double mutants, gonad disorganization was observed in few animals within 24 hours after they reached the adult or arrested L4 stage, 2 days later a higher fraction of animals had disorganized gonads (20°C). Animals' age after hatching is indicated below the chart. Statistical analysis is described in Materials and Methods.

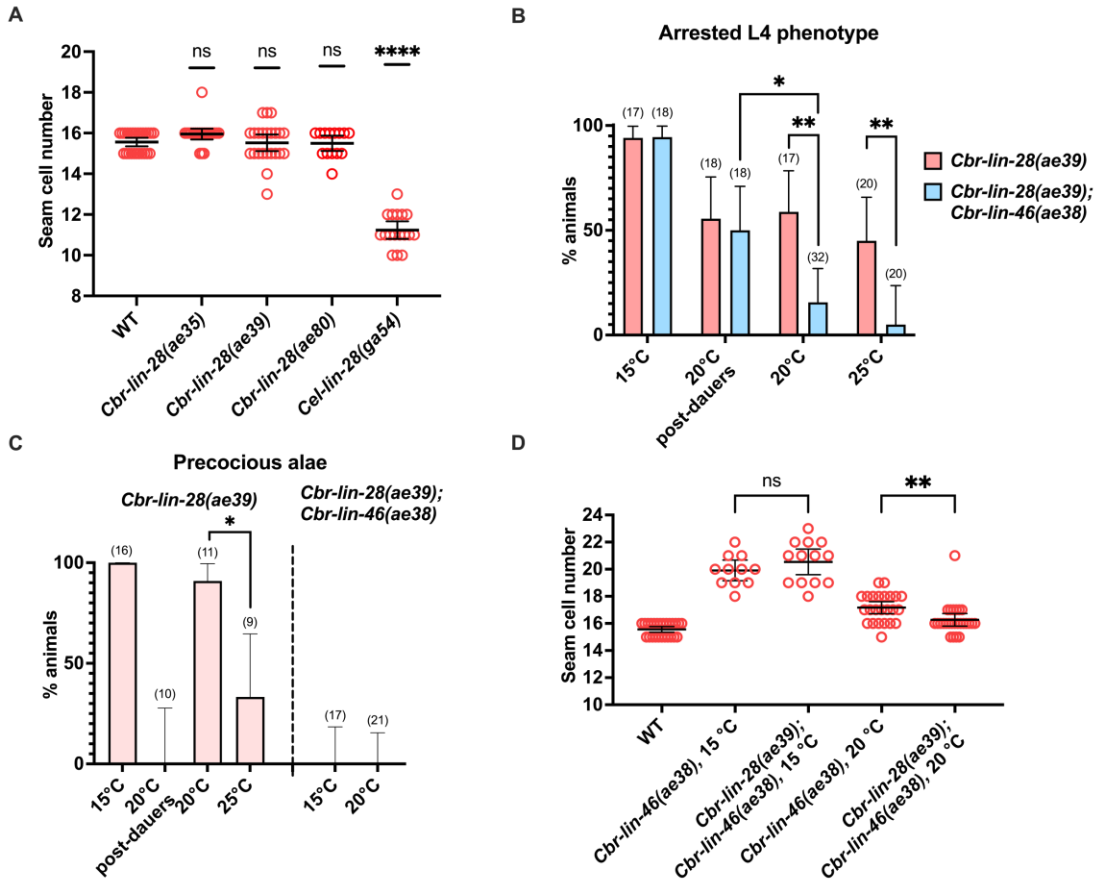



*Figure 3.12. Cbr-lin-28(0)* mutant phenotypes and their suppression by *Cbr-lin-46(0)* allele. (A) *Cbr-lin-28(0)* mutants do not have a reduced number of seam cells at 20°C, unlike *Cel-lin-28(0).* (B) L4 developmental arrest was more penetrant in the *Cbr-lin-28(0)* mutant at 15°C than at 20°C (both dauers and post-dauers, $p \leq 0.05$). It also was not suppressed by *Cbr-lin-46(0)* mutation at 15°C in contrast to 20°C and 25°C. Those animals that did not arrest their development at L4, had "successfully molted" or intermediate phenotypes. Sample sizes are specified in parenthesis above the bars. (C) Precocious alae of *Cbr-lin-28(0)* occurred less often at 25°C and were suppressed by dauer pathway and *Cbr-lin-46(0)* mutation at 15°C and 20°C. (D) Increased seam cell

number of *Cbr-lin-46(0)* mutant was suppressed by *Cbr-lin-28(0)* mutation at 20$^{o}$C but not at 15$^{o}$C. Statistical analysis is described in Materials and Methods.

*Cbr-lin-28*'s mutant phenotype was cold-sensitive. At 15°C, most animals became arrested L4s and all had precocious alae (n = 16) (Fig. 3.12B and C). Moreover, patches of precocious alae were longer and some animals had complete precocious alae. Additionally, the strain could not be maintained at 15°C. Some animals produced eggs at this temperature but they did not hatch, although eggs placed at 15°C after the mothers were grown at 25°C were viable and did hatch, suggesting a maternal effect embryonic problem at cold temperatures.

To confirm that the *Cbr-lin-28* mutant phenotypes that we observed are indeed those of null alleles, we generated a deletion (*ae80*) that removed 78% of the 206 amino-acid coding region. This deletion starts within the CSD RNA-binding domain and deletes both CCHC zinc knuckles, so that the 46 remaining amino acids contain none of *Cbr-lin-28*'s known functional domains.

*Cbr-lin-28(ae80)* L4 larvae had 15.5+/-0.7 (n = 14) seam cells, and 42% (n = 12) had precocious alae patches. After 24 hours, all of 30 L4 larvae picked to a separate plate had vulvae arrested at early L4 stages (L4.2-L4.3 according to Mok *et al.*, 2015), produced oocytes (most of them also had embryos), had L4 cuticles (60% had precocious alae patches, n = 15), and disorganized gonads.

Therefore, the phenotypes of *Cbr-lin-28(ae80)* worms were not significantly different from those of other alleles (*ae35* and *ae39*). Heterochronic defects remained weak, and

L2 seam cell divisions were not skipped. The penetrance of the "arrested L4" phenotype might be higher in this strain since no "successfully molted" worms were observed among the 30 isolated L4, although some advanced-stage worms (with adult-like vulva) were rarely observed on plates as well as worms with protruding vulva.

Overall, *Cbr-lin-28(0)* has only a minor resemblance to *Cel-lin-28(0)*, pleiotropic effects, and variable penetrance and expressivity for most phenotypes.

### **Cbr-lin-28** ***is Expressed at All Stages in*** **C. briggsae** ***and Down-Regulated in Seam Cells After the L1 and L3 Stages***

In *C. elegans, lin-28* shows a characteristic “on early, off late” expression pattern that parallels its function in controlling L2 fates, and this temporal down-regulation is a consequence of miRNAs acting via its 3’UTR (Moss *et al.* 1997; Tsialikas *et al.* 2017). Because the phenotype of mutant *Cbr-lin-28* differed from that of its *C. elegans* ortholog, we examined the expression of *Cbr-lin-28* to see if that was different as well.

We employed a transgenic approach that had been successfully used in *C. elegans* which creates multicopy extrachromosomal arrays of plasmids (Stinchcomb *et al.* 1985; Mello *et al.* 1991). A full-length translational fusion with GFP that included intact 5’ and 3’ regulatory regions was constructed (Fig. 3.13) The construct was injected into wild-type *C. briggsae,* producing a stable extrachromosomal array *aeEx45*.

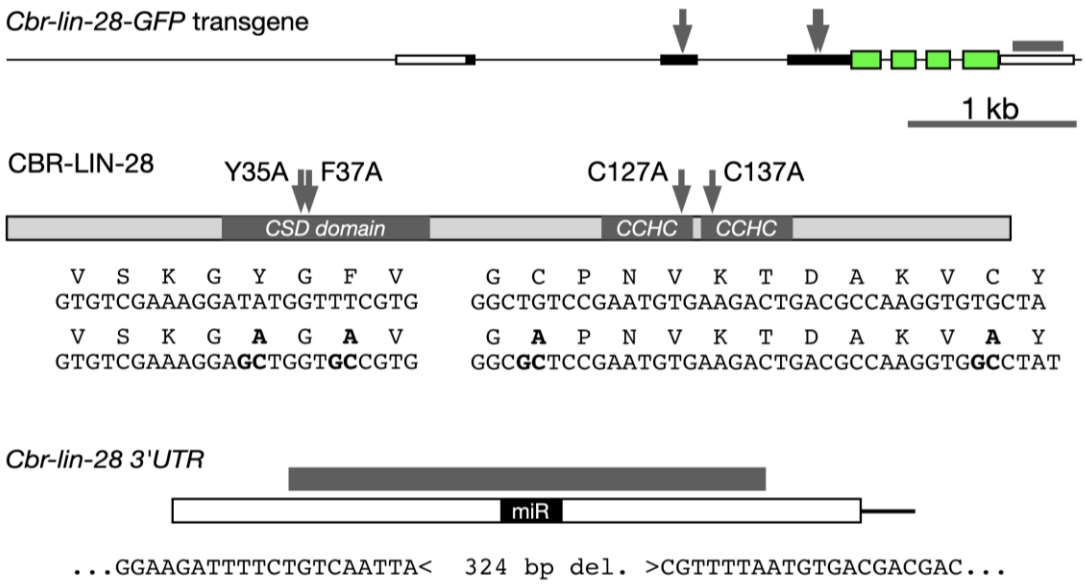



*Figure 3.13. Cbr-lin-28::GFP* transgenes. Top: An approximately 5.5 kb genomic sequence containing the *Cbr-lin-28* coding region and 5' and 3' regulatory regions was cloned into a plasmid. A nematode-optimized GFP was fused in-frame upstream of the stop codon (green). Arrows show locations of mutations in CSD and CCHC domains that inactivate the protein. Gray box above the 3'UTR shows the extent of the deletion that removes the miRNA sites. Middle: A schematic of the CBR-LIN-28 protein showing the locations of inactivating mutations in the CSD domain and CCHC motifs. Sequences of wild type and mutant transgenes are shown. Bottom: A schematic of the *Cbr-lin-28* 3'UTR showing the location of the recognition sites for let-7 and lin-4 miRNAs (miR). Grey bar shows the extent of the deletion removing the miRNA sites. The sequences flanking the deletion are shown.

In transgenic animals, GFP fluorescence was observed in head and tail neurons, motor neurons, muscles (including the pharynx), intestinal cells, and seam cells (Fig. 3.14), which is similar to *C. elegans*, except that *Cel-lin-28::GFP* had obvious expression throughout the hypodermis (Moss *et al.* 1997). In contrast to *C. elegans*, most GFP expression did not appreciably decline with age.

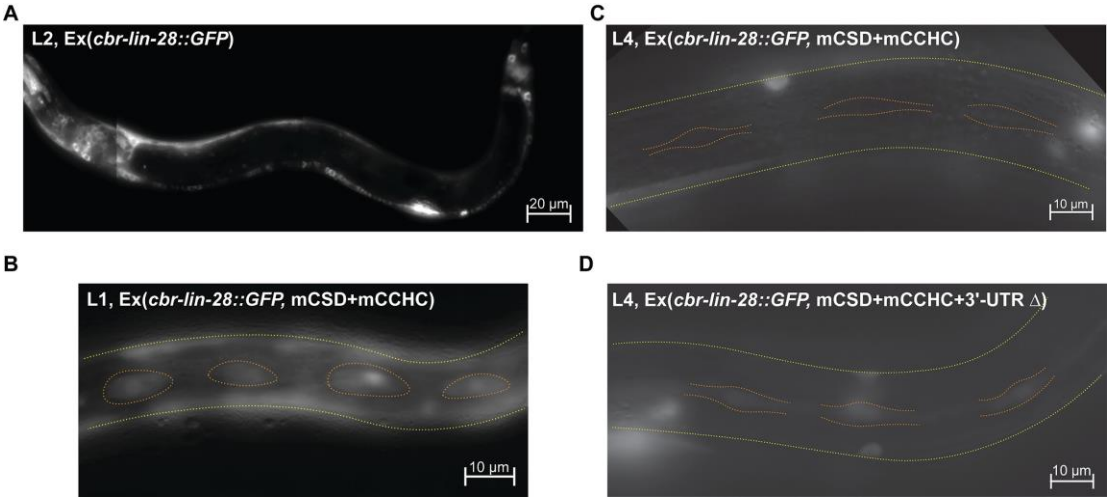



*Figure 3.14.* The expression of LIN-28 is down-regulated during *C. briggsae* development. Fluorescent microscopy images, green channel. All animals are oriented anterior end left, dorsal side up. Yellow dotted line indicates sides of animals, orange dotted line outlines seam cells. (A) An AF16 L2 larva from a brood of animals carrying *Cbr-lin-28::GFP* on an extrachromosomal array. The expression is visible in neurons, pharynx, and P-cells. (B) Fluorescing seam cells in an L1 larva expressing *Cbr-lin-28::GFP* with mutated CSD and CCHC domains. (C) Seam cells not glowing in an L4 larva expressing *Cbr-lin-28::GFP* array with mutated CSD and CCHC domains and an

intact 3’UTR. (D) Seam cells glowing in an L4 larva with *Cbr-lin-28::GFP* array with mutated CSD and CCHC domains and a 3'UTR deletion.

About 30% of fluorescing animals also had alae gaps. Extrachromosomal arrays might exceed wild-type levels of expression since stable transgenes contain several copies of the gene (Mello *et al.* 1991). Thus, it is possible that our construct resulted in overexpression of *Cbr-lin-28*—possibly by overcoming miRNA repression—to cause a weak reiterative phenotype. Furthermore, we observed that embryos showing very bright GFP fluorescence failed to hatch or died soon after hatching; only 2 larvae hatched out of 34 brightly fluorescing eggs, suggesting that very high *Cbr-lin-28* expression causes an embryonic lethal phenotype.

To address the possibility that overexpression of the transgene affected *Cbr-lin-28* regulation, we generated *aeEx46* transgene with mutations in the *lin-28* protein’s two functional domains, the CSD (Y35A, F37A) and the CCHC zinc fingers (C127A, C137A) (Fig. 3.13). The GFP fluorescence was still observed at all stages and did not decline except for seam cells. In seam cells, robust fluorescence was observed mostly at the L1 stage. Weaker fluorescence was observed at L2 and L3 stages and occurred less often than at the L1 stage, and no fluorescence was observed at the L4 stage (Table 3.3).

**Table 3.3**

*Percents of Animals with Fluorescent Seam Cells in Strains with a Cbr-lin-28::GFP Extrachromosomal Array*

| Array | | Stage | | | | |
|---|---|---|---|---|---|---|
| | | L1 | L2 | L3 | L4 | young adult |
| *Cbr-lin-28-GFP mCSD+mCCHC* | 1 | 73% (15) | 42% (12) | 28% (18) | 0% (24) | 0% (11) |
| | 2 | 70% (20) | 18% (17) | 11% (19) | 0% (19) | 0% (5) |
| | 3 | 40% (15) | 41% (17) | 31% (13) | 0% (28) | 0% (2) |
| *Cbr-lin-28-GFP mCSD+mCCHC+3'-UTR del* | | 75% (16) | 50% (14) | 35% (20) | 17% (23) | 11% (18) |

*Note.* The number in parentheses is the total number of fluorescent animals observed; percents are those with at least one fluorescent seam cell. mCSC: mutations in the Cold Shock Domain; mCCHC: mutations in the CCHC zinc fingers (see Fig. 3.13).

We further modified the transgene construct to contain a deletion in the 3'UTR to remove miRNA sites (Fig. 3.13). Animals with *aeEx48* transgene encoding a mutant protein and a 3'UTR deletion did not show any differences in the place and timing of *Cbr-lin-28* expression, except for seam cells. Fluorescing seam cells were observed less often after the L1 stage, similar to arrays with an intact 3'UTR. Nevertheless, they were observed in L4 and young adult animals in this strain, in contrast to 3 independently generated strains bearing the CSD/CCHC mutant with an intact 3'UTR (Table 3.3, Fig. 3.14B, C, and D). Thus, *Cbr-lin-28* is down-regulated in seam cells in late larval development in part via its 3'UTR.

Overall, the marked difference in expression between *C. elegans* and *C. briggsae* parallels the differences in phenotype, where it seems *Cbr-lin-28* has a broader role in the

animal than *Cel-lin-28*. Nevertheless, 3'UTR-dependent down-regulation in seam cells occurs in both species.

### **Cbr-lin-46(0)** ***Mutants are Similar to*** **Cel-lin-46(0)** ***Mutants***

In *C. elegans, lin-46* was discovered as a suppressor of *lin-28(0)* phenotype (Pepper *et al.* 2004). *lin-46(0); lin-28(0)* double mutants appear mostly as wild type, whereas *lin-46(0)* single mutants have alae gaps and an increased number of seam cells, with both defects being cold-sensitive. *lin-46* encodes an unusual protein with protein-protein interaction activity.

A *Cbr-lin-46(0)* allele was generated by targeting the second exon: the *Cbr-lin-46(ae38)* mutation is an insertion causing a frameshift that results in a premature stop codon. A second allele, *Cbr-lin-46(ae44)*, has a deletion removing the start-codon (Table 3.1; Fig. 3.7).

The null mutant has a weak reiterative phenotype similar to *Cel-lin-46(0)* mutants. In *C. briggsae*, around 65% of the animals had alae gaps at either 20°C or 25°C, and over 80% at 15°C (Fig. 3.15B). The penetrance at higher temperatures is higher than is seen for *Cel-lin-46(0)* animals, but the cold sensitivity is shared (Pepper *et al.* 2004). In both species, the number of seam cells was significantly higher at 15°C (Fig. 3.15C). There were also slight egg-laying defects, and vulvae were often abnormally shaped in *C. briggsae* (Fig. 3.15A, Fig. 3.16). Thus, *Cbr-lin-46(0)* mutants have similar defects and cold sensitivity as *Cel-lin-46(0)* mutants, suggesting that the orthologs have similar functions. Furthermore, the fact that null alleles are cold sensitive in both species implies that a process that is exposed by the loss of *lin-46* has inherent cold sensitivity.

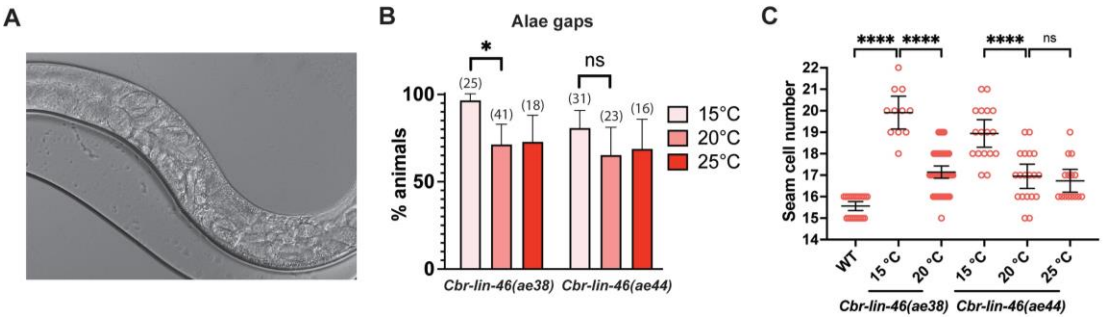



*Figure 3.15.* Null mutants of Cbr-lin-46 have a reiterative phenotype. (A) A DIC photomicrograph of a cbr-lin-46(ae38) adult animal with an egg-laying defect and accumulated embryos at 20°C. The worm is oriented anterior end left, dorsal side up. (B) Alae gaps in Cbr-lin-46(0) mutants are slightly more frequent at 15°C. Sample sizes are specified in parenthesis above the bars.(C) Cbr-lin-46(0) mutants have increased numbers of seam cells that are also cold-sensitive.

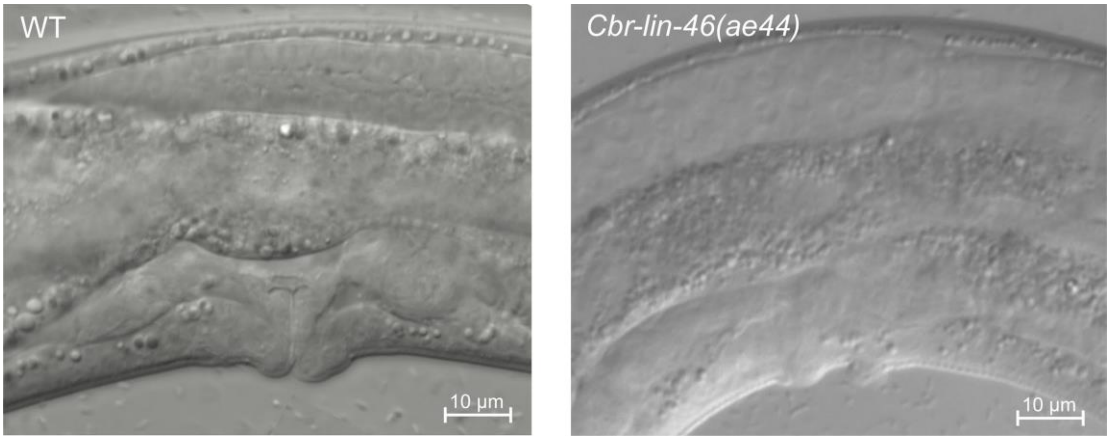



*Figure 3.16. Cbr-lin-46(0)* mutants have slight vulval developmental defects. A DIC microphotograph of a *Cbr-lin-46(ae44)* animal with vulval protrusions (20°C) compared to the vulva of a wild-type animal.

**Cbr-lin-46(0) *Partially Suppresses the* Cbr-lin-28(0) *Phenotype***

To see whether the relationship between *Cbr-lin-28* and *Cbr-lin-46* is conserved despite the drift in *lin-28's* role, we constructed a *Cbr-lin-46(ae38)*; *Cbr-lin-28(ae39)* double null mutant. Surprisingly, we found that *Cbr-lin-46(0)* suppressed not only the precocious alae defect of *Cbr-lin-28(0)* mutants, but that the L4 developmental arrest was partly suppressed (although not at 15°C), and that the gonad disorganization was partly suppressed at all temperatures (Fig. 3.12B and C, Fig. 3.11, Fig. 3.17).

Interestingly, *Cbr-lin-46(0)* did not suppress the L4 arrest phenotype when the double-mutant passed through dauer: the fraction of L4-arrested animals at 20°C in *Cbr-lin-28(0); Cbr-lin-46(0)* double mutants that had passed through dauer was comparable to the fraction of "arrested L4" animals in *Cbr-lin-28(0)* post-dauers at 20°C (Fig. 3.12B). This suggests that different downstream effectors exist for *Cbr-lin-28* in continuous development and dauer development.

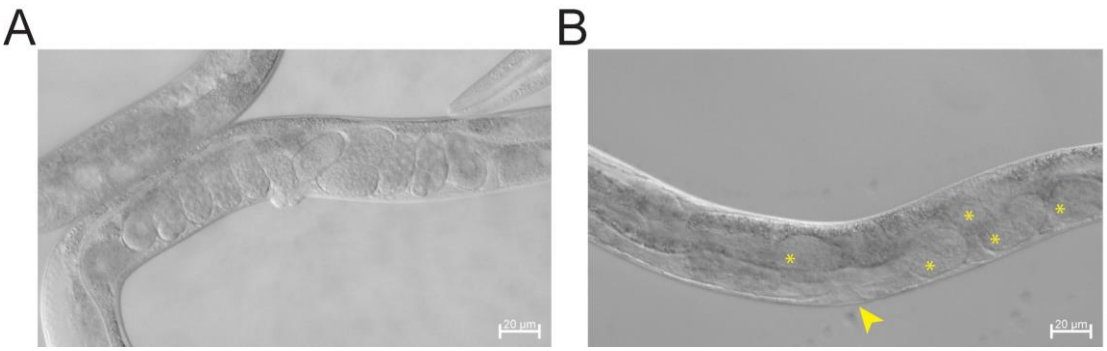



*Figure 3.17. Cbr-lin-28(0); Cbr-lin-46(0)* double mutants sometimes arrest at L4. DIC microphotographs showing (A) a successfully molted *Cbr-lin-28(ae39); Cbr-lin-46(ae38)* animal with adult vulva and eggs; (B) a *Cbr-lin-28(ae39); Cbr-lin-46(ae38)*

animal with an arrested L4 phenotype. Arrowhead points at the L4-shaped vulva and asterisks indicate embryos.

We also observed some reciprocal suppression: the increased number of seam cells in *Cbr-lin-46(0)* mutants was suppressed by the *Cbr-lin-28(0)* mutation at 20°C, although not at 15°C (Fig. 3.12D). By contrast, successfully molted double mutants had alae gaps at comparable rates to the *Cbr-lin-46(0)* single mutants (63.1%, n = 19 at 20°C and 61.5%, n = 13 at 25°C, Fig. 3.15B). Thus, some of the reiterative traits of *Cbr-lin-46(0)* were not suppressed by *Cbr-lin-28(0)*, which is in contrast to what occurs in *C. elegans* (Pepper *et al.* 2004).

**The Cbr-lin-46 *5'UTR Mutant Phenotype Differs from that of a* Cbr-lin-28(0) *Mutant***

In *C. elegans*, the *lin-46* 5'UTR is a regulatory region through which *lin-28* acts to inhibit *lin-46* expression; small deletions in this sequence causes a phenotype that resembles the *Cel-lin-28(lf)* phenotype (Ilbay *et al.* 2021). This 36-nt 5'UTR is conserved among all species of *Caenorhabditis* and is identical between *C. elegans* and *C. briggsae*. We created a 6-bp deletion (allele *ae43*) in this region using CRISPR/Cas9 (Table 3.1, Fig. 3.18A). The *Cbr-lin-46* 5'UTR mutants had protruding vulvae and either full or gapped precocious alae at the L4 stage; however, they had the same number of seam cells as the wild type (Fig. 3.18B, C, and D). This phenotype resembles the heterochronic traits of *Cbr-lin-28(0)*, however, the penetrance and expressivity of the alae defect are more severe in the *Cbr-lin-46(gf)* mutant. Significantly, the *Cbr-lin-46* 5'UTR mutant lacks the larval-arrest and gonad disintegration defects of the *Cbr-lin-28(0)* mutants. Thus, the role

of *Cbr-lin-46* in developmental timing resembles that of *Cel-lin-46*, but the fact that the phenotype of the *Cbr-lin-46* 5'UTR deletion differs substantially from that of *Cbr-lin-28(0)* suggests that the relationship between *lin-28* and *lin-46* has drifted as these species evolved.

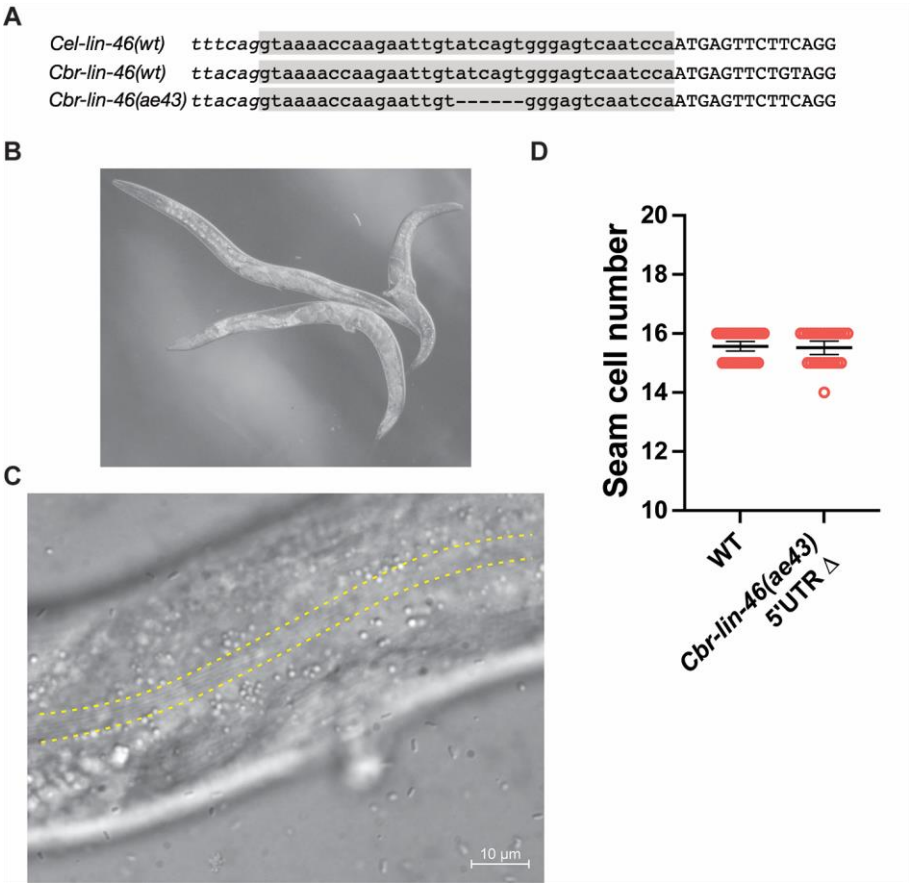



*Figure 3.18.* A deletion in *Cbr-lin-46*'s 5'UTR causes a precocious phenotype. (A) The 5'UTR of *C. elegans* and *C. briggsae lin-46* and the *Cbr-lin-46(ae43)* allele. The gray box highlights the conserved 36-nt 5'UTR, with a splice acceptor for a trans-spliced leader to the left and the start of the ORF to the right. (B) DIC microphotograph of *Cbr-lin-46* 5'UTR deletion mutants, showing protruding vulvae and accumulated eggs. (C)

DIC microphotograph of a cuticle of *Cbr-lin-46(ae43)* L4 larva with precocious alae (outlined by a dotted line). (D) *lin-46* 5'UTR deletion mutants do not skip L2 stages (20°C).

## mir-241, mir-48, *and* mir-84 *Have a Conserved Function in* C. elegans *and* C. briggsae

In *C. elegans*, three *let-7*-family miRNAs, *mir-48*, *mir-84*, and *mir-241* (the "*3let-7s*"), redundantly control *hbl-1* and *lin-28*: a strong phenotype appears when all 3 are knocked out, causing reiteration of L2-specific cell fates, whereas single mutants have little or no effect (Abbott *et al.* 2005; Tsialikas *et al.* 2017). By contrast, in *C. briggsae*, a *Cbr-mir-48(0)* mutation alone yielded a strong phenotype: *Cbr-mir-48(ae65)* mutant burst at the vulva at the end of the L4 molt, had an increased number of seam cells, and incomplete adult alae (Table 3.1, Fig. 3.19A and B). We saw that 95% of mutant adults had less than half of the normal amount of adult alae, and 11% lacked alae entirely (n = 44).

The *Cbr-mir-48* and *Cbr-241* genes are within 3kb of each other on linkage group V. A deletion that removed both *Cbr-mir-48* and *Cbr-mir-241* (allele *ae73*, Table 3.1) had a more severe phenotype than *Cbr-mir-48(ae65)* alone; 92% of adult animals lacked alae altogether (n = 13). However, mutants that had developed through the dauer pathway did not burst at the vulva and appeared wild-type, indicating that these mutations, like their *C. elegans* counterparts, are suppressed by the dauer developmental pathway (data not shown).

Deleting all three miRNAs resulted in animals that could not be maintained as homozygotes because most were sterile. However, the frequency of alae patches in *Cbr-mir-48 Cbr-mir-241(ae73); Cbr-mir-84(ae70)* animals segregating from heterozygotes was similar to that of the double mutant: 92% lacked alae (n = 13). Furthermore, this triple mutant did not show an increase in the number of seam cells compared to the *Cbr-mir-48(0)* single mutant (Fig. 3.19C).

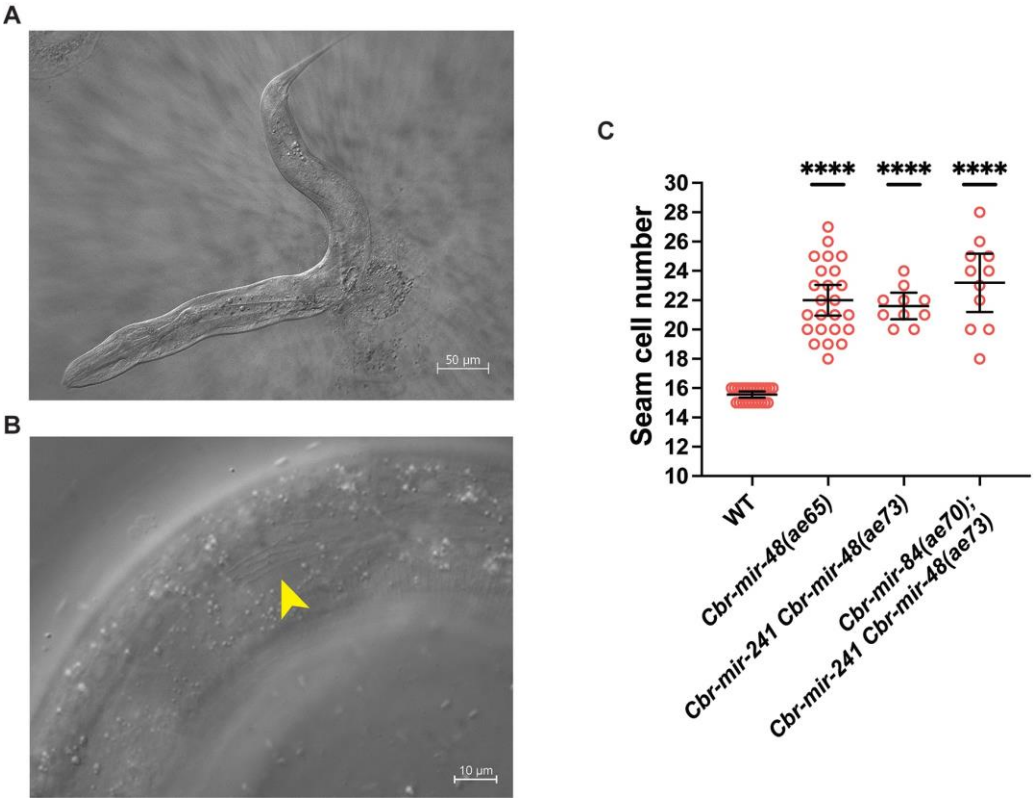



*Figure 3.19. Cbr-3let-7s* mutants have reiterative phenotypes. (A) DIC micrograph of a *Cbr-mir-48(ae65)* mutant that has burst at vulva after reaching adulthood. (B) *Cbr-mir-48(ae65)* young adults develop alae patches (indicated by an arrowhead). (C) *Cbr-mir-48(ae65)* have an increased number of seam cells, but adding *Cbr-mir-241(0)* and *Cbr-*

*mir-84(0)* mutations does not cause further increase in the seam cell number. The difference between mutant groups was not statistically significant ($p > 0.05$).

In other aspects, the *Cbr-mir-241(0)* and *Cbr-mir-84(0)* single null mutants and the *Cbr-mir-241(0); Cbr-mir-84(0)* double null mutants had few differences from the wild type. There were no alae gaps and the number of seam cells was close to normal at both 20°C and 15°C (Fig. 3.20). A few animals had egg-laying defects and abnormal vulvae (Fig. 3.21), and some of these egg-laying defective animals appeared to be stuck in lethargus (not pumping). Finally, a small percentage of sterile animals was observed in these strains (Table 3.4).

**Table 3.4**

*Phenotypes of* Cbr-mir-241(0) *and* Cbr-mir-84(0) *Mutants*

| | Egl | Sterile | Lethargic | N |
|---|---|---|---|---|
| Wild type | 2.5% (7) | 0 | 0.7% (2) | 283 |
| Wild type | 0 | 0 | 0 | 174 |
| *Cbr-mir-241(ae64)* | 3% (11) | 1.1% (4) | 1.4% (5) | 363 |
| *Cbr-mir-84(ae68)* | 6% (11) | 0 | 1.6% (3) | 182 |
| *Cbr-mir-84(ae69)* | 8% (25) | 0.32% (1) | 1.3% (4) | 313 |
| *Cbr-mir-241(ae64); Cbr-mir-84(ae69)* | 13.1% (23) | 0 | 1.7% (3) | 175 |
| *Cbr-mir-241(ae64); Cbr-mir-84(ae70)* | 10.5% (17) | 0 | 1.85% (3) | 162 |

*Note.* Egg-laying defective (Egl) animals accumulated eggs in the uterus. Lethargic animals had no pharyngeal pumping. Animals with both traits were included in both columns. Wild-type animals were observed twice. On less crowded plates, no Egl animals were observed, on a more crowded plate 2.5% were Egl.

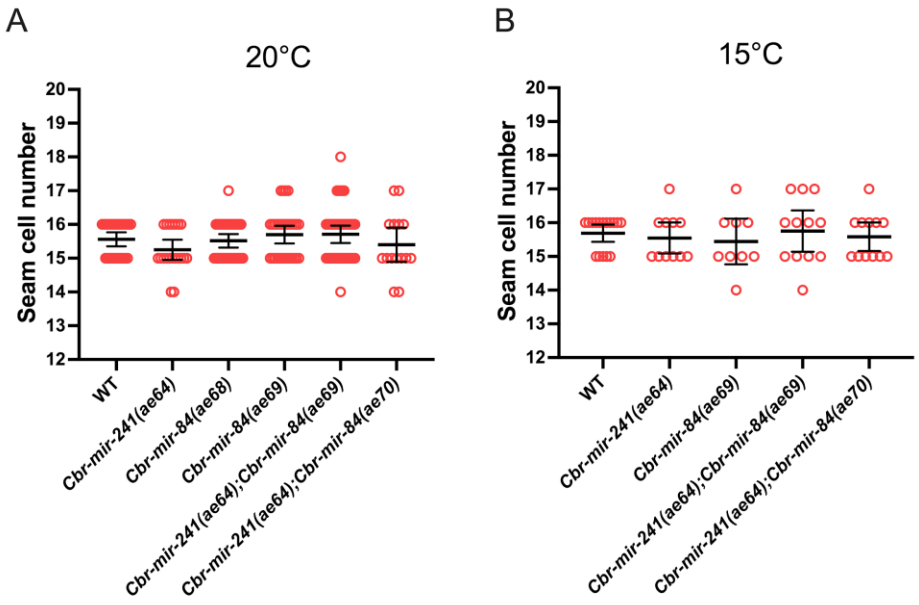



*Figure 3.20. Cbr-mir-241(0), Cbr-mir-84(0),* single and double mutants do not have reiterations of L2 stages at (A) 20°C or (B) 15°C. There was no statistically significant difference between WT and any other group at both temperatures (*p-value* > 0.05). Statistical analysis is described in Materials and Methods.

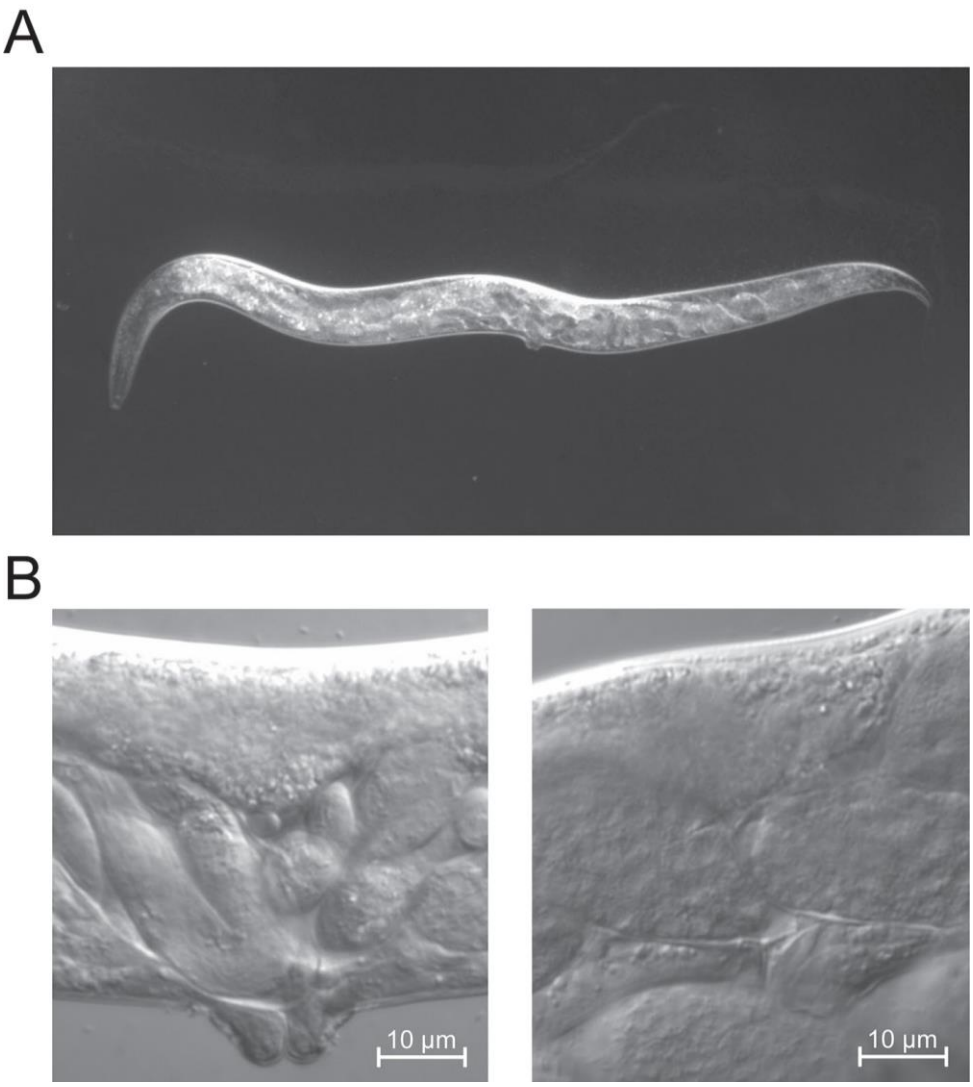



*Figure 3.21*. *Cbr-mir-241(0)*; *Cbr-mir-84(0)* double mutants occasionally develop egg-laying defects. DIC microphotographs showing (A) a *Cbr-mir-241(ae64); Cbr-mir-84(ae70)* double mutant with accumulated eggs; other *Cbr-mir-241(0)*, *Cbr-mir-84(0)* single and double mutants similar occasional phenotypes; (B) abnormal vulvae shapes of the *Cbr-mir-241(ae64); Cbr-mir-84(ae70)* double mutants; other *Cbr-mir-241(0)*, *Cbr-mir-84(0)* single and double mutants occasionally had similar abnormal vulvae.

In *C. elegans*, mutations in *lin-4* and the three *let-7*-related miRNAs *mir-48*, *mir-84*, and *mir-241*, have distinct phenotypes: deletion of *lin-4* causes reiteration of L1-specific fates and deletion of all of the *3let-7s* causes reiteration of L2-specific fates (Chalfie *et al.* 1981; Abbott *et al.* 2005). All four of these miRNA genes have been shown to act together in stage-specifically downregulating *lin-28* to ensure appropriate expression of L2 fates (Tsialikas *et al.* 2017). Because we found that mutations in *Cbr-lin-4* and *Cbr-3let-7s* both cause reiteration of L2 fates, we tested whether these mutations enhanced one another, leading to a reiteration of the earlier L1 fates. To our knowledge, the equivalent mutant of *C. elegans* has not been reported. A *Cbr-lin-4* deletion was introduced into *Cbr-mir-241(0), mir-48(0), mir-84(0)* mutant background (also containing *Cbr-hbl-1::AID; TIR1(F79G)*; see below). The quadruple mutants were vulvaless with gapped alae at adulthood (data not shown). However, in contrast to *Cbr-lin-4(0)* mutants, late L2 larvae of the quadruple mutant had a lower number of seam cells (mean = 12.4+/-1.3, n = 14), suggesting that some seam cells reiterated L1 fates. The phenotype resembles the *Cbr-lin-14* 3'UTR deletion, suggesting that *Cbr-3let-7s* participate in the downregulation of *Cbr-lin-14*, which contains let-7 sites in its 3'UTR.

### *Auxin-Inducible Degron System in* C. briggsae

In *C. elegans*, certain heterochronic genes have pleiotropic phenotypes that include embryonic lethality or infertility. Anticipating that these genes might have similar pleiotropies in *C. briggsae*, we used the auxin-inducible degron system to generate conditional alleles. We produced lines of *C. briggsae* expressing *TIR1(F79G)* from extrachromosomal (*aeEx44)* and attached (*aeIs15)* arrays (Hills-Muckey *et al.* 2021). The

attached transgene (*aeIs15*) was located by crossing with marker strains and found to be on LGII.

Unexpectedly, both the extrachromosomal and attached *TIR1(F79G)* arrays caused a reduction in the number of intestinal nuclei (Table 3.5). However, no other abnormalities were observed and the animals appeared to develop normally and be healthy. The reason for this reduction in intestinal nuclei is unclear. The intestinal nuclei glowed brightly, indicative of high array expression.

**Table 3.5**

*Intestinal Nuclei Number in Strains Expressing TIR1(F79G)*

| Genotype | Average number of intestinal nuclei at L3 and older |
|---|---|
| Wild type | 33.1+/-1.8 (n = 60) |
| *TIR1(F79G) II* without auxin | 25.7+/-3.3 (n = 17) |
| *TIR1(F79G) II*; *Cbr-hbl-1::AID* on 5-Ph-IAA | 22.6+/-2.5 (n = 10) |
| *TIR1(F79G) II*; 3let-7s, *Cbr-hbl-1::AID* on 5-Ph-IAA | 25.7+/-2.7 (n = 15) |

**Cbr-hbl-1(lf) *Causes a Precocious Phenotype like* Cel-hbl-1(lf)**

In *C. elegans, hbl-1* encodes an Ikaros-family transcription factor involved in hypodermis development where null alleles are embryonic lethal and weak alleles have a heterochronic phenotype with reduced number of seam cells, precocious alae, and a protruding vulva, resembling *lin-28(0)* (Fay *et al.* 1999; Lin *et al.* 2003; Abrahante *et al.* 2003). To study *Cbr-hbl-1* loss-of-function while avoiding potential embryonic lethality,

the locus was tagged with an auxin-inducible degron (AID) using CRISPR/Cas9 (Table 3.1). The *hbl-1::AID* strain was then crossed with lines bearing the *TIR1(F59G)* transgene to generate lines in which *Cbr-hbl-1* activity could be reduced in response to the auxin analog 5-Ph-IAA (Hills-Muckey *et al.* 2021).

Adult animals were placed on plates with 0.01-0.02 µmol of 5-Ph-IAA, and the phenotypes of the next generation were characterized. We saw that 47% of animals on those plates had fully precocious alae, and the remainder had gapped precocious alae (n = 30). There was no embryonic lethality. However, 82% of adult animals remained stuck in the L4 molt, and 27% of mutants had a precocious vulval differentiation (reaching the "christmas tree", or L4.4-L4.5 according to Mok, *et al.*, 2015, stage of morphogenesis by the end of the L3 stage) or a protruding vulva (Fig. 3.22B). As in *C. elegans hbl-1(lf)*, the protruding vulva developed during the L4 stage. Other animals had normal vulval development and a functional vulva. There was a slight reduction in seam cell number in some *Cbr-hbl-1(lf)* animals. However, the reduction was not as significant as in *Cel-hbl-1::AID* under similar conditions (Fig. 3.22C). These observations show that the functions of *hbl-1* are at least partly conserved between *C. elegans* and *C. briggsae*.

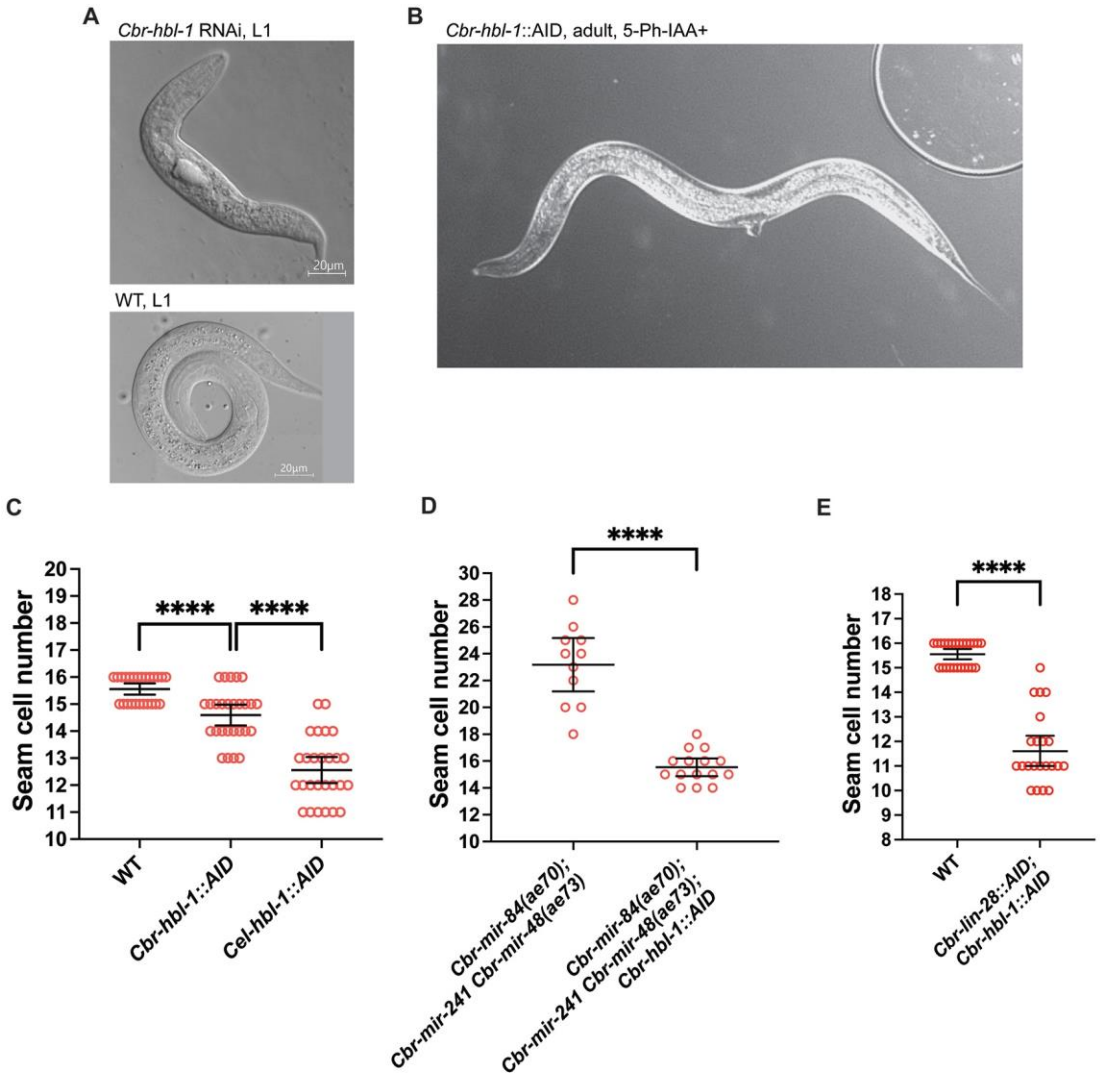



*Figure 3.22. Cbr-hbl-1* is required for early development and promotes L2 seam cell fates. (A) DIC micrograph of a wild-type L1 larva and deformed L1 larva from *Cbr-hbl-1* dsRNA injections. These animals do not survive and develop into adults. (B) A *Cbr-hbl-1::AID* young adult grown on 5-Ph-IAA. Notice a protruding vulva. (C) Some *Cbr-hbl-1::AID* mutants have a slightly reduced number of seam cells in the presence of 5-Ph-IAA, but the reduction is not as large as for *Cel-hbl-1::AID*, which may due to differences in *hbl-1* orthologs functions or a difference in the degradation efficiency. (D) *Cbr-hbl-1* depletion on 5-Ph-IAA suppresses increased seam cell numbers in the *Cbr-*

*3let-7s* triple mutant. *Cbr-3let-7s* seam cell counts are the same data set as in Fig. 6,C. (E) Combined depletion of *Cbr-lin-28::AID* and *Cbr-hbl-1::AID* on 5-Ph-IAA causes a reduced number of seam cells.

To test whether a more drastic reduction of *Cbr-hbl-1* activity could cause embryonic lethality like that seen in *C. elegans*, we performed RNAi. A dsRNA representing 668bp from exon 4 of the *Cbr-hbl-1* ORF was injected into wildtype *C. briggsae*, and we observed dead L1 larvae in the next generation (Fig. 3.22A). Their terminal phenotype appeared to be more developmentally advanced than that observed in similar *C. elegans* experiments with none of the eggs failing to hatch (Fay *et al.* 1999). No older larvae or adults with heterochronic phenotypes were observed, potentially indicating that all animals receiving RNAi failed to proceed with development after hatching. This result suggests that *Cbr-hbl-1* may have a role in embryonic development but one that differs slightly from *Cel-hbl-1*.

### ***Reiterative Phenotype*** **of Cbr-3let-7s** ***is Suppressed by*** **Cbr-lin-28(0)** ***and*** **Cbr-hbl-1(lf)**

The *3let-7s* play an important role in downregulating *lin-28* and *hbl-1* in *C. elegans*, a conclusion supported by the fact that *lin-28(0)* and *hbl-1(lf)* are epistatic to loss of the *3let-7s* (Abbott *et al.* 2005). Likewise, we found that *Cbr-lin-28(0)* and *Cbr-hbl-1(lf)* were epistatic to the reiterative phenotype caused by *Cbr-3let-7s(0)*. The *Cbr-hbl-1::AID; Cbr-3let-7s(0); TIR1(F79G)* strain had a number of seam cells close to normal (Fig. 3.22D), precocious alae (100%, n = 14), and protruding vulvae (43%, n = 14) when grown on 5-Ph-IAA plates. *Cbr-lin-28(ae39); Cbr-mir-48(ae65)* double mutants had a

normal number of seam cells (15.8+/-0.6, n = 13), precocious alae patches (66.7%, n = 12) at L4, a nearly complete adult alae, although some had a gap (25%, n = 8), and protruding vulvae (24%, n = 21).

These observations suggest that both *Cbr-lin-28* and *Cbr-hbl-1* act downstream of the *Cbr-3let-7s* and are necessary for the reiteration of the L2 stage caused by these three mutants, as in *C. elegans*. This is surprising since *Cbr-lin-28(0)* and *Cbr-hbl-1(0)* single mutants mostly did not show a reduction in seam cell numbers.

### *Simultaneous Reduction of* Cbr-lin-28 *and Cbr-hbl-1 Activities Shows that* Cbr-lin-28 *Acts in the L2*

In *C. elegans*, both *lin-28* and *hbl-1* are needed for L2 fates to occur. Because *C. briggsae lin-28(0)* mutants showed no L2 defect, we investigated whether it might still be involved at this stage by testing whether mutations would enhance the precocious phenotype caused by loss of *Cbr-hbl-1* activity. To do this, we generated a *Cbr-lin-28::AID; Cbr-hbl-1::AID* strain in a *TIR1(F79G)* background. When grown on 5-Ph-IAA plates, these animals had a reduction in seam cell numbers that was more severe than *Cbr-hbl-1:aid* alone (Fig. 3.22E; compare with Fig. 3.22C). They also developed gapped alae at the L3 stage (100% of animals had some precocious alae at L3), and complete alae by the L4. Thus, the reduction of both *Cbr-lin-28* and *Cbr-hbl-1* activity resembled the *Cel-lin-28(0)* phenotype. These double mutants also had a prolonged L3 stage and became stuck in the L3 molt: 24 hours after L3 animals were selected, some still had L3 cuticles with gapped alae and non-reflexing gonads (Fig. 3.23). In other animals, the gonads migrated closer to the pharynx and anus than normal before reflexing, and occasionally, the distal tips cells

leading the gonad arms migrated in unexpected directions. Finally, the vulvae were either protruding or stuck in an L4-like (pre-"christmas tree", or L4.2-L4.3 according to Mok *et al.*, 2015) shape. Interestingly, all of the *Cbr-lin-28::AID; Cbr-hbl-1::AID* animals grown on 5-Ph-IAA were sterile and had disorganized gonads.

These observations show that *Cbr-lin-28* is involved with *Cbr-hbl-1* in promoting L2 cell fates, as in *C. elegans*. But in *C. elegans*, both genes are necessary, and in *C. briggsae* they are partially redundant.

### **Cbr-let-7(0)** ***Mutants Have Additional Molts but No Heterochronic Defects***

*C. elegans let-7(0)* mutants have delayed adult alae formation due to reiteration of L3-specific developmental events, and they burst at the vulva upon reaching adulthood (Reinhart *et al.* 2000; Vadla *et al.* 2012). Two mutant alleles of *Cbr-let-7* were generated: an insertion (*ae47*) and deletion (*ae48*), both of which eliminate *Cbr-let-7* activity (Table 3.1).

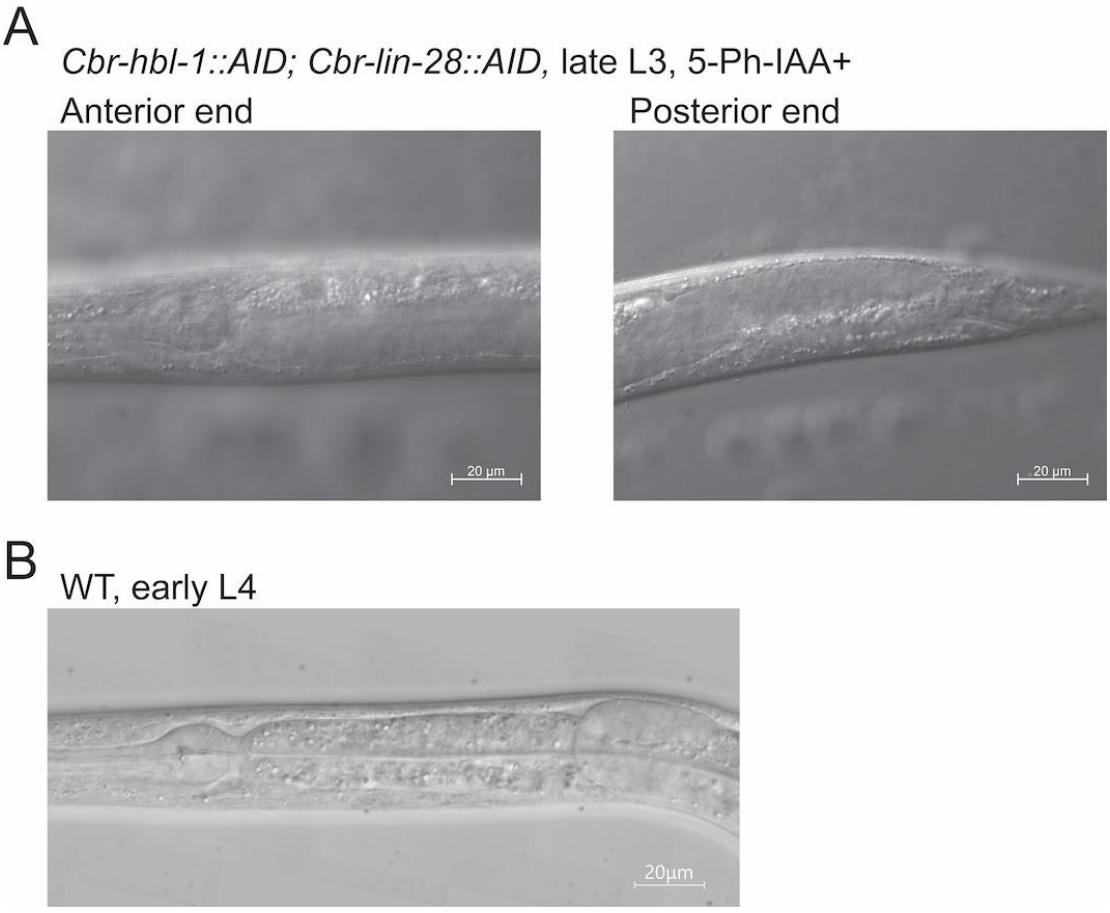



*Figure 3.23.* Simultaneous depletion of *Cbr-lin-28* and *Cbr-hbl-1* leads to gonad migration defects. DIC microphotographs showing (A) gonad arms in a *Cbr-hbl-1::AID; Cbr-lin-28::AID* late L3 worm on 5-Ph-IAA; gonad arms are extended towards the ends instead of reflexing compared to (B) wild-type early L4 animal with normal gonad reflexion. Also, compare to Fig. 3.10A. Animals are oriented anterior end left, dorsal side up.

Both *Cbr-let-7(0)* mutants displayed egg-laying defects, slightly abnormal vulvae shapes, and at least one extra molt (Fig. 3.24). The vulvae of these animals were slightly protruding, and adults burst at the vulvae on microscope slides, suggesting defects in

vulval development or structure. In contrast to *Cel-let-7(0)*, the *Cbr-let-7(0)* mutants developed normal adult alae at the L4 molt. The alae looked thinner than the wild type, perhaps due to a cuticle defect or the formation of overlying cuticle during an extra molt (Fig. 3.25). Some *Cbr-let-7(0)* mutants may have two extra molts: A plate containing four L4 larvae were placed at 20°C and the number of shed cuticles were counted the next day: 6 cuticles were found and 2 adult animals (with eggs) were stuck in cuticles (Fig. 3.26). The *Cbr-let-7(0)* mutants had a slightly increased number of seam cells at low temperatures, but it is unclear whether this is a heterochronic defect (Fig. 3.27).

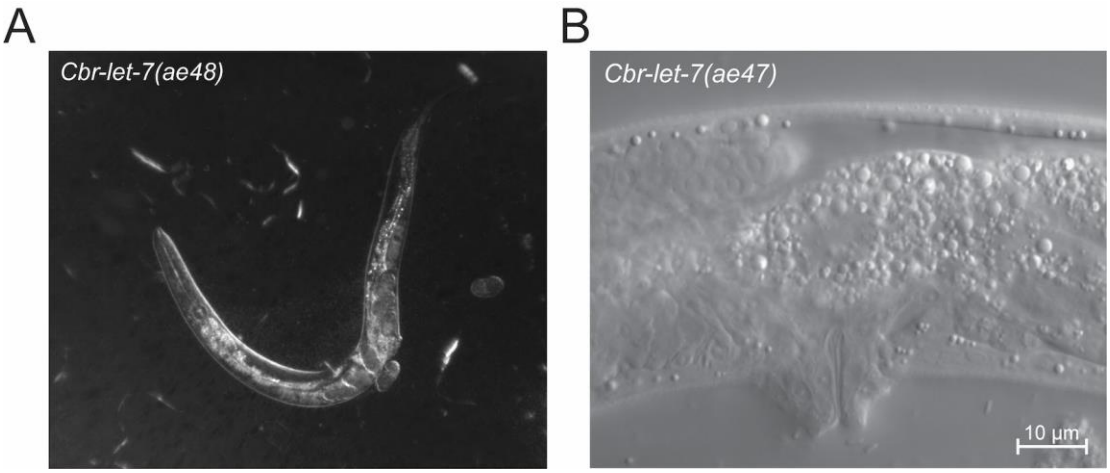



*Figure 3.24. Cbr-let-7(0)* mutants have egg-laying defects. DIC micrographs of (A) a *Cbr-let-7(ae48)* mutant with accumulated eggs. (B) Vulva of *Cbr-let-7(ae47)*. Compare to Fig. 3.16B. Mutant vulvae protrude more. Animals are oriented anterior end left, dorsal side up.

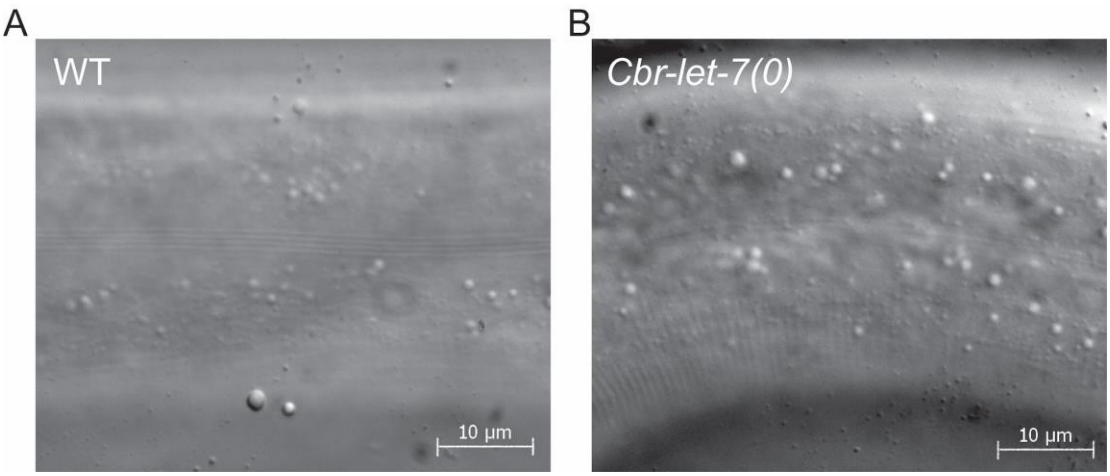



*Figure 3.25. Cbr-let-7(0)* mutants have thinner alae than the wild type. DIC micrographs of (left) Wild type *C. briggsae* adult alae, and (right) *Cbr-let-7(ae47)* adult alae in animals of approximately the same age. In *Cbr-let-7(0)* mutants adult alae appear to be thinner. Animals are oriented anterior end left, dorsal side up.

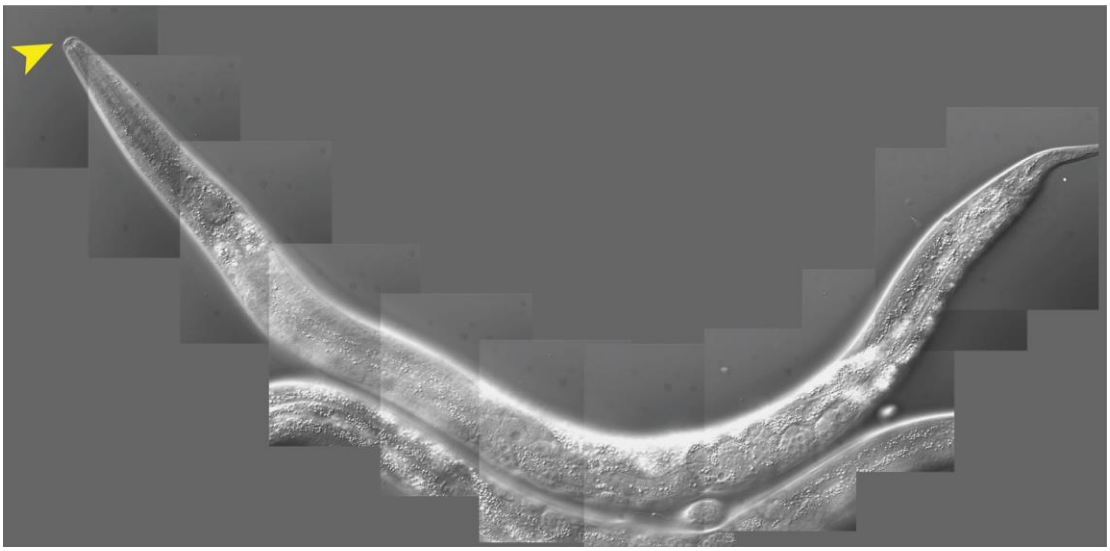


*Figure 3.26. Cbr-let-7(0)* mutants have extra molts. DIC micrograph of an adult *Cbr-let-7(ae47)* worm with cuticle coming off at the anterior end, indicated by the arrowhead.

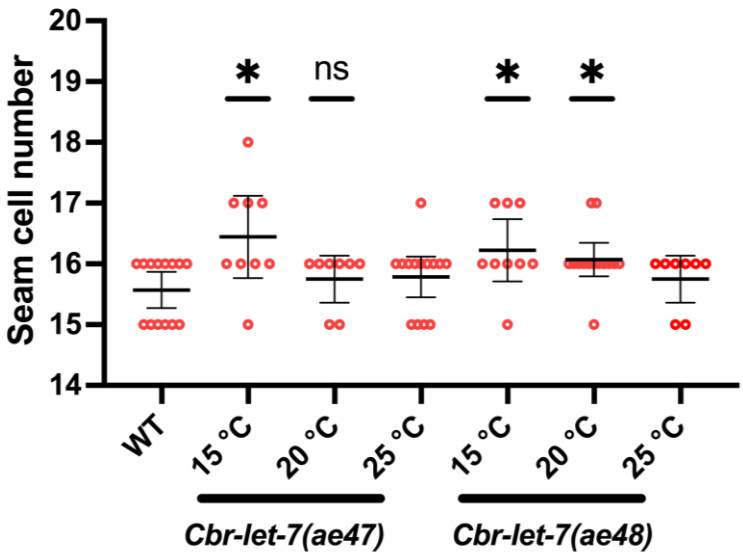



*Figure 3.27.* Seam cell number in *Cbr-let-7(0)* mutants is similar to wild type. Although there is an increase in the seam cell number in some animals at lower temperatures, we cannot conclude that this is due to reiteration of L2 cell fates as opposed to other reasons. Statistical analysis is described in Materials and Methods.

### *Cbr-let-7(0) Mutation Suppresses Later Defects of Cbr-lin-14(0) and Cbr-lin-28(0) Mutants*

Despite *Cbr-let-7(0)* mutants not reiterating late larval stage seam cell fates (as assessed by alae formation), we tested whether they could nevertheless suppress the precocious alae defect of *Cbr-lin-28(0)*, as occurs in *C. elegans* (Slack *et al.* 2000; Vadla *et al.* 2012). Examining *Cbr-lin-28(ae39); Cbr-let-7(ae47)* animals, we found that *Cbr-let-7(0)* mutation completely suppressed the "arrested L4" phenotype and gonad disorganization of the *Cbr-lin-28(0)* mutant, which implies that this aspect of the *Cbr-lin-28(0)*

phenotype is in part due to the inappropriate upregulation of *Cbr-let-7* and presumably the subsequent silencing of the miRNA's targets.

Some *Cbr-lin-28(0); Cbr-let-7(0)* animals looked wild-type, whereas others had egg-laying defects and resembled *Cbr-let-7(0)* mutants. The double mutants had slightly abnormal vulvae similar to *Cbr-let-7(0)* single mutants, and sometimes developed protruding vulvae (data not shown). The double mutants also underwent extra molts, however, unlike the *Cbr-let-7(0)* single mutants, they usually did not complete those molts and remained stuck in the cuticle (Fig. 3.28). Four L4 larvae from the *Cbr-lin-28(0); Cbr-let-7(0)* strain were isolated on a separate plate, and cuticles were scored next day; 4 cuticles were found and all of the animals were adults stuck in the cuticle while molting. Thus, loss of *Cbr-lin-28* slightly mitigates this *Cbr-let-7(0)* phenotype, suggesting that some functions of *Cbr-let-7* depend on the activity of *Cbr-lin-28.*

Interestingly, although some *Cbr-lin-28(0); Cbr-let-7(0)* double mutants developed precocious alae at the L4 stage at both 15°C (84.2%, n = 38) and at 20°C (25%, n = 8), the frequencies were lower than in *Cbr-lin-28(0)* single mutant (compare to Fig. 3.12C). Alae patches were usually located on the head and just behind the pharynx, but short patches were also observed in other areas. The results suggest that precocious alae formation in *Cbr-lin-28(0)* mutants is in part caused by premature *Cbr-let-7* upregulation.

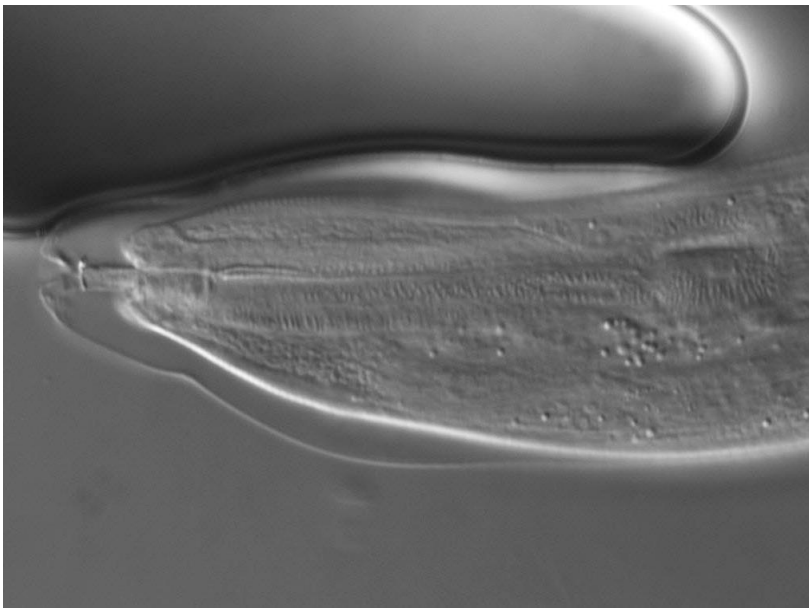


*Figure 3.28. Cbr-lin-28(0); Cbr-let-7(0)* double mutants have extra molts. DIC micrograph showing an *Cbr-lin-28(ae39); Cbr-let-7(ae47)* adult with a partly shed cuticle. Incomplete extra molts were common for this strain.

Finally, we examined a *Cbr-lin-14(ae51) Cbr-let-7(ae50)* double mutant. The *Cbr-let-7(0)* mutation restored fertility to *Cbr-lin-14(0)* mutants and suppressed precocious alae formation. This observation suggests that sterility and precocious alae occur in *Cbr-lin-14(0)* mutants because of the inappropriate *Cbr-let-7* expression. In *C. elegans*, *lin-14* acts in part through *lin-28* to control late-stage events, so the sterility and precocious alae of *Cbr-lin-14(0)* could be due to the downregulation of *Cbr-lin-28*, although we have not tested that hypothesis here (Seggerson *et al.* 2002; Tsialikas *et al.* 2017).

### *The* Cbr-hbl-1(lf) *Phenotype is Partly Epistatic to that of* Cbr-let-7(0)

Work in *C. elegans* suggests that *hbl-1* acts downstream of *let-7* (Lin *et al.* 2003; Abrahante *et al.* 2003; Abbott *et al.* 2005; Vadla *et al.* 2012). We tested the ability of *Cbr-hbl-1::AID* to suppress *Cbr-let-7(0)* phenotype by constructing the double mutant. Of double mutant animals grown on 5-Ph-IAA plates, 81% (n = 21) developed patches of precocious alae at the L3 molt, although no animals were stuck in the L4 molt. These results indicate that the *Cbr-hbl-1(lf)* mutant phenotype is partly epistatic to that of *Cbr-let-7(0)*, as seen in *C. elegans* (Abrahante *et al.* 2003).

### *A Cbr-lin-41(lf) Mutation Causes a Developmental Arrest at the L4 Stage*

*C. elegans lin-41* has multiple roles in the animal: null mutants are sterile (due to a germline defect) and hypomorphs have a heterochronic phenotype (Slack *et al.* 2000; Tocchini *et al.* 2014). We chose to tag the *C. briggsae* ortholog with the auxin-inducible degron so its level of activity could be controlled. We inserted the *aid* sequence near the start codon of *Cbr-lin-41* with CRISPR/Cas9. In this process, we also generated two loss-of-function alleles, including a potential null allele (Table 3.1). This allele, *Cbr-lin-41(ae77)*, is an incorrect *aid* insertion creating a false ORF that did not contain any *Cbr-lin-41* exons. A potential start codon that was in-frame with the remaining *Cbr-lin-41* ORF was located 181 bp downstream of the original start. Because homozygous *Cbr-lin-41(ae77)* animals were sterile, the allele was balanced with the marker *Cbr-spe-8(v142)* that is also sterile when homozygous (R. Ellis, pers. comm.). Thus, only heterozygous animals reproduced. *Cbr-lin-41(ae77)* segregating from the heterozygous strain were identified as having a shorter body and Dpy-like phenotype.

Homozygous *Cbr-lin-41(ae77)* animals had a developmental delay at the end of the L4 stage similar to that observed in *Cbr-lin-28(0)* mutants. Around 52 hours post hatching, when wild-type animals complete the L4 molt, develop alae, adult vulva, and both types of gametes, the *Cbr-lin-41(ae77)* animals also had oocytes and spermatozoa but did not have alae, and their vulvae were stuck at the pre-"christmas tree", or L4.2-L4.3 according to Mok *et al.*, 2015, stage of morphogenesis (Fig. 3.29). Sometimes they had disorganized gonads similar to *Cbr-lin-28(0)* mutants. However, older *Cbr-lin-41(ae77)* animals had alae and adult-shaped vulvae in contrast to *Cbr-lin-28(0)* mutants, which sometimes failed to continue development after the L4 arrest. This suggests that the "arrested L4" state in *Cbr-lin-28(0)* mutants cannot be due solely to *Cbr-lin-41* downregulation. Notably, in contrast to *Cel-lin-41* mutants, *Cbr-lin-41(ae77)* animals did not have precocious alae.

The *Cbr-lin-41(ae76)* allele is a small insertion that creates a frameshift and early stop codon. Another in-frame start codon occurred 170 bp downstream of the original, which might allow some expression of functional protein (Table 3.1). This allele caused a weaker phenotype than *ae77* and could be maintained as a homozygous line. The *Cbr-lin-41(ae76)* mutants had these additional defects: 17.9% successfully molted into adults, 67.9% were stuck in the L4 molt, and 14.2% had an "arrested L4" phenotype with disorganized gonads (n = 28). None had precocious alae.

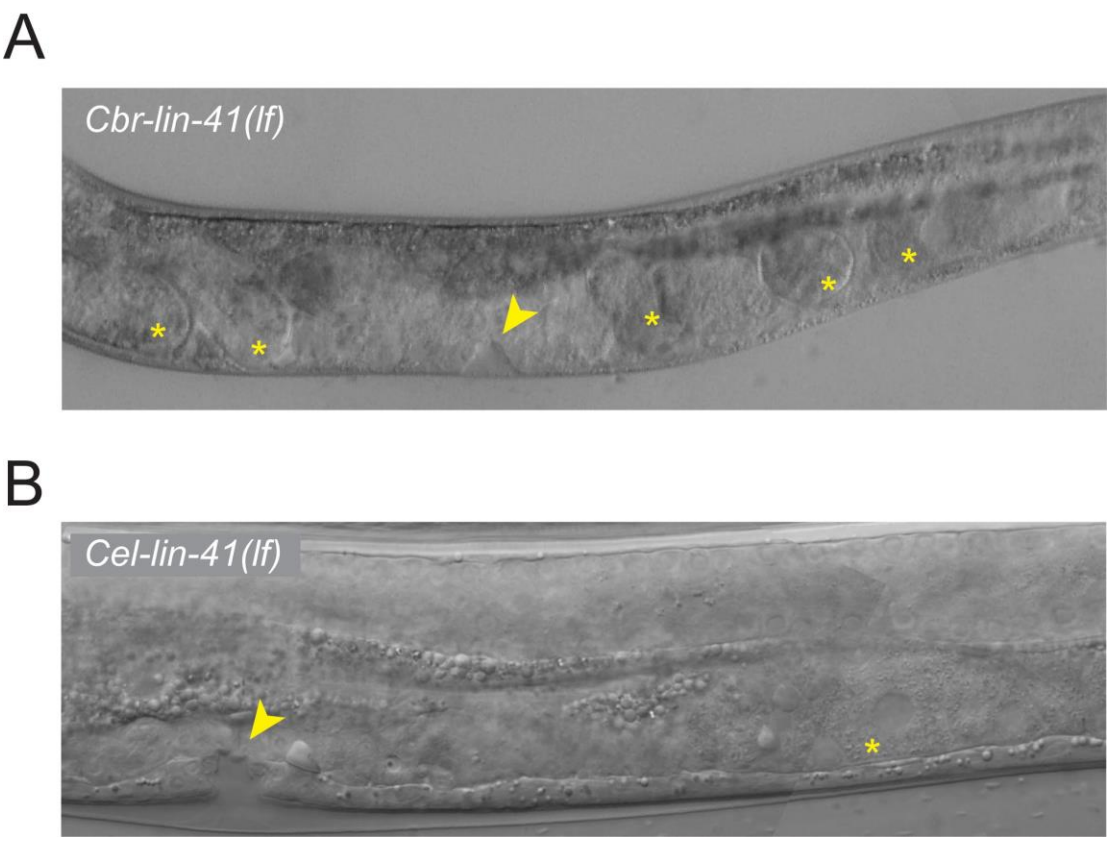



*Figure 3.29.* Both *Cel-lin-41(lf)* and *Cbr-lin-41(lf)* have L4 developmental delay or arrest. DIC micrographs of (top) *Cbr-lin-41(ae76)* and (bottom) *Cel-lin-41::AID(aeIs10)* animals grown on 5-Ph-IAA. In both, the vulva is arrested at an L4 stage of development (arrowheads) and the germ line is producing embryos or oocytes (asterisks). Animals are oriented anterior end left, dorsal side up.

Animals with a degron-tagged *Cbr-lin-41* and *TIR1(F79G)* expressed from an attached array grown on 5-Ph-IAA produced oocytes and sperm before the soma completed development: the animals had vulvae still undergoing morphogenesis and no alae. The *Cbr-lin-41::AID* animals also had slightly abnormal vulvae, with asymmetric shapes and protrusions, and had egg-laying defects. There were no sterile animals on 5-Ph-IAA, and

no precocious alae. The observations reveal similar drifts in the roles of *lin-28* and *lin-41* during the evolution of the two species, with more explicit control of developmental timing per se in *C. elegans* and less in *C. briggsae*.

***Depletion of C. elegans lin-41 Causes L4 Developmental Arrest***

Although a variety of defects caused by loss of *Cel-lin-41* activity have been observed (Slack *et al.* 2000; Tocchini *et al.* 2014), to our knowledge, L4 arrest like we observed in *Cbr-lin-41(lf)* mutants, have not been reported. To test whether *C. elegans lin-41(lf)* mutants can arrest in the L4 stage, we generated a *Cel-lin-41::AID* strain and crossed it with a strain expressing *TIR1(F79G)* allele (Hills-Muckey *et al.* 2021). Synchronized L1 larvae of wild-type *C. elegans* and *Cel-lin-41::AID* were placed on plates with or without 5-Ph-IAA. After 57 hours of development, 71% (n = 14) of wild-type animals were molting or had already shed cuticles, and the remainder were still late L4 larvae. Most molting animals had mature vulvae and were still producing sperm. Only 14% of the animals were already producing oocytes (one young adult and one molting animal). Of the *Cel-lin-41::AID* animals, 40% (n = 15) were molting, only 13% had mature vulvae (2 of the molting animals), and the others had L4-shaped vulvae. Surprisingly, 73% of the animals already had both sperm and oocytes, including animals that appeared like "late L4" larvae. Moreover, when we looked at *Cel-lin-41::AID* animals on 5-Ph-IAA at 72 hours of development, 13% of animals had an arrested L4 phenotype characterized by an L4-like vulva ("christmas tree"-like, or L4.4-L4.5 according to Mok, *et al.*, 2015, morphology), lack of adult alae, the absence of the final molt, and the presence of oocytes (Fig. 3.29). These observations indicate that reduction of *Cel-lin-41* activity can produce

an L4 developmental delay or arrest similar to that observed for both *Cbr-lin-41(lf)* and *Cbr-lin-28(0),* resulting in asynchrony between the germline and soma.

### *The* Cbr-lin-41 *Gene Acts Downstream of* Cbr-let-7 *in the Heterochronic Pathway*

In *C. elegans, lin-41* is a primary target of *let-7* in the heterochronic pathway (Slack *et al.* 2000). To test whether *Cbr-lin-41* acts downstream of *Cbr-let-7*, we made the *Cbr-lin-41(ae76)*; *Cbr-let-7(ae48)* double mutant. Of the double mutants observed (n = 33), 97% of the animals were stuck in the L4 molt and 3% had an "arrested L4" phenotype (L4-shaped vulva, no alae, produced embryos). The difference in the penetrance of the "arrested L4" phenotype was not significant compared to the *Cbr-lin-41(ae76)* single mutant ($p > 0.05$, Fisher's exact test). Because the double mutant displayed the *Cbr-lin-41(lf)* mutant characteristics and none of the *Cbr-let-7(0)* mutant features were displayed, the *Cbr-lin-41(lf)* phenotype was epistatic to that of *Cbr-let-7(0),* suggesting that *Cbr-lin-41* acts downstream of *Cbr-let-7*, as in *C. elegans.*

### *A* Cbr-lin-41(0) *Mutation Enhances the* Cbr-hbl-1(lf) *Phenotype*

In *C. elegans, lin-41* and *hbl-1* both appear to control L3 cell fates, and the animals lacking the activities of both genes generate some precocious alae at the L2 molt (Abrahante *et al.* 2003; Vadla *et al.* 2012). To test whether depletion of both these genes would cause a more severe phenotype in *C. briggsae*, a *Cbr-lin-41(ae76); Cbr-hbl-1::AID* double mutant was generated. Double mutants carrying the *TIR1(F79G)* unattached array were placed on 5-Ph-IAA-containing plates, and their phenotypes were analyzed. Double mutants resembled *Cbr-lin-28::AID; Cbr-hbl-1::AID* animals grown on 5-Ph-IAA: they had an L3 developmental delay, gonads with delayed reflexion, and

abnormal DTC migration after the reflection. Moreover, precocious alae patches appeared at the L2 molt in 69% of animals (n = 26), and 100% of animals had gapped (68.4%) or complete (31.6%) precocious alae at the L3 molt (n = 19). The double mutants also failed to shed L3 cuticles. The number of seam cells was slightly reduced (14.5+/-0.8, n = 26) compared to wild type (15.6+/-0.5, n = 25), but was similar to that observed in *Cbr-hbl-1::AID* strain grown on 5-Ph-IAA (14.8+/-0.8, n = 23, Fig. 3.22C). Thus, these counts do not show an effect of *Cbr-lin-41(lf)* on seam cell number. Our observations suggest that *Cbr-lin-41* and *Cbr-hbl-1* also control L3 hypodermal cell fates redundantly, but the fact that seam cell duplication occurs normally shows that *lin-41* does not control L2 fates.

### *The* Cbr-lin-29(0) *Phenotype Resembles the* Cel-lin-29(0) *Phenotype*

The *lin-29* gene encodes a zinc finger transcription factor that directly regulates the larval-to-adult adult switch in the *C. elegans* hypodermis; in its absence, the seam cells fail to differentiate, whereas precocious alae are formed because of early *lin-29* activity (Ambros and Horvitz 1984; Ambros 1989; Rougvie and Ambros 1995; Azzi *et al.* 2020).

A *Cbr-lin-29(0)* mutant allele was made by targeting the 6th exon, where orthologous *Cel-lin-29* mutations are located (Rougvie and Ambros 1995). The deletion *ae75* is a frameshift which leads to a premature stop codon (Table 3.1). The *Cbr-lin-29(ae75)* mutants did not develop alae and had delayed vulval development that caused them to burst at the adult stage (n = 15, Fig. 3.30). Some animals retained a patch of L4 cuticle around the vulva, which occasionally prevented bursting and allowed some animals to survive and produce eggs. On average, *Cbr-lin-29(0)* rarely produced larvae and could

not be maintained as homozygotes. We therefore balanced *Cbr-lin-29(ae75)* with *Cbr-trr-1(v76)* (Guo *et al.* 2013). Overall, the phenotypes of *Cel-lin-29(0)* and *Cbr-lin-29(0)* mutants are very similar, suggesting conserved function and relationship to targets.

*Figure 3.30.* DIC micrograph of a typical *Cbr-lin-29(ae75)* adult. The abnormal vulva is typical for this mutant, but the tail is not.

**Cbr-lin-28 *and* Cbr-lin-41 *Act Through* Cbr-lin-29**

In *C. elegans, lin-29* acts at the end of the heterochronic gene hierarchy and is necessary for the late-stage phenotypes of earlier acting heterochronic genes (Ambros 1989). To test whether the L4 developmental arrest in particular of *Cbr-lin-28(0)* and *Cbr-lin-41(lf)* mutants required *Cbr-lin-29* activity, we made the *Cbr-lin-28(ae39); Cbr-lin-29(ae75)* and *Cbr-lin-41(ae76); Cbr-lin-29(ae75)* double mutants.

Double homozygotes of these alleles could not be maintained because they burst at the vulva at adulthood and had very few progeny. Strains that were heterozygous for *Cbr-lin-29(ae75)* and homozygous for *Cbr-lin-28(ae39) or Cbr-lin-41(ae76)* (determined by PCR genotyping) segregated mostly worms (more than 50%) that lacked developmental arrest and disorganized gonads, suggesting that loss of one copy of *Cbr-lin-29* is sufficient to suppress these phenotypes. These strains segregated worms phenotypically similar to *Cbr-lin-29(0),* which lacked adult alae and burst at the vulva (Fig. 3.31), as well as worms that phenotypically resembled *Cbr-lin-41(lf)* or *Cbr-lin-28(0)* respectively. Among *Cbr-lin-41(ae76); Cbr-lin-29(ae75)/+* animals, 60% of worms resembled wild type (a normal L4 molt, vulva and adult alae), 19% were stuck in the L4 molt and looked like *Cbr-lin-41(ae76)* single mutants, and 21% looked like *Cbr-lin-29(ae75)* single mutants (n = 48). *Cbr-lin-28(ae39); Cbr-lin-29(ae75)/+* animals segregated L4 larvae that had precocious alae patches (12 of 14 animals examined), and adults that had the *Cbr-lin-29(0)* phenotype (burst vulvae) lacked alae completely (n = 11). These observations suggest that loss of *Cbr-lin-29* is epistatic to loss of either *Cbr-lin-28* or *Cbr-lin-41*. Thus, *Cbr-lin-28* and *Cbr-lin-41* act through *Cbr-lin-29*, as they do in *C. elegans*, and interestingly, the arrested L4 phenotype also depends on *Cbr-lin-29*.

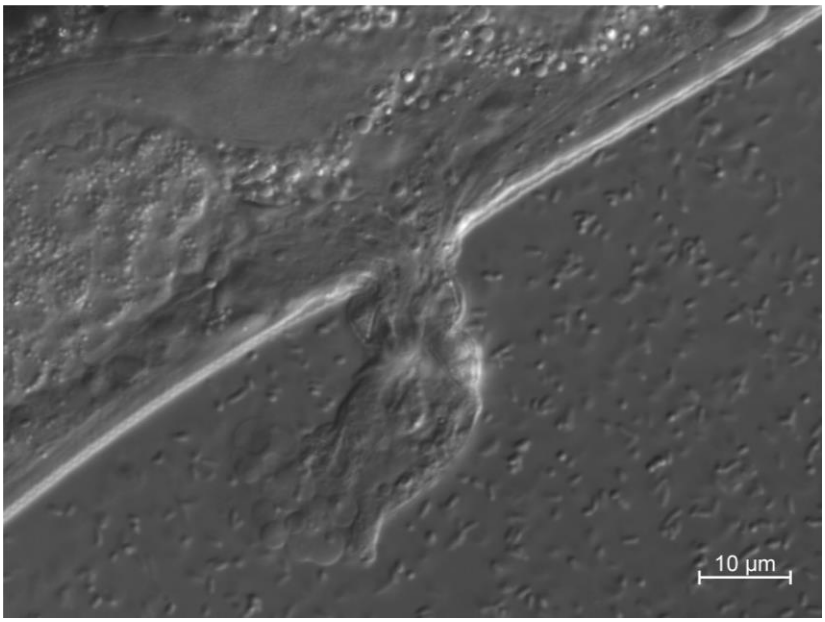



*Figure 3.31*. DIC micrograph showing a typical vulva of adult *Cbr-lin-28(ae39); Cbr-lin-29(ae75)* animals. The defect is similar to *Cbr-lin-28(ae39)* single mutant. Anterior end is to the left.

**Discussion**

We characterized 11 *C. briggsae* orthologs of *C. elegans* heterochronic genes using a total of 35 genetic lesions and 18 double and triple mutants and found that their mutant phenotypes differ in significant ways from those of *C. elegans*. Although most orthologs displayed defects in developmental timing, some of the phenotypes differed in which stages were affected, the penetrance and expressivity of the phenotypes, or by having pleiotropic effects that were not obviously connected to developmental timing. However, when examining pairwise epistasis and synergistic relationships, we found those reflected the relationships between their *C. elegans* orthologs, suggesting that the arrangements of these genes in functional modules is conserved, but the modules' relationships to each

other and/or to their targets has drifted since the time of the species' last common ancestor.

A previous comparison of *C. elegans* and *C. briggsae* orthologs by RNA-interference (RNAi) showed that only a small fraction (91 of 1333 orthologs) have significantly different loss-of-function phenotypes in the two species (Verster *et al.* 2014). This study included the protein-coding heterochronic gene orthologs, except *Cbr-lin-28*, but in general did not detect the degree of divergence that we observed. However, the level of analysis was limited and included only larval or embryonic lethality, growth rate, morphology, and fertility.

Our observations suggest that the level of functional divergence in the heterochronic gene orthologs is greater than previously thought. Despite this, the fundamental structure of the heterochronic pathway is largely conserved between *C. elegans* and *C. briggsae*. Since these species have nearly indistinguishable larval development and post-embryonic cell lineages, the differences in single-gene mutant phenotypes and some pairwise relationships indicate a significant degree of developmental systems drift has occurred while giving rise to essentially the same anatomy and life history.

### *Conservation of Key Regulatory Modules*

As has been done in the analysis of other complex developmental pathways, the heterochronic pathway can be divided into subcircuits or modules that govern individual aspects of the phenotype (Verd *et al.* 2019). The modules are defined by enhancement or epistasis relationships among genes that act either together or in opposition to control cell

fates at specific larval stages. Our findings suggest that these modules are largely conserved between *C. elegans* and *C. briggsae*.

The *lin-4/lin-14* module in *C. elegans* specifies L1 cell fates and controls the transition to L2 fates (Chalfie *et al.* 1981; Ambros and Horvitz 1987; Wightman *et al.* 1993). *lin-14* acts to specify L1 cell fates then is down-regulated by *lin-4*, allowing L2 and later fates to occur. We found the same is true in *C. briggsae*: *Cbr-lin-14* has nearly the same role and is required for the reiterative mutant phenotype of mutant *Cbr-lin-4*. Taken with the fact that the miRNA sites in *Cbr-lin-14*'s 3'UTR are conserved, and their deletion also causes a reiterative phenotype, we find that the regulatory relationship between *lin-4* and *lin-14* is conserved between the two species.

The genes *lin-28, lin-46, 3let-7s,* and *hbl-1* comprise a complex regulatory module that, in *C. elegans*, specifies L2 cell fates and the transition to the L3 (Pepper *et al.* 2004; Vadla *et al.* 2012; Tsialikas *et al.* 2017; Ilbay and Ambros 2019; Ilbay *et al.* 2021). In *C. briggsae*, the relationships among these genes is conserved, although relative roles within the module have drifted. As in *C. elegans*, in *C. briggsae* both *lin-28* and *hbl-1* are required for L2 fates, *lin-46* acts downstream of *lin-28* while *lin-28* also has *lin-46*-independent activity, the *3let-7*s act upstream of *hbl-1*, and the *3let-7*s are needed for the transition to L3 fates.

The transition from L3 to L4 and subsequently to adult cell fates is regulated by the *let-7/lin-41/lin-29* module in *C. elegans* (Slack *et al.* 2000; Vadla *et al.* 2012; Azzi *et al.* 2020). Like *lin-28*, *let-7*'s individual role has drifted, but its relationship to other genes is largely conserved: In both species, *let-7* generally acts downstream of *lin-14*, *lin-28*, and

*hbl-1* and upstream of *lin-41*. *lin-41*'s role has also drifted, but it appears to be a regulatory target of *let-7* and acts to negatively regulate *lin-29* in both species. *lin-29* is the furthest downstream of all the genes in both species, at least with regard to the larva-adult transition. Thus, the core relationships in the *let-7/lin-41/lin-29* regulatory module are conserved.

It is also worth noting the high phenotypic variability that we observed, which is greater in *C. briggsae* than in *C. elegans* for some mutants. This fact speaks to the high degree of genetic buffering that these modules provide when fully intact, a condition that would accommodate considerable developmental systems drift.

***Evolutionary Drift in Regulatory Relationships***

Despite the fact that key regulatory relationships are conserved between the species, the single-gene phenotypes and some double mutant effects reveal substantial drift in the relationships of these genes to downstream targets. This drift is manifested in two ways: a shift in the role of the gene in the developmental timing of cell fates (their heterochronic roles), and the uncovering of additional roles that are not obviously related to cell fate timing, specifically the completion of larval development and gonad integrity.

Two aspects of the role of the *lin-4*/*lin-14* module in *C. briggsae* compared to *C. elegans* are significant. First, the fact that *Cbr-lin-4* primarily affects the transition from L2 to L3 suggests either that it alone is not sufficient to repress *Cbr-lin-14*, or that *Cbr-lin-14* is not sufficient to specify L1 fates. Second, the fact that *Cbr-lin-4(0)* suppresses—but is not epistatic to—the precocious *Cbr-lin-14(0)* indicates drift in *lin-4*'s relationship to its targets. Specifically, whereas in *C. elegans lin-4*'s primary target is *lin-14* and

secondarily the L2 regulators *lin-28* and *hbl-1*, in *C. briggsae lin-4* may play a more significant role regulating *lin-28* and *hbl-1* than it does with *lin-14*.

In both species, both *lin-14* and *lin-28* have sites for *lin-4* and *let-7* family miRNAs in their 3'UTRs. The regulation of *lin-28* by both miRNA families was described previously and it was shown that the *lin-4/lin-14* module controls the expression of the *3let-7s* (Tsialikas *et al.* 2017). The drift we see in the role of *lin-4* may reflect differences in the relative strengths of multiple miRNAs from the *lin-4* and *let-7* families in down-regulating their multiple targets. Developmental systems drift arises due to the accumulation of small changes—in this case, perhaps, in miRNA expression, abundance, or target sensitivity—and how those changes are compensated for by other small changes that keep developmental outcomes the same (True and Haag 2001).

Such subtle shifts in relative strengths is more apparent among the *3let-7*s. In *C. elegans*, mutations in *mir-48, mir-84,* and *mir-241* individually have very weak or undetectable phenotypes but when combined cause a strong L2 reiterative defect (Li *et al.* 2005; Abbott *et al.* 2005). These miRNAs all resemble *let-7* in having the same 8-nt seed sequence and therefore have the potential to regulate the same targets (Lau *et al.* 2001; Lim *et al.* 2003). In *C. elegans*, *mir-48* is largely redundant with the other two genes, but in *C. briggsae mir-48* is mostly responsible for work of all three genes for this phenotype (loss of *mir-48* has nearly the same heterochronic effect as that of the triple mutant). Thus, small evolutionary changes in the relative levels of these miRNAs, or differences in the regulation caused by changes in 3'UTRs of their target genes, may be offset by compensatory changes in other miRNAs or target sites, leading to identical outputs of the regulatory module for the different species.

A reciprocal example of drift involving redundancy is *lin-28* and *hbl-1*. In *C. elegans*, each gene is needed to specify L2 cell fates (Ambros and Horvitz 1984; Lin *et al.* 2003; Abrahante *et al.* 2003). By our observations, the regulatory module involving these genes is conserved, but in *C. briggsae*, *lin-28*'s role in specifying L2 fates is barely detectable until *Cbr-lin-28(lf)* is combined with *Cbr-hbl-1(lf)*, indicating its redundancy with *Cbr-hbl-1*. The substantial difference in *lin-28*'s solo role in the two species may reflect a drift in two parallel components of this regulatory module, which are the negative regulators of *hbl-1*: the *3let-7*s and *lin-28's* direct target, *lin-46*. In *C. elegans*, the effect of deleting *lin-46*'s 5'UTR is similar to, although weaker than, *lin-28*'s null phenotype (Ilbay *et al.* 2021). It is therefore surprising that deletion of the presumed *Cbr-lin-28* regulatory site in the 5'UTR of *Cbr-lin-46* does not phenocopy *Cbr-lin-28(0)* at all, but rather looks more like *Cbr-hbl-1(lf)*. Furthermore, the role of *lin-46* appears not to have drifted during the divergence of the two species. It is possible that *Cbr-lin-28* may not be the only gene acting via the 5'UTR of *Cbr-lin-46*, but that alone would not explain *Cbr-lin-28*'s redundancy with *Cbr-hbl-1*. A shift in the relative strength of *lin-28*'s regulation of *let-7* or another target may be responsible.

Significant drift was also found in the *let-7/lin-41/lin-29* module. On their own, neither *Cbr-let-7* nor *Cbr-lin-41* influence the developmental timing of cell fates, in striking contrast to what happens in *C. elegans*. However as in *C. elegans*, in *C. briggsae*, *lin-41* shows some redundancy with *hbl-1* in controlling seam cell differentiation, demonstrating that its regulatory relationships with other heterochronic regulators is conserved. *let-7*, on the other hand, does not have the same suppressor interactions with the early-acting heterochronic genes *lin-14* and *lin-28* in *C. briggsae* as it has in *C.*

*elegans*, although it does have a role in cessation of the molting cycle in both species. This "split" phenotype is consistent with the analysis of Azzi and colleagues who showed a branching of the pathway in the control of *lin-29* isoforms that affect different aspects of terminal differentiation in the hypodermis (Azzi *et al.* 2020).

By far, the most significant difference between *C. elegans* and *C. briggsae* is *lin-28*'s phenotype, which shows only minor heterochronic defects in *C. briggsae* while at the same time displaying significant late larval arrest and gonad integrity problems, two unexpected phenomena that are not well understood even in *C. elegans*. This was surprising given *lin-28*'s broad conservation and role in timing among animals (Moss and Tang 2003; Balzer *et al.* 2010; Romer-Seibert *et al.* 2019). We have demonstrated here that a reduction in *lin-41* activity can also lead to L4 arrest in *C. elegans*. Perhaps a difference in *lin-28*'s regulatory relationship with *lin-41* (possibly via its direct regulation of *let-7*) is responsible for *lin-28*'s different influence on the completion of larval development in the two species. Additionally, we found significant differences in the time and place of *lin-28*'s expression between the two species—including different degrees of significance of 3'UTR regulation—which may also account for the drift in pleiotropies over evolutionary time.

Our comparison of the heterochronic genes of *C. elegans* to those of their orthologs in *C. briggsae* revealed less drastic changes than has been seen in the sex determination pathways. Sex determination pathways evolve rapidly: *C. elegans* and *C. briggsae* developed hermaphroditism independently, since their common ancestor was dioecious (Ellis 2017; Haag *et al.* 2018). Some sex determination genes have conserved roles in *Caenorhabditis* species, such as *tra-1*, *tra-2*, the *fem* genes, and *fog-3* whereas others

have quite different functions, including genes involved in sex determination in one species but not the other. Whether there exist genes in *C. briggsae* that have primary roles in developmental timing but are not among the orthologs studied here can be determined by forward genetic screens in these species for mutants with developmental timing defects.

### *Insights into the Heterochronic Pathway of* C. elegans

Our investigation of the heterochronic gene orthologs of *C. briggsae* revealed new relationships between this pathway and other aspects of the animal's growth that may be relevant to both species. In particular, we found that a *Cel-lin-41* mutation can cause a developmental arrest similar to that caused by *Cbr-lin-28* and *Cbr-lin-41* mutations. It is possible that redundancy that has not yet been uncovered in *C. elegans* could connect the heterochronic genes to completion of larval development and gonad integrity. Given the number of heterochronic gene orthologs directly involved gonad integrity—either because mutations in them cause disintegration (*Cbr-lin-28*, *Cbr-hbl-1*, *Cbr-lin-41*) or mutations suppress that disintegration (*Cbr-lin-46*, *Cbr-let-7*)—it would not be surprising to find that further investigation of heterochronic genes in *C. elegans* uncovers such a connection.

The majority of developmental systems drift in the heterochronic pathway appears to have occurred in redundant and parallel components of regulatory modules. Parallel regulatory branches may have different "weights" in determining phenotypic outcome in different species. Even in *C. elegans*, we do not yet fully understand the nature of these parallel branches—such as non-*lin-4* regulation of *lin-14*, *lin-46*-independent regulation

of *hbl-1* by *lin-28*, and the different contributions of the three *let-7* miRNAs to the repression of multiple heterochronic genes. Further investigation using multiple *Caenorhabditis* species may reveal why the pathway is organized as it is and why it has evolved the ways it has.

# Chapter 4

## Heterochronic Genes Act in a Sequence at Certain Times in Development to Promote Stage-Specific Developmental Events


### Abstract

The heterochronic genes of *C. elegans* control the sequence and timing of developmental events. The four core heterochronic genes *lin-14, lin-28, hbl-1,* and *lin-41* act in a sequence to specify cell fates associated with each of the four larval stages. It was previously shown that *lin-14* has two activities separated in time that promote L1 and L2 developmental events, respectively. Using the auxin-inducible degron system I showed that *lin-28* and *hbl-1* have two activities separated in time promoting L2 and L3 developmental events. Thus, these two genes function much like *lin-14*, but later in development. It was not possible to determine if *lin-41* had two different activities. Nevertheless, I showed that the core heterochronic genes act in a sequence that reflects their relative positions in the heterochronic pathway.


### Introduction

The heterochronic genes act in a hierarchy to control the sequence of developmental events during the post-embryonic stages of *C. elegans*. Four main heterochronic genes – *lin-14, lin-28, hbl-1,* and *lin-41* – promote the transitions between L1, L2, L3, and L4 seam cell fates. Although the functions of each heterochronic gene have been well-characterized, little is known about precisely when they act relative to each other and to the events they control. Outstanding questions are: Do genes that control the same cell fate events act at the same time? And: Do the genes act during the execution of the event they control, or prior to that event?

Ambros and Horvitz used temperature-sensitive alleles of *lin-14* to determine its time of action (Ambros and Horvitz, 1987). They shifted worms from permissive to restrictive temperature (or *vice versa*) at specific times after hatching and showed that *lin-14* had two activities - one that promoted L1 seam cell divisions (*lin-14a*) and another that promoted L2 seam cell divisions (*lin-14b*). The presence of two separate activities and their timing could not have been deciphered from the observations of null mutants.

Similar studies have not been carried out for other core heterochronic genes – lin-28, hbl-1, and lin-41. It is known that *lin-28* and *hbl-1* regulate L2 seam cell fates and there is some evidence that they also might regulate seam cell fates after the L2 stage. *lin-28(0)* mutants skip L2 seam cell fates and develop precocious alae one stage earlier, and sometimes precocious alae patches are observed two stages earlier (Vadla, 2012). *hbl-1(ve18)*, a hypomorphic allele, causes a precocious differentiation of seam cells, and although it does cause skipping of L2 seam cell fates, it suppressed the increased seam cell number in *mir-48, mir-241, mir-84* mutants (Abrahante, 2003, Abbott et al., 2005). *lin-41(0)* mutants develop incomplete adult alae precociously, although it is unclear what stage *lin-41* regulates (Slack et al., 2000).

I chose to apply a novel method for a rapid degradation of target proteins – the auxin-inducible degron system. This system was adapted from *Arabidopsis thaliana*. It requires a strain of worms expressing transgenic enzyme atTIR-1 and an AID tag fused with a target protein. Then, upon addition of a plant hormone 3-indoleacetic acid (IAA, auxin), TIR-1 binds to the tag and recruits an ubiquitination complex that attaches polyubiquitin to the tag targeting it for the proteasomal degradation (Zhang et al., 2015). This system was upgraded recently – a modified TIR-1 was designed that lacks the background

degradation, works with a lower concentration of auxin analog 5-Ph-IAA and has a shorter response time (Hills-Muckey et al., 2022). This approach would allow us to study the times of action of other heterochronic genes similarly to how it was done with *lin-14* temperature-sensitive mutants.

## Results

### *The Times that* lin-14 *Acts as Determined by* lin-14:aid *Match Those Determined by Temperature-Sensitive Alleles*

In *lin-14(0)* mutants, seam cell division patterns characteristic of the L2 stage occur at the L1 stage, L3 patterns occur in the L2, and precocious adult alae develop at the L3 molt. The studies using temperature-sensitive alleles of *lin-14* showed that it has two activities that act at different times: an early activity named *lin-14a* that occurs in the beginning of the L1 stage and promotes L1 developmental events, and a second activity, *lin-14b* that occurs in the middle of the L1 stage and promotes L2 developmental events (Ambros and Horvitz, 1984). When only *lin-14a* activity is defective, seam cells execute L2 cell fates at the L1 stage and then repeat them again at the L2 stage, with later stages occurring normally. When only *lin-14b* activity is defective, seam cells skip L2 developmental events and have only 3 larval stages that results in a reduced number of seam cells and precocious alae.

To test the usefulness of the auxin-inducible system for determining the times of action for heterochronic genes, I analyzed the phenotypes of *lin-14::AID* worms transferred to and from 5-Ph-IAA at different time points after synchronization. Shifting animals onto

5-Ph-IAA should be equivalent to shifting temperature-sensitive (*ts*) alleles to the non-permissive temperature.

I first assessed *lin-14*'s control of L2 events (the *lin-14b* activity) by assessing seam cell number at adulthood of animals shifted at different times (Table 4.1, experiments 1 and 2). If normal L2 seam cell linages are skipped, and L3 patterns occur in their place, adult seam cell numbers should be reduced (Ambros and Horvitz, 1987). I observed that the number of seam cells at adulthood was the lowest when *lin-14::AID* animals were shifted to 5-Ph-IAA at 4-8 hours of development (Fig. 4.1A). Larvae transferred to 5-Ph-IAA at 10 and 12 hours in development had an intermediate number of seam cells, and most larvae executed normal seam cell division patterns when they were transferred to 5-Ph-IAA at 14 hours in development or later (Fig. 4.1A).

Larvae transferred to 5-Ph-IAA at 6 hours had full precocious alae, but when transferred between 10 and 12 hours in development, they displayed partial or no precocious alae, and alae developed at the normal time when they were transferred to 5-Ph-IAA at 14 hours in development or later (Fig. 4.1B).

Most *lin-14::AID* animals grown with or without 5-Ph-IAA entered L1 lethargus between 16-18 hours in development, and L1 seam cell divisions were observed at 4-7 hours in development. Thus, the end of the time period requiring *lin-14b* activity as determined by the AID system was the mid- to late- L1 stage. This period was similar to that defined using temperature-sensitive *lin-14* alleles (Ambros and Horvitz, 1984).

It is important to note that *lin-14::AID* animals grown continuously on 5-Ph-IAA had a reduced number of seam cells, indicating that *lin-14* activity was not completely reduced

to null levels (Fig. 4.1A). Because *lin-14(0)* animals lack both *lin-14a* and *lin-14b* activities, they execute the L2 lineage pattern (a double cell division) once, precociously in the L1, and thus have a normal adult seam cell count (Ambros and Horvitz, 1984). Because continuous growth of this strain on 5-Ph-IAA does not result in wildtype seam cell numbers, I suspect that the degron does not completely inactivate *lin-14*, but rather results in a partial loss-of-function instead of a null phenotype. However, this distinction should not affect the interpretation of these timing experiments.

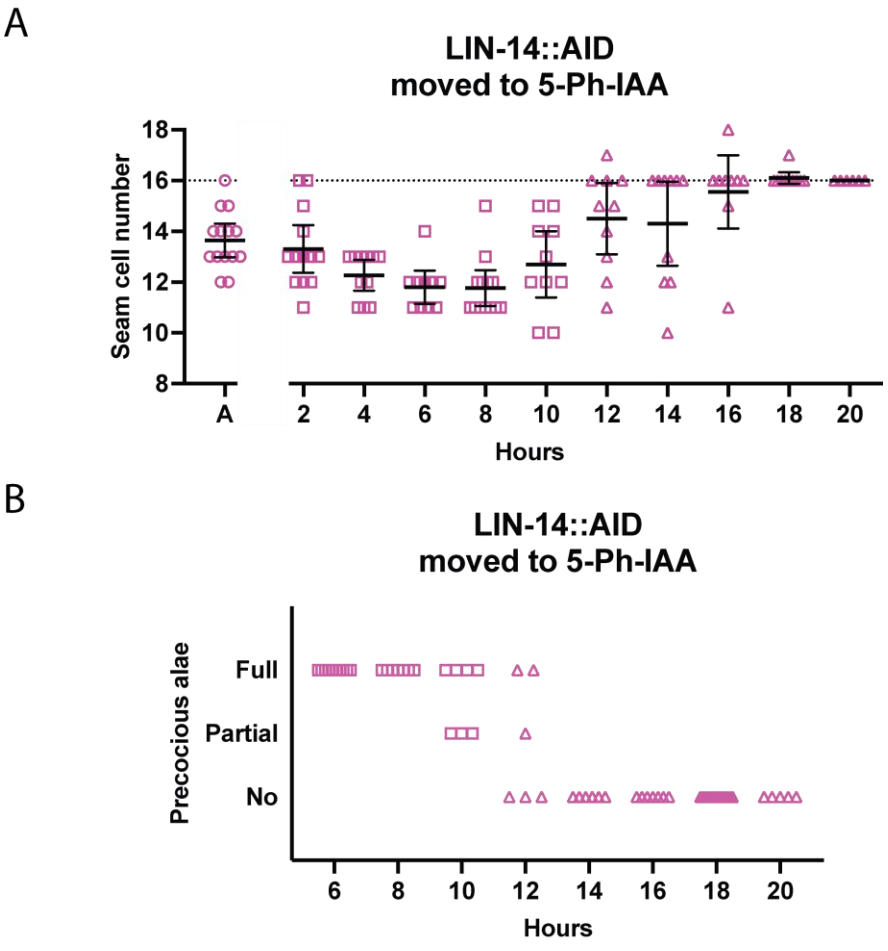



*Figure 4.1. lin-14* acts during the L1 stage to promote L2 seam cell fates. (A) Numbers of seam cells in *lin-14::AID* adults moved to 5-Ph-IAA at the indicated times after

synchronization. Horizontal dotted line indicates the wildtype number of seam cells, 16. "A" indicates worms grown continuously on 5-Ph-IAA. Bars indicate averages and 95% CI. (B) Alae in *lin-14::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. Alae data was recorded only for L4 larvae. Different dot shapes indicate different groups of synchronized worms that were necessary to cover all the times. Several animals at 14 hours had fewer than 16 seam cells, those animals also were at the late L3 stage and the alae data is not known for them and not shown in the second plot. In general, the L1 lethargus began between 16 and 18 hours.

Shifting *lin-14::AID* animals off of 5-Ph-IAA should be the equivalent of shifting *ts*-alleles to the permissive temperature. Most *lin-14::AID* larvae removed from 5-Ph-IAA in the first hour in development had more seam cells than the wild type but no precocious alae (Table 4.1, experiment 12, Fig. 4.2A and B). A similar phenotype was observed by Ambros and Horvitz (1984) and was a result of worms executing L2 fates in the L1 with normal lineages after that (*lin-14a*$^{-}$*b*$^{+}$). As a consequence, the L2 fates are executed twice, both in the L1 and L2, resulting in a higher than normal number of seam cells. Shifting animals at 2 hours resulted in a nearly wild-type level of seam cells and some precocious alae. These data indicate that the auxin-sensitive period for the *lin-14a* activity is at 1 hour or prior and that the auxin-sensitive period for the *lin-14b* begins around 2 hours.

Intestinal nuclei divisions occur during the L1 lethargus, and these are also under the control of *lin-14*. *C. elegans* L1s hatch with 20 intestinal nuclei, but some of these undergo mitosis during the L1 molt to yield 30-34 intestinal nuclei by adulthood. My own counts of the *lin-14::AID* strain showed that early L1 larvae had 20 ± 0.5 (n = 14)

intestinal nuclei, and animals after L2 seam cell divisions had 30 ± 2.3 (n = 12) intestinal nuclei. Consistent with prior observations, I observed that *lin-14(0)* mutants or *lin-14::AID* worms grown continuously on 5-Ph-IAA have only 21.8 ± 2.4 (n = 15) intestinal nuclei at adulthood.

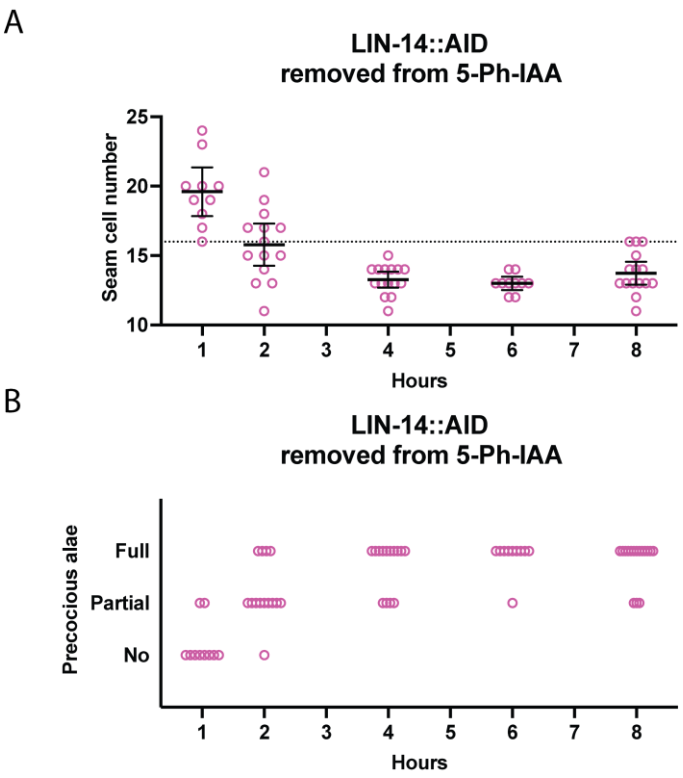



*Figure 4.2*. The restoration of *lin-14* activity early in development causes a reiteration of the L2 cell fates. (A) Numbers of seam cells in *lin-14::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization. Horizontal dotted line indicates the exact wild-type number of seam cells. Bars indicate averages and 95% CIs. (B) Alae in *lin-14::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization.

Next, I determined the auxin-sensitive period for *lin-14*'s control of intestinal nuclear divisions. Intestinal nuclei numbers were reduced in *lin-14::AID* worms transferred to 5-Ph-IAA before 16 hours. L1 lethargus was observed at 16-18 hours, and the worms in lethargus (without pharyngeal pumping) that were transferred to 5-Ph-IAA at 18 hours developed a normal number of intestinal nuclei (experiment 3, Table 4.1, Fig. 4.3). The

number of intestinal nuclei increased in worms transferred to 5-Ph-IAA at 13-14 hours in development as compared to those always grown on 5-Ph-IAA, yet was still less than the wild-type number, indicating that *lin-14* activity promoting intestinal nuclei divisions was not completed at that time. Thus, this activity of *lin-14* in the intestine has a slightly different time frame compared to its activities promoting seam cell fates. As a result, although the control of intestinal nuclei divisions and the *lin-14a* activity both occur in the L1 stage, these activities have different time frames and might be independent.

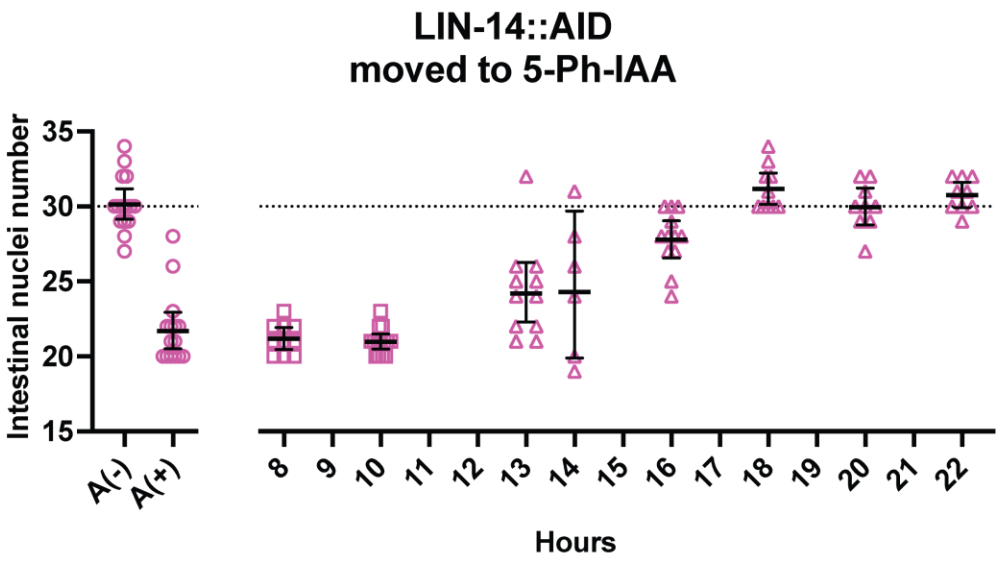



*Figure 4.3. lin-14* has a separate timeframe for the activity that promotes intestinal nuclei divisions. Intestinal nuclei numbers in *lin-14::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. The horizontal dotted line indicates the average number of intestinal nuclei in wild-type worms. "A(-)" indicates worms grown without 5-Ph-IAA. "A(+)" indicates worms grown on 5-Ph-IAA. Bars indicate averages and 95% CIs. Different dot shapes indicate different groups of synchronized worms.

### **lin-28** ***and*** **hbl-1** ***Act at the End of the First Larval Stage and During the Second Larval Stage to Promote L2 and L3 Developmental Events***

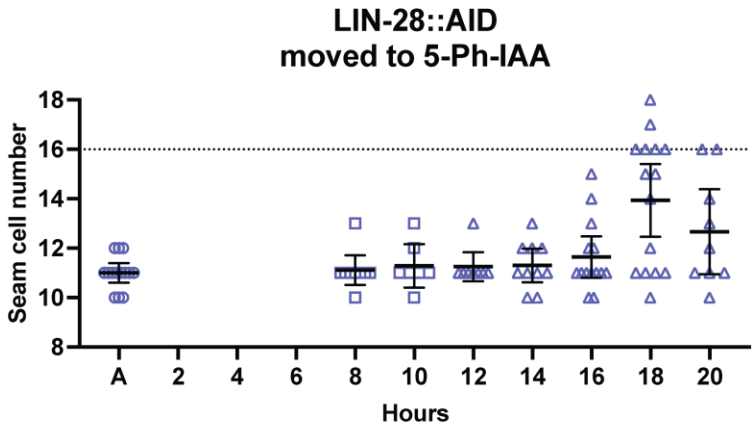
Having established for *lin-14* that the auxin-sensitive periods match the established temperature-sensitive periods, I employed this system to study *lin-28* and *hbl-1*.

Seam cell numbers in *lin-28::AID* worms increased in animals transferred to 5-Ph-IAA during the L1 lethargus at 18 hours, as compared with worms transferred earlier (Fig. 4.4A). This time of action is later than was defined while studying *lin-14::AID* worms (experiments 1 and 2, Table 4.1, Fig. 4.4A). Thus, the time that *lin-28* acts to promote L2 seam cell fates appears to be separate from the time for *lin-14b,* and occurs later in development.

In the next sets of experiments (4 and 5, Table 4.1), the *lin-28(lf)* phenotype was weaker when *lin-28::AID* worms were moved to 5-Ph-IAA at 16-20 hours in development and was not observed in those moved at 22 hours or later. However, some *lin-28::AID* worms transferred to 5-Ph-IAA at 22 hours and later had 17 seam cells. The reason is unclear (Fig. 4.5A).

The time frame for the end of *hbl-1* activity promoting L2 seam cell fates was almost identical to that of *lin-28*, except that the weakening in severity of the phenotype was observed slightly later – in worms moved to 5-Ph-IAA at 18 hours. The majority of *hbl-1::AID* worms developed a normal number of seam cells when they were moved to 5-Ph-IAA at 24 hours in development or later (Fig. 4.5B).

A




B

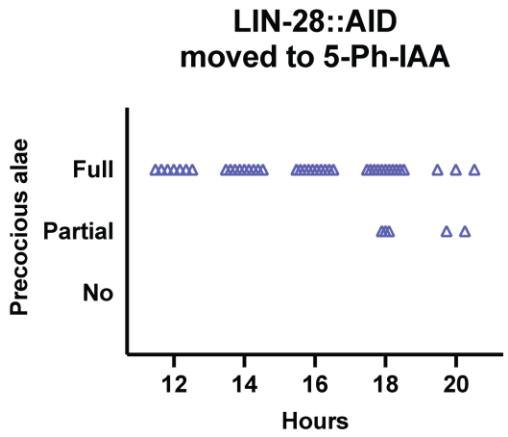



*Figure 4.4.* The activity of *lin-28* that promotes L2 seam cell fates occurs later than the similar activity of *lin-14*. (A) Numbers of seam cells in *lin-28::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. The horizontal dotted line indicates the wild-type number of seam cells. "A" indicates worms grown on 5-Ph-IAA. Bars indicate averages and 95% CIs. (B) Alae in *lin-28::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. Different dot shapes indicate different groups of synchronized worms.

The time at which the *lin-28* and *hbl-1* activities that promote L2 seam cell fates weakens coincides with the time that the L2 seam cell divisions occur in most wild-type (RG733) and degron strains (*lin-28::AID* and *hbl-1::AID*) grown without 5-Ph-IAA; these divisions start during the L1 molt and continue for around 2 hours after the molt. Considering the time needed for the process of degradation to start working, we conclude that the activities of *lin-28* and *hbl-1* are required right before the L2 seam cell divisions.

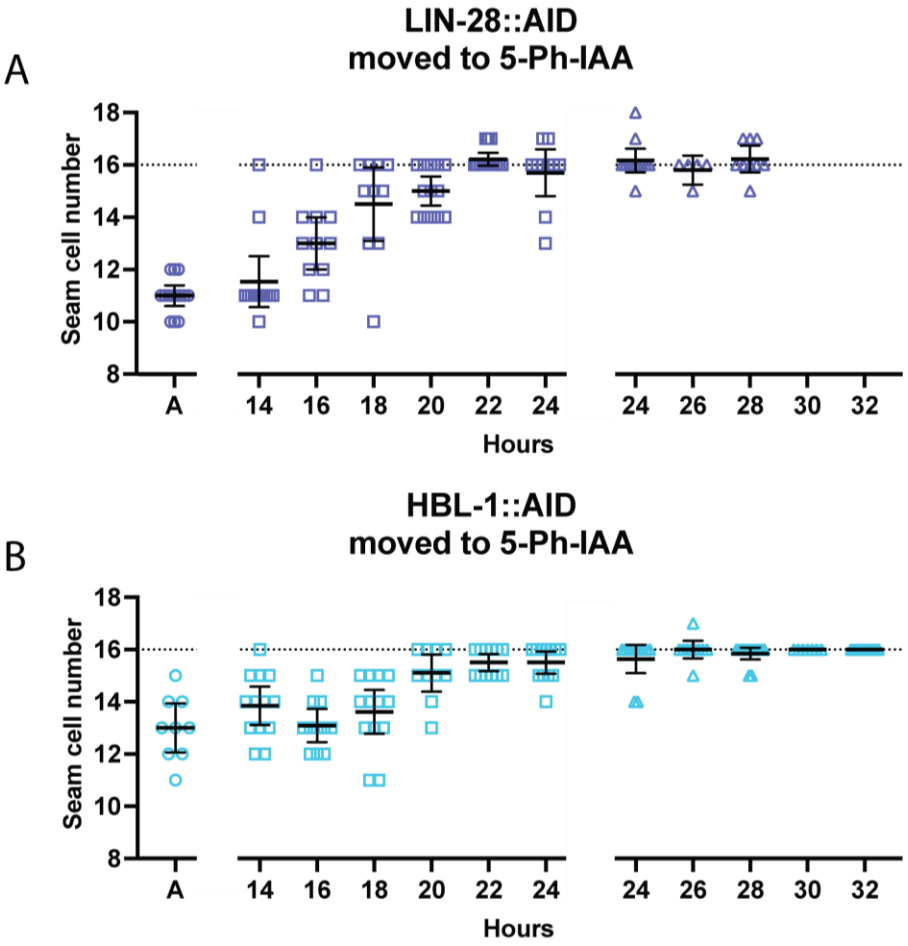



*Figure 4.5*. The activities of *lin-28* and *hbl-1* that promote L2 seam cell fates coincide in time. (A) Numbers of seam cells in *lin-28::AID* worms moved to 5-Ph-IAA at the

indicated times after synchronization. (B) Numbers of seam cells in *hbl-1::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. Horizontal dotted lines indicate the wild-type number of seam cells. "A" indicates worms grown continuously on 5-Ph-IAA. Bars indicate averages and 95% CIs. Different dot shapes indicate different groups of synchronized worms.

For both *lin-28* and *hbl-1*, the time frame for specifying precocious alae development was slightly later than the time for the reduced number of seam cells. Some worms transferred to 5-Ph-IAA at 20, 22, and 24 hours had normal numbers of seam cells and complete or gapped precocious alae (Fig. 4.6), which indicates that they executed the L2 seam cell fates normally and then skipped a stage. Thus, both *lin-28* and *hbl-1* have two activities separated in time, much like *lin-14*.

For *lin-28*, the activity promoting L3 seam cell stages completed between 24 and 26 hours in development (Fig. 4.6, A). For *hbl-1*, this activity completed at 28-30 hours in development (Fig. 4.6, B).

Precocious alae also had different appearances in *lin-28::AID* and *hbl-1::AID* strains with weaker phenotypes (Fig. 4.7). In *lin-28::AID* worms, alae were clear even though they had gaps, in *hbl-1::AID*, alae were “weak”—more transparent or thinner and difficult to see. It was not possible to estimate an approximate percent of coverage in most cases, because it was difficult to distinguish if they were complete or partial. This observation also indicates that *lin-28* and *hbl-1* control the final differentiation of seam cells differently.

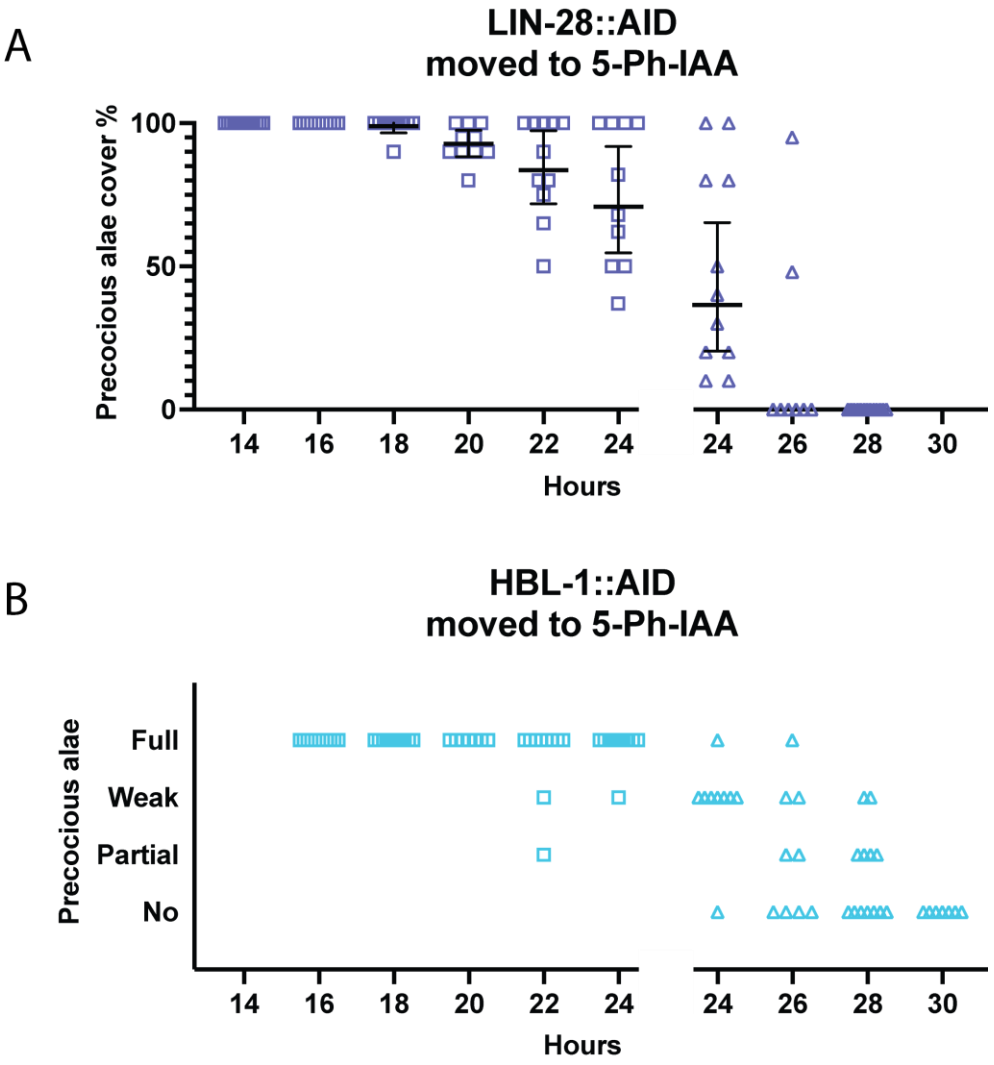



*Figure 4.6. lin-28* and *hbl-1* have activities that promote seam cell fates after the L2 stage. (A) Precocious alae in *lin-28::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. The percent was calculated as the fraction of seam cells that developed alae versus the total number of seam cells. Bars indicate averages and 95% CIs. (B) Precocious alae in *hbl-1::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. Alae were scored as "full" when they could be easily followed from head to tail, "weak" when alae did not have clear gaps but also could not be followed completely, and "partial" when gaps were observed. A gap in the graph

separates different experiments. Different dot shapes indicate different groups of synchronized worms.

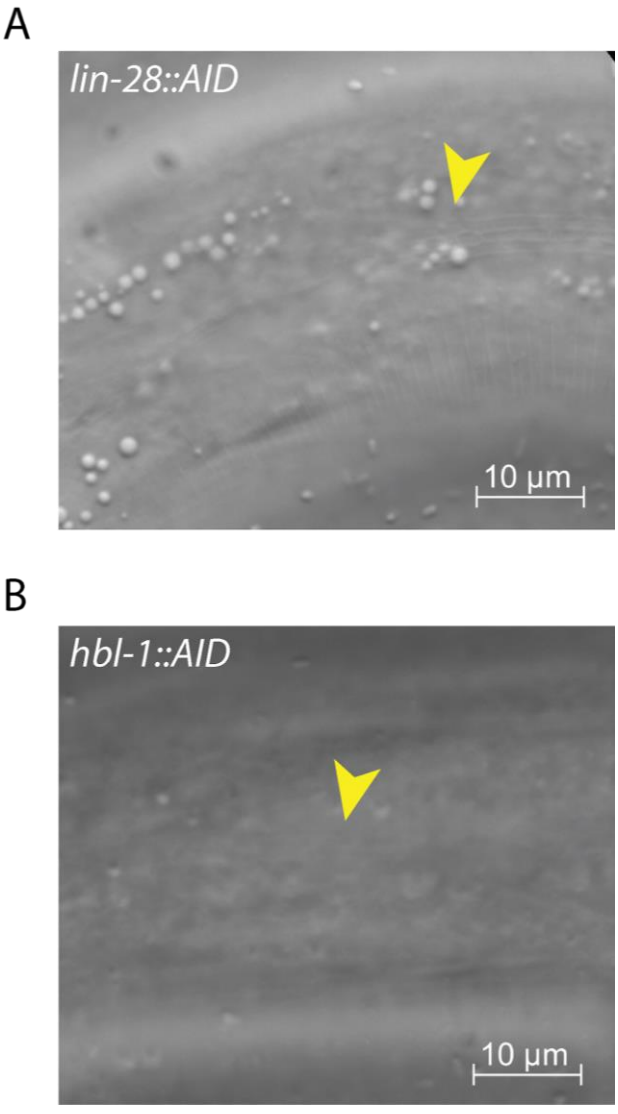



*Figure 4.7*. Precocious alae appearances differ in weak loss-of-function phenotypes of *lin-28::AID* and *hbl-1::AID* worms. DIC microphotographs of L4 worms grown at 20°C. Worms are oriented anterior to the left, dorsal side up. (A) *lin-28::AID* worms develop clear alae patches when moved to 5-Ph-IAA at times close to the end of the second

activity. The yellow arrowhead indicates the edge of an alae patch. (B) *hbl-1::AID* worms develop thin and transparent alae when moved to 5-Ph-IAA at times close to the end of the second activity, the edges of alae patches are hard to discern. The yellow arrowhead indicates alae.

### **lin-28** *is Regulated through a Positive Feedback Loop*

Removing *lin-28::AID* worms from 5-Ph-IAA did not cause a restoration of *lin-28* activity. L1 larvae that were placed on 5-Ph-IAA after synchronization and removed in 1 hour developed a null-like phenotype with reduced numbers of seam cells and precocious alae (11 ± 0.5 seam cells, 87% with full precocious alae, n = 8). This result indicates that *lin-28* might have a positive feedback loop in its regulation, so that its expression cannot be restored once the activity of the protein is reduced.

### **hbl-1** *has Two Activities Separated in Time*

To identify the time frame for the restoration of *hbl-1* activity, synchronized *hbl-1::AID* worms were placed on 5-Ph-IAA plates and then transferred to regular plates at 4 hour intervals (experiments 8 and 9, Table 4.1). There was a drop in seam cell number in worms removed from 5-Ph-IAA at 24 hours in development and further (Fig. 4.8).

To identify the *hbl-1* times of action more precisely, *hbl-1::AID* worms were transferred to 5-Ph-IAA or removed from it in 2-hour intervals (experiments 10 and 11, Table 4.1).

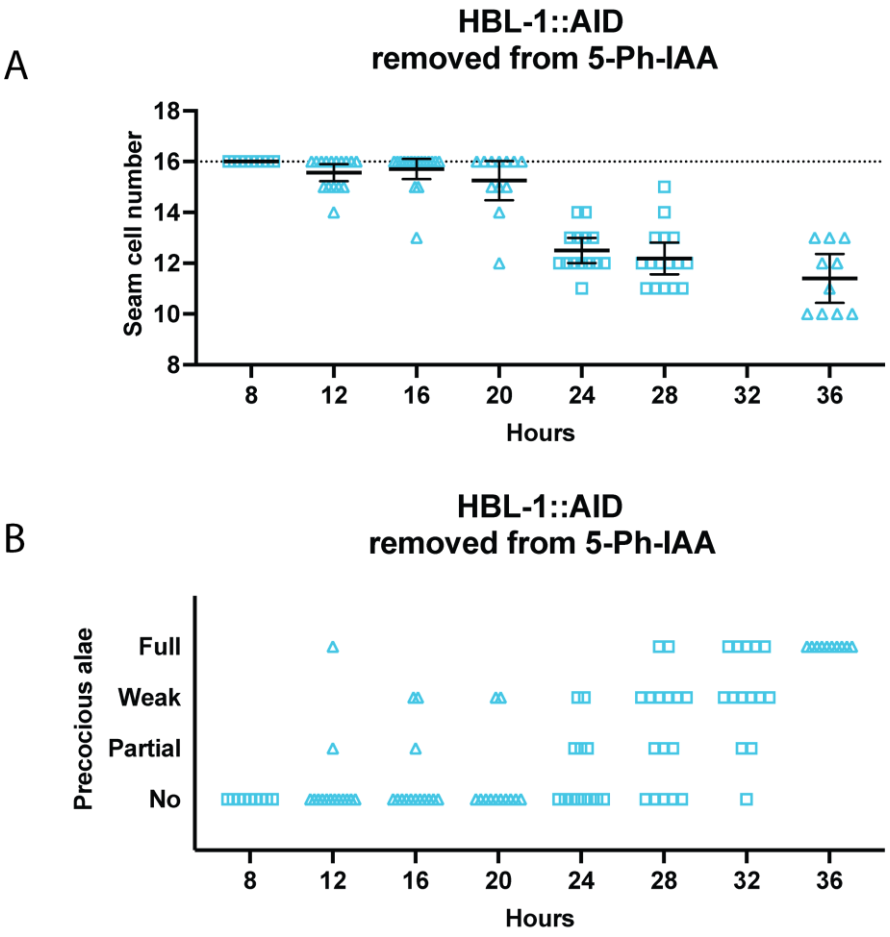



*Figure 4.8. hbl-1* activity is restored after removal from 5-Ph-IAA. (A) Numbers of seam cells in *hbl-1::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization. Horizontal dotted lines indicate the wild-type number of seam cells. Bars indicate averages and 95% CIs. (B) Precocious alae in *hbl-1::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization. Different dot shapes indicate different groups of synchronized worms.

As previously seen, the number of seam cells was normal in worms transferred to 5-Ph-IAA at 22 ± 2 hours in development (Fig. 4.9, A). In the reciprocal experiment, the number of seam cells remained normal in worms removed from 5-Ph-IAA before 18 hours in development and showed a strong loss-of-function phenotype in those removed at 24 hours or later (Fig. 4.9, B). These results indicate that the activity of *hbl-1* can still promote L2 seam cell fates if restored before 22 hours in development (assuming about two hours for 5-Ph-IAA washout from the tissues).

L2 seam cell divisions occur between 21-24 hours in wildtype animals, as well as in *hbl-1::AID* worms. In *hbl-1::AID* animals grown continuously on 5-Ph-IAA L2 divisions were observed at the same time. This finding suggests that the first *hbl-1* activity occurs right before or during the L2 seam cell divisions.

Full precocious alae were observed in *hbl-1::AID* worms that had been transferred to 5-Ph-IAA before 24 hours in development. The development of precocious alae became less frequent in later transfers, until they disappeared altogether in worms transferred at 30 hours in development (Fig. 4.10, A). Similarly, in worms removed from 5-Ph-IAA at 24-26 hours in development, precocious alae sometimes appeared (Fig. 4.10, B). Since most worms did not develop precocious alae when they were moved to 5-Ph-IAA at 28 hours or later, and most worms formed precocious alae if they were removed from 5-Ph-IAA at the same time, this must be shortly after the time that *hbl-1* acts to control later events. Thus, *hbl-1*'s activity promoting seam cell fates after the L2 (24-26 hours) is separated in time from its earlier activity controlling seam cell divisions (18-22 hours). This second activity ceases in 4-6 hours after the L2 divisions are completed, which is approximately 4-6 hours before the L2 molt.

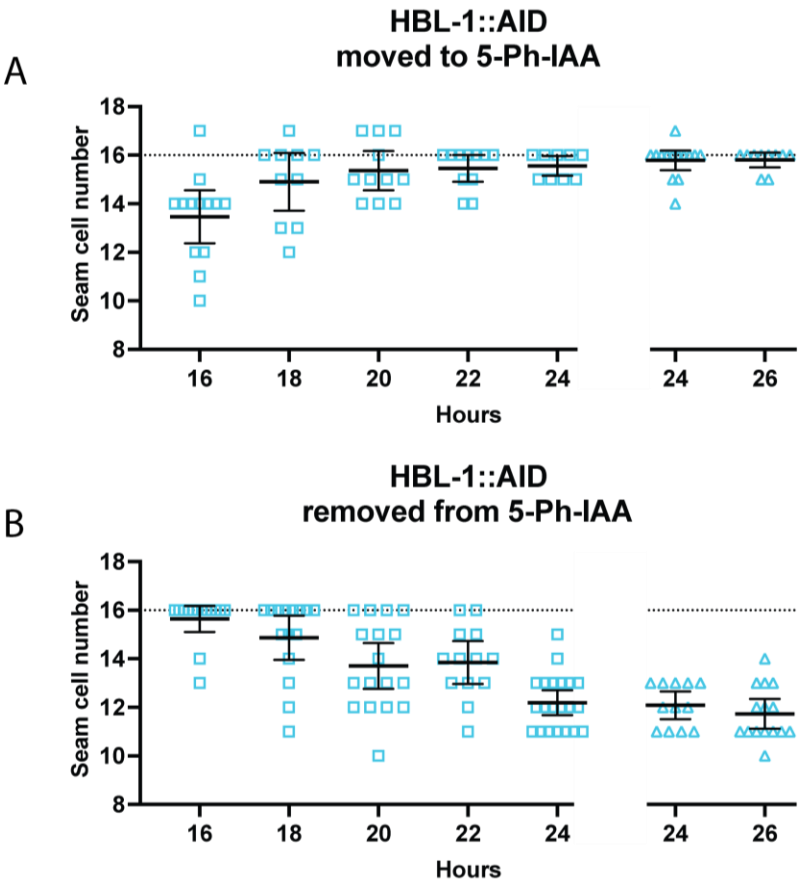



*Figure 4.9. hbl-1* has a narrow time frame for promoting L2 seam cell fates. (A) Numbers of seam cells in *hbl-1::AID* worms transferred to 5-Ph-IAA at the indicated times after synchronization. (B) Numbers of seam cells in *hbl-1::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization. Horizontal dotted lines indicate the exact wild-type number of seam cells. Bars indicate averages and 95% CIs. Gaps in the graphs separate different experiments. Different dot shapes indicate different groups of synchronized worms.

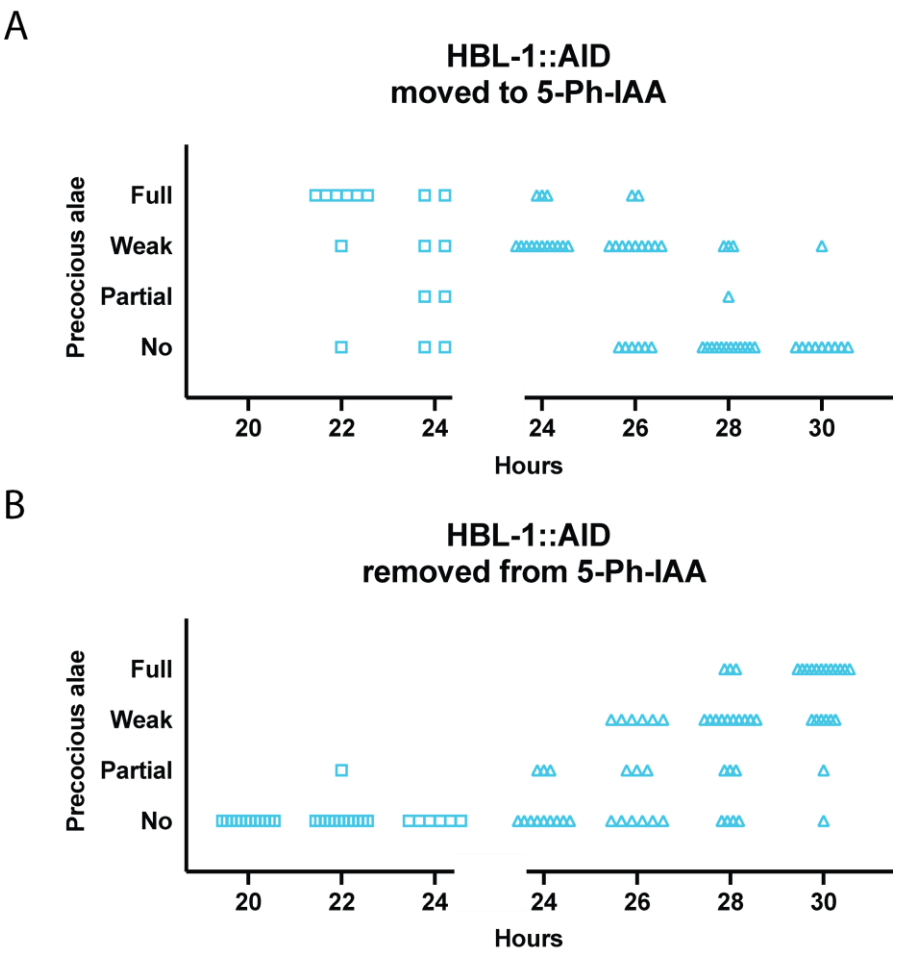



*Figure 4.10*. The time of the second *hbl-1* activity is distinct from the first. (A) Precocious alae in *hbl-1::AID* worms moved to 5-Ph-IAA at the indicated times after synchronization. (B) Precocious alae in *hbl-1::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization. Alae were scored as "full" if they could be easily followed from head to tail, "weak" if they did not have clear gaps but could not be easily followed, and "partial" when gaps were observed. Gaps in graphs separate different experiments. Different dot shapes indicate different groups of synchronized worms.

### **lin-41** ***Acts During the L3 Stage***

Short precocious alae patches (less than 50% coverage) were observed in around 38% of *lin-41::AID* worms grown on 5-Ph-IAA (Fig. 4.11, A). These worms also had a Dpy phenotype.

The fraction of worms with precocious alae patches was smaller in animals transferred to 5-Ph-IAA at 20 hours in development or later, compared to 16 and 18 hours (experiments 12, 13, and 14, Table 4.1, Fig. 4.11, A). This result indicates that *lin-41* might have some activity before the L1 or L2 molt, although it is unclear if it regulates seam cell divisions at different stages. After 18 hours, the fraction of worms with alae patches remained at approximately the same level until 38 hours in development, when it was no longer observed. Fisher's exact test indicated that there was a difference between the combined 16-18 hours group and the 20-40 hours group ($p < 0.05$).

In worms removed from 5-Ph-IAA, alae patches were observed at a rate similar to those grown on 5-Ph-IAA at 32.5 hours in development (n = 19) or later.

In wild-type worms and *lin-41::AID* worms grown with or without 5-Ph-IAA, the L3 seam cell divisions were observed at 36 hours in development and the L3 molt around 40 hours in development. Thus, *lin-41* acts during the L3 stage to promote L3 seam cell fates.

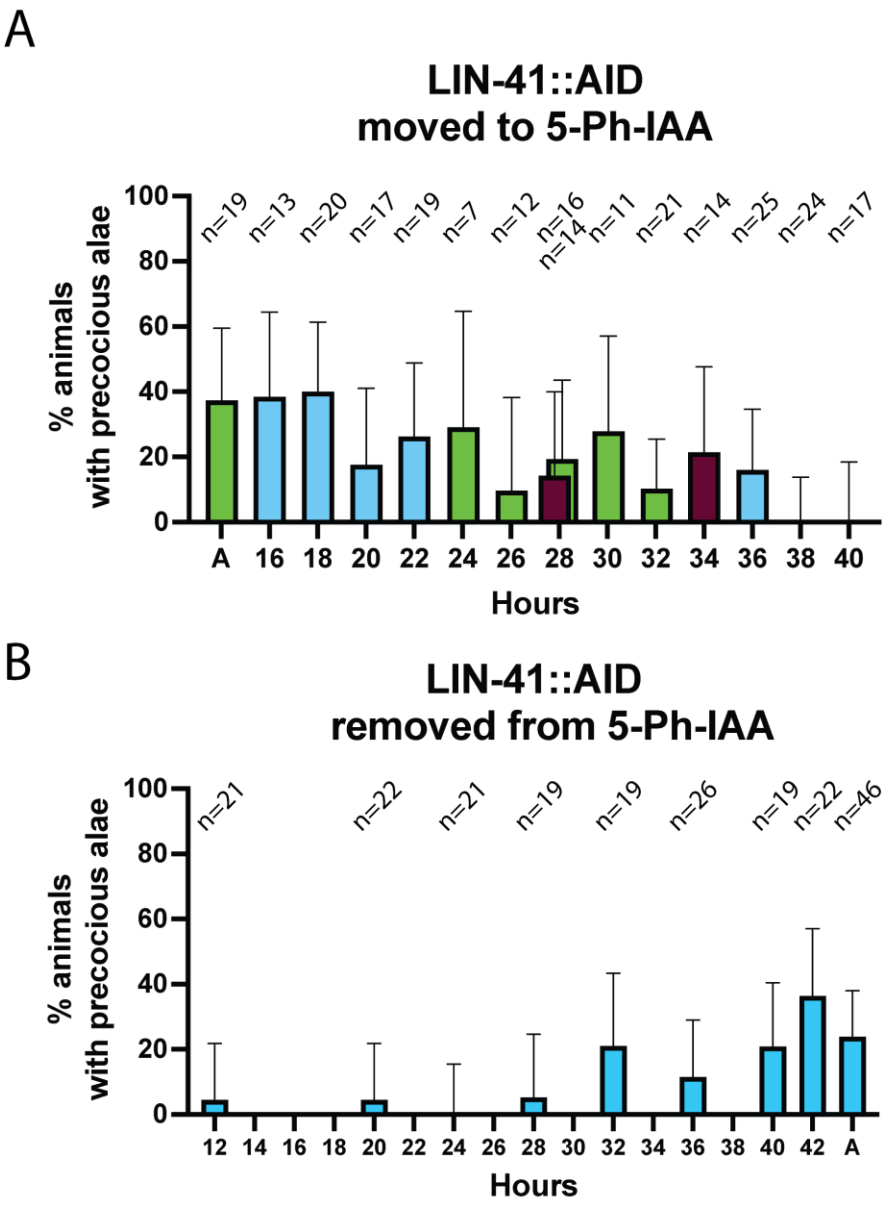



*Figure 4.11. lin-41* acts during the L3 stage but also has some activity during the L1 stage. (A) Percent of animals with precocious alae in *lin-41::AID* worms transferred to 5-Ph-IAA at the indicated times after synchronization. Different bar colors indicate different groups of synchronized worms. (B) Percent of animals with precocious alae in *lin-41::AID* worms removed from 5-Ph-IAA at the indicated times after synchronization.

Error bars indicate 95% CIs. Different groups of synchronized worms are not indicated in this plot. The total number of observed worms is indicated above the bars.

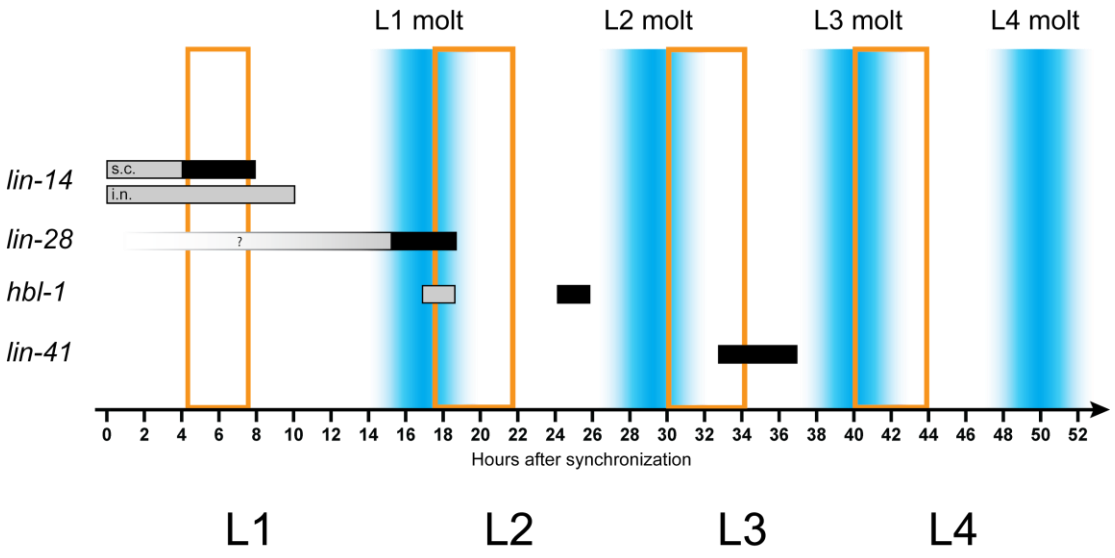



*Figure 4.12.* Times of actions for heterochronic genes in *C. elegans* development. The axis shows hours in development, the "0" point is the time when synchronized worms are transferred to food. Blue boxes indicate the molts. Orange boxes indicate seam cell divisions. Gray bars - the activities of proteins influencing the number of seam cells (L2 symmetric divisions), time frame for the strong (lf-like) phenotype, black bars - the activities of proteins influencing the final differentiation of seam cells, time frame for the strong (lf-like) phenotype, the start time of an activity is determined as the first time point when the most worms had a phenotype divergent from WT either in backward or forward transfers (except *lin-41*). S.c. - *lin-14* activities related to seam cells, i.n. - *lin-14* activities related to intestinal nuclei, only seam cells activities are shown for other proteins.

**Table 4.1**

*List of Experiments*

| Experiment number | Strains synchronized | Direction of transfers | Times in development covered |
|---|---|---|---|
| 1 | *lin-14::AID, lin-28::AID* | forward | 2, 4, 6, 8, 10 |
| 2 | *lin-14::AID, lin-28::AID* | forward | 12, 14, 16, 18, 20 |
| 3 | *lin-14::AID* | forward | 8, 10 |
| 4 | *lin-28::AID, hbl-1::AID* | forward | 14, 16, 18, 20, 22, 24 |
| 5 | *lin-28::AID, hbl-1::AID* | forward | 24, 26, 28, 30, 32 |
| 6 | *lin-14::AID* | forward | 13, 14, 16, 18, 20, 22 |
| 7 | *hbl-1::AID, lin-41::AID* | forward | 24, 26, 28, 30, 32 |
| 8 | *hbl-1::AID, lin-41::AID* | forward, backward | 12, 16, 18, 20, 22, 36, 38, 40 |
| 9 | *hbl-1::AID, lin-41::AID* | forward, backward | 8, 24, 28, 32, 34 |
| 10 | *hbl-1::AID* | forward, backward | 14, 16, 18, 20, 22, 24 |
| 11 | *hbl-1::AID* | forward, backward | 24, 26, 28, 30 |
| 12 | *lin-14::AID* | forward, backward | 1, 2, 4, 6, 8 |
| 13 | *lin-41::AID* | forward | 36, 38, 40, 42, 44 |
| 14 | *lin-41::AID* | backward | 40, 42 |

*Note.* Strains in each experiment were sync. Forward - synchronized worms put on regular plates, then transferred to 5-Ph-IAA; Backward - synchronized worms put on

plates with 5-Ph-IAA, then transferred to regular plateshronized at the same time and transfers were carried out at similar time points.

**Discussion**

My first goal in this study was to demonstrate that the auxin-inducible system could be used to study the time of actions of heterochronic genes in *C. elegans*. The experimental design was based on studies of *lin-14* temperature-sensitive alleles (Ambros and Horvitz, 1987). In those studies, the authors observed individual larvae grown at 15℃ or 25℃ and shifted to the reciprocal temperature at specific times in development. Their data were based on scoring specific cell lineage events known to be under *lin-14* control, to determine the times at which *lin-14* acted to control different cell fates. All data were plotted on a scale of 20°C development. Similarly, I observed changes in seam cell lineages such as different numbers of seam cells and their times of differentiation following shifts to or from plates with 5-Ph-IAA.

I synchronized L1 larvae by starvation at hatching. Newly hatched L1 larvae soon arrest development if they lack nutrients. However, the time of placing synchronized larvae on food that we consider a "0" or "start" point is not the same as the time of hatching. Furthermore, the full degradation of a target protein is usually achieved within 1 hour of placing adult worms on auxin (Zhang, 2015), although it might occur more rapidly in larvae due to their smaller size. Even the incomplete degradation of a protein might be enough to cause a loss of function. Thus, the response time in our experiments could be less than 1 hour. We maintained all worms and carried out the experiments at 20°C.

The time for recovery of target protein expression after the removal from auxin was not described for the new 5-Ph-IAA system, but for the original AID system it was 24 hours for adult worms (Zhang, 2015). Considering that we used a significantly smaller concentration of the inducer (0.005μM of 5-Ph-IAA vs 25μM of auxin in the original) and smaller size of larvae we might expect that recovery would occur more rapidly.

Our results for *lin-14* are similar to those obtained using temperature-sensitive alleles. We observed the end of *lin-14a* activity between 2 and 4 hours, slightly earlier than Ambros and Horvitz who observed it at 4 hours, which is close considering the factors described above. For *lin-14b* activity, we observed a strong phenotype until 8 hours in development and then it was intermediate until 14-16 hours in development. Ambros and Horvitz observed it until 11-12 hours in development.

However, they considered animals with 30% or fewer seam cells expressing L3 patterns equivalent to those grown on 15°C (permissive temperature). In our experiments, 70% of worms transferred to 5-Ph-IAA at 12 hours had 14 or more seam cells, meaning that they had less than 30% of seam cells expressing L3 patterns. Thus they would fall in the "wild type" category and we could consider the "12 hours" point as the end of *lin-14b* activity, which closely corresponds to the results of Ambros and Horvitz.

Although we were not able to observe a time frame for the restoration of *lin-14a* activity, *lin-14b* activity could be fully restored when worms were removed from 5-Ph-IAA plates at 1 hour after start, and at 2 hours in some worms, whereas Ambros and Horvitz observed restoration following shifts up until 6-7 hours in development. Thus, the recovery from 5-Ph-IAA is not as rapid as the forward experiments for *lin-14*. But

overall, these results showed that the AID system could be used to determine the times of actions for heterochronic genes and that times obtained in forward experiments are precise.

We were not able to observe recovery of *lin-28* activity after the removal from 5-Ph-IAA. By contrast, *hbl-1* activity could be fully restored, the removal from auxin at 2-4 hours prior to the L2 seam cell divisions still promoted L2 patterns. If we assume that the recovery of activity occurs nearly immediately after the removal from 5-Ph-IAA, then it indicates that *hbl-1* activity is required 2-4 hours before L2 seam cell divisions. If we assume that the recovery of the activity occurs in 2-4 hours after the removal from 5-Ph-IAA, then it indicates that *hbl-1* activity is required immediately before L2 seam cell divisions. In any case, the time frames for *hbl-1* activities are narrow, and it must promote L3 seam cell fates in the middle of the L2 stage.

Nevertheless, I showed that *lin-28* and *hbl-1* each have two times of actions, promoting different aspects of seam cell fates at two different stages, much like *lin-14*. Having two activities separated in time might be necessary if heterochronic genes act on different sets of targets, or if the first activities of proteins promote the accumulation of their further targets. It remains unclear if heterochronic genes execute both activities doing the same molecular function at two different times, or two different functions.

Interestingly, there is a time delay between the activities of upstream and downstream genes controlling the same stage. For example, *lin-14b* occurs before the first activity of *lin-28*, which occurs before the first activity of *hbl-1*, all of which control L2 seam cell divisions. Those delays might reflect the activity of other components of the

heterochronic pathway that connect the mentioned genes. Plausible candidates are miRNAs *lin-4* and *3-let-7s* that downregulate *lin-28* and *hbl-1*, and *lin-46* that negatively controls *hbl-1*.

The AID system failed to show clearly if *lin-41* has two different activities and it would require additional experiments to find this out. For example, observations of male tail seam cell lineages that differ at L3 and L4 stages could be valuable. In general, *lin-41* activity was required between 32-38 hours in development, which is the time period when L3 seam cell divisions occur. However, the precocious alae phenotype is more penetrant in worms grown continuously on 5-Ph-IAA or moved to 5-Ph-IAA before 20 hours in development than later. That suggests that *lin-41* might have some activity before the L2 seam cell divisions.

# Chapter 5

## Discussion

My work has added new two layers to our understanding of the heterochronic pathway: I have shown that the core genes of the pathway are all conserved in Caenorhabditis, but some of their functions and interactions have begun to undergo evolutionary change, and I have shown how the activities of four major heterochronic genes are distributed in time in *C. elegans*.

Studying the heterochronic genes' orthologs in *C. briggsae* revealed a significant drift in the functions of some genes. Would the history of the heterochronic pathway be the same if Sydney Brenner had chosen *C. briggsae* as a model organism instead of *C. elegans*? It seems likely that *lin-4* and *lin-14* would still have been identified since they each have strong heterochronic phenotypes in *C. briggsae*. Perhaps *hbl-1* would have been identified too since its loss-of-function phenotype caused by the targeted degradation is close to that of *C. elegans*, and its hypomorphic alleles would probably have had an observable heterochronic phenotype. However, it might have taken more time to find that *lin-28*, *let-7*, and *lin-41* are related to the heterochronic pathway, since their effects on the hypodermal seam cell lineages are weak or undetectable in *C. briggsae*. Thus, there is a chance we would still have been unaware of their heterochronic functions, especially the regulation of L2 seam cell fates by *lin-28*. These speculations raise an important point - perhaps there are also important genes in the *C. elegans* heterochronic pathway that have not yet been discovered, because their mutant phenotypes are mild or undetectable. Although some genes have been identified through suppressor screens, our findings

suggest that a simple forward mutant screen in *C. briggsae* might detect new or unique heterochronic genes.

As was noted in other studies (Haag, 2005), high sequence conservation does not always indicate conserved functions, and vice versa. LIN-28, which has a highly conserved amino acid sequence in both species, also has the most divergent functions among the heterochronic proteins. In *C. briggsae* it has pleiotropic effects and regulates the gonad development, phenotypes that were not observed in *C. elegans lin-28* mutants. There is some evidence that *Cel-lin-28* is also involved in the gonad regulation, but in this species it acts redundantly with *Cel-hbl-1*. For example, in *C. elegans*, double mutants with *lin-28(0)* and hypomorphic *hbl-1* alleles develop disorganized gonads similar to those observed in *C. briggsae lin-28(0)* animals (Minutillo M., pers. comm.). Intriguingly, *Cbr-lin-28* acts redundantly with *Cbr-hbl-1* in the regulation of L2 seam cell fates, as can be observed in double loss of function mutants. These results imply that ancestral species had LIN-28 and HBL-1 acting redundantly to regulate several processes, and that during evolution the role of each gene has drifted and become either more or less important. This mechanism of preserving genetic redundancy is called piggyback. Two genes that initially have multiple overlapping functions, each can lose some functions during evolution leaving them to another gene alone but if further mutations deactivate both redundant and non-redundant functions, such mutations are eliminated from populations preserving the redundancy (Vavouri et al., 2008) (Vavouri *et al.* 2008).

Another example evolutionary change with redundant genes involves the family of *let-7* miRNAs. All three family members downregulate *lin-28* and *hbl-1* in *C. elegans*. Only

removing all three of them results in a strong reiterative phenotype, whereas a loss of only *mir-48* causes a strong phenotype in *C. briggsae*.

Nevertheless, heterochronic gene orthologs form conserved modules that regulate stage-specific developmental events in *C. elegans* and *C. briggsae* – the *lin-4/lin-14* module regulates L1 and L2 seam cell fates, and the *3-let-7s/lin-28/hbl-1* module regulates L2 and L3 seam cell fates. There is also some evidence that the lin-41/let-7 module is involved in the regulation of L3 and L4 seam cell fates, and in terminal differentiation via conserved *lin-29*.

Furthermore, the study of heterochronic pathway conservation is not complete. Some heterochronic genes such as *sel-7, daf-12,* and *mir-237* do not have crucial functions in *C. elegans* and therefore were not studied in my work. However, it is possible that their orthologs might be more important in the developmental regulation in *C. briggsae*. Besides, there might be other genes involved in the heterochronic pathway in *C. briggsae* that do not have such functions in *C. elegans*. Carrying out mutagenesis screens and mutating other known orthologs related to the heterochronic pathway in *C. briggsae* could be a future direction in exploring the evolutionary drift in the developmental timing regulation.

Another direction of my work was studying the times of actions of heterochronic genes. I showed that four main heterochronic genes *lin-14, lin-28, hbl-1,* and *lin-41* act in a sequence that reflects the chain of their regulatory relationships and that *lin-28* and *hbl-1* have two activities separated in time, similarly to *lin-14*. The two activities of *lin-28* and *hbl-1* were also observed in *C. briggsae,* where *Cbr-lin-28(0)* and *Cbr-hbl-1(lf)* single

mutants skip a stage after the L2, but *Cbr-lin-28(0); hbl-1(0)* double mutant larvae lack L2 seam cell patterns.

Having heterochronic genes that each act at two different points in time might be necessary if they act on different sets of targets. These targets might be available only at certain time points, for example, if the first activity of a gene induces the production of the second set of targets. Having each heterochronic gene promoting two stages is also more efficient than having a separate gene for each stage since that would also require having separate repressors and would create more potential points for destabilization. Finally, if each gene acts at more than one time point, and their activities partially overlap, they can specify many more times as a group than if each gene acted only once.

Most likely, the times of actions do not differ significantly for *lin-14, lin-28* and *hbl-1* orthologs since they control similar stages and the timing of *lin-28* expression is similar in both species. However, it looks like *lin-41* and *let-7* might act later in *C. briggsae* since their mutations cause later defects.

Finding the times of actions for the heterochronic genes orthologs of *C. briggsae* could be another subject for future research and would elucidate if the times of actions are conserved for the orthologs that regulate similar stages but differ in their significance for certain developmental events.

## References


Abbott, A.L., Alvarez-Saavedra, E., Miska, E.A., Lau, N.C., Bartel, D.P., Horvitz, H.R., Ambros, V., 2005. The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in Caenorhabditis elegans. Dev. Cell 9, 403–414. https://doi.org/10.1016/j.devcel.2005.07.009

Aboobaker, A., Blaxter, M., 2003. Hox gene evolution in nematodes: novelty conserved. Curr. Opin. Genet. Dev. 13, 593–598. https://doi.org/10.1016/j.gde.2003.10.009

Abrahante, J.E., Daul, A.L., Li, M., Volk, M.L., Tennessen, J.M., Miller, E.A., Rougvie, A.E., 2003. The Caenorhabditis elegans hunchback-like gene lin-57/hbl-1 controls developmental time and is regulated by microRNAs. Dev. Cell 4, 625–637. https://doi.org/10.1016/s1534-5807(03)00127-8

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

Ambros, V., Horvitz, H.R., 1984. Heterochronic mutants of the nematode Caenorhabditis elegans. Science 226, 409–416. https://doi.org/10.1126/science.6494891

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

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

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712. https://doi.org/10.1126/science.1138140

Bettinger, J.C., Lee, K., Rougvie, A.E., 1996. Stage-specific accumulation of the terminal differentiation factor LIN-29 during Caenorhabditis elegans development. Dev. Camb. Engl. 122, 2517–2527. https://doi.org/10.1242/dev.122.8.2517

Brown K., 2001 Comparative Studies of Vulva Development in C. briggsae

Chalfie M., H. R. Horvitz, and J. E. Sulston, 1981 Mutations that lead to reiterations in the cell lineages of C. elegans. Cell 24: 59–69.

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

Chen, C., Fenk, L.A., de Bono, M., 2013. Efficient genome editing in Caenorhabditis elegans by CRISPR-targeted homologous recombination. Nucleic Acids Res. 41, e193. https://doi.org/10.1093/nar/gkt805

Cho, S.W., Kim, S., Kim, J.M., Kim, J.-S., 2013. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31, 230–232. https://doi.org/10.1038/nbt.2507

Coghlan, A., Wolfe, K.H., 2002. Fourfold faster rate of genome rearrangement in nematodes than in Drosophila. Genome Res. 12, 857–867. https://doi.org/10.1101/gr.172702

Choi S., and V. Ambros, 2019 The C. elegans heterochronic gene lin-28 coordinates the timing of hypodermal and somatic gonadal programs for hermaphrodite reproductive system morphogenesis. Dev. Camb. Engl. 146. https://doi.org/10.1242/dev.164293

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823. https://doi.org/10.1126/science.1231143

Cosmides, L.M., Tooby, J., 1981. Cytoplasmic inheritance and intragenomic conflict. J. Theor. Biol. 89, 83–129. https://doi.org/10.1016/0022-5193(81)90181-8

Cuevas, E., Rybak-Wolf, A., Rohde, A.M., Nguyen, D.T.T., Wulczyn, F.G., 2015. Lin41/Trim71 is essential for mouse development and specifically expressed in postnatal ependymal cells of the brain. Front. Cell Dev. Biol. 3.

Cutter, A.D., Wasmuth, J.D., Washington, N.L., 2008. Patterns of Molecular Evolution in Caenorhabditis Preclude Ancient Origins of Selfing. Genetics 178, 2093–2104. https://doi.org/10.1534/genetics.107.085787

Dokshin G. A., K. S. Ghanta, K. M. Piscopo, and C. C. Mello, 2018 Robust genome editing with short single-stranded and long, partially single-stranded DNA donors in Caenorhabditis elegans. Genetics 210: 781–787.

Eberhard, W.G., 1980. Evolutionary consequences of intracellular organelle competition. Q. Rev. Biol. 55, 231–249. https://doi.org/10.1086/411855

Ellis R. E., 2017 “The persistence of memory”—Hermaphroditism in nematodes. Mol. Reprod. Dev. 84: 144–157.

Ellis, R.E., 2022. Sex Determination in Nematode Germ Cells. Sex. Dev. Genet. Mol. Biol. Evol. Endocrinol. Embryol. Pathol. Sex Determ. Differ. 1–18. https://doi.org/10.1159/000520872

Euling, S., Ambros, V., 1996. Heterochronic genes control cell cycle progress and developmental competence of C. elegans vulva precursor cells. Cell 84, 667–676. https://doi.org/10.1016/s0092-8674(00)81045-4

Fay, D.S., Stanley, H.M., Han, M., Wood, W.B., 1999. A Caenorhabditis elegans homologue of hunchback is required for late stages of development but not early embryonic patterning. Dev. Biol. 205, 240–253. https://doi.org/10.1006/dbio.1998.9096

Félix, M.-A., Duveau, F., 2012. Population dynamics and habitat sharing of natural populations of Caenorhabditis elegans and C. briggsae. BMC Biol. 10, 59. https://doi.org/10.1186/1741-7007-10-59

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811. https://doi.org/10.1038/35888

Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J.A., 2013. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nat. Methods 10, 741–743. https://doi.org/10.1038/nmeth.2532

Frøkjær-Jensen, C., Davis, M.W., Hopkins, C.E., Newman, B., Thummel, J.M., Olesen, S.-P., Grunnet, M., Jorgensen, E.M., 2008. Single copy insertion of transgenes in C. elegans. Nat. Genet. 40, 1375–1383. https://doi.org/10.1038/ng.248

Ghanta K. S., and C. C. Mello, 2020 Melting dsDNA donor molecules greatly improves precision genome editing in Caenorhabditis elegans. Genetics 216: 643–650.

Greene, S., Huang, J., Hamilton, K., Tong, L., Hobert, O., Sun, H., 2023. The heterochronic LIN-14 protein is a BEN domain transcription factor. Curr. Biol. CB 33, R217–R218. https://doi.org/10.1016/j.cub.2023.02.016

Grishok, A., 2005. RNAi mechanisms in Caenorhabditis elegans. FEBS Lett. 579, 5932–5939. https://doi.org/10.1016/j.febslet.2005.08.001

Groenen, P.M., Bunschoten, A.E., van Soolingen, D., van Embden, J.D., 1993. Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis; application for strain differentiation by a novel typing method. Mol. Microbiol. 10, 1057–1065. https://doi.org/10.1111/j.1365-2958.1993.tb00976.x

Guo Y., X. Chen, and R. E. Ellis, 2013 Evolutionary change within a bipotential switch shaped the sperm/oocyte decision in hermaphroditic nematodes. PLoS Genet. 9: e1003850.

Ha, I., Wightman, B., Ruvkun, G., 1996. A bulged lin-4/lin-14 RNA duplex is sufficient for Caenorhabditis elegans lin-14 temporal gradient formation. Genes Dev. 10, 3041–3050. https://doi.org/10.1101/gad.10.23.3041

Haag, E.S., 2005. The evolution of nematode sex determination: C. elegans as a reference point for comparative biology. WormBook Online Rev. C Elegans Biol. 1–14. https://doi.org/10.1895/wormbook.1.120.1

Haag E. S., D. H. Fitch, and M. Delattre, 2018 From “the worm” to “the worms” and back again: the evolutionary developmental biology of nematodes. Genetics 210: 397–433.

Hammell C. M., and G. J. Hannon, 2016 Inducing RNAi in Caenorhabditis elegans by Injection of dsRNA. Cold Spring Harb. Protoc. 2016: pdb. prot086306.

Hedgecock E. M., and J. G. White, 1985 Polyploid tissues in the nematode Caenorhabditis elegans. Dev. Biol. 107: 128–133.

Hill R. C., C. E. de Carvalho, J. Salogiannis, B. Schlager, D. Pilgrim, *et al.*, 2006 Genetic flexibility in the convergent evolution of hermaphroditism in Caenorhabditis nematodes. Dev. Cell 10: 531–538.

Hills-Muckey, K., Martinez, M.A.Q., Stec, N., Hebbar, S., Saldanha, J., Medwig-Kinney, T.N., Moore, F.E.Q., Ivanova, M., Morao, A., Ward, J.D., Moss, E.G., Ercan, S., Zinovyeva, A.Y., Matus, D.Q., Hammell, C.M., 2022. An engineered, orthogonal auxin analog/AtTIR1(F79G) pairing improves both specificity and efficacy of the auxin degradation system in Caenorhabditis elegans. Genetics 220, iyab174. https://doi.org/10.1093/genetics/iyab174

Hodgkin, J., 1987. Primary sex determination in the nematode C. elegans. Dev. Camb. Engl. 101 Suppl, 5–16. https://doi.org/10.1242/dev.101.Supplement.5

Hodgkin, J., 1980. More sex-determination mutants of Caenorhabditis elegans. Genetics 96, 649–664. https://doi.org/10.1093/genetics/96.3.649

Hodgkin, J., Barnes, T.M., 1991. More is not better: brood size and population growth in a self-fertilizing nematode. Proc. Biol. Sci. 246, 19–24. https://doi.org/10.1098/rspb.1991.0119

Hodgkin, J.A., Brenner, S., 1977. Mutations causing transformation of sexual phenotype in the nematode Caenorhabditis elegans. Genetics 86, 275–287.

Hoe, N., Nakashima, K., Grigsby, D., Pan, X., Dou, S.J., Naidich, S., Garcia, M., Kahn, E., Bergmire-Sweat, D., Musser, J.M., 1999. Rapid molecular genetic subtyping of serotype M1 group A Streptococcus strains. Emerg. Infect. Dis. 5, 254–263. https://doi.org/10.3201/eid0502.990210

Holland, P.W.H., 2013. Evolution of homeobox genes. Wiley Interdiscip. Rev. Dev. Biol. 2, 31–45. https://doi.org/10.1002/wdev.78

Hong, Y., Lee, R.C., Ambros, V., 2000. Structure and function analysis of LIN-14, a temporal regulator of postembryonic developmental events in Caenorhabditis elegans. Mol. Cell. Biol. 20, 2285–2295. https://doi.org/10.1128/MCB.20.6.2285-2295.2000

Hristova M., D. Birse, Y. Hong, and V. Ambros, 2005 The Caenorhabditis elegans heterochronic regulator LIN-14 is a novel transcription factor that controls the developmental timing of transcription from the insulin/insulin-like growth factor gene ins-33 by direct DNA binding. Mol. Cell. Biol. 25: 11059–11072. https://doi.org/10.1128/MCB.25.24.11059-11072.2005

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.-R.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227–229. https://doi.org/10.1038/nbt.2501

Ilbay, O., Ambros, V., 2019. 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. Dev. Camb. Engl. 146, dev183111. https://doi.org/10.1242/dev.183111

Ilbay, O., Nelson, C., Ambros, V., 2021. C. elegans LIN-28 controls temporal cell fate progression by regulating LIN-46 expression via the 5′ UTR of lin-46 mRNA. Cell Rep. 36, 109670. https://doi.org/10.1016/j.celrep.2021.109670

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., Nakata, A., 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J. Bacteriol. 169, 5429–5433. https://doi.org/10.1128/jb.169.12.5429-5433.1987

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., Doudna, J., 2013. RNA-programmed genome editing in human cells. eLife 2, e00471. https://doi.org/10.7554/eLife.00471

Johnson, S.M., Lin, S.Y., Slack, F.J., 2003. The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. Dev. Biol. 259, 364–379. https://doi.org/10.1016/s0012-1606(03)00202-1

Klass, M., Wolf, N., Hirsh, D., 1976. Development of the male reproductive system and sexual transformation in the nematode Caenorhabditis elegans. Dev. Biol. 52, 1–18. https://doi.org/10.1016/0012-1606(76)90002-6

Koonin, E.V., Makarova, K.S., 2022. Evolutionary plasticity and functional versatility of CRISPR systems. PLoS Biol. 20, e3001481. https://doi.org/10.1371/journal.pbio.3001481

Lau N. C., L. P. Lim, E. G. Weinstein, and D. P. Bartel, 2001 An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 858–862.

Lee, R.C., Feinbaum, R.L., Ambros, V., 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843–854. https://doi.org/10.1016/0092-8674(93)90529-y

Li M., M. W. Jones-Rhoades, N. C. Lau, D. P. Bartel, and A. E. Rougvie, 2005 Regulatory mutations of mir-48, a C. elegans let-7 family MicroRNA, cause developmental timing defects. Dev. Cell 9: 415–422.

Lim L. P., N. C. Lau, E. G. Weinstein, A. Abdelhakim, S. Yekta, *et al.*, 2003 The microRNAs of Caenorhabditis elegans. Genes Dev. 17: 991–1008.

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

Liu Z. C., and V. Ambros, 1989 Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in Caenorhabditis elegans. Genes Dev. 3: 2039–2049.

Liu Z., and V. Ambros, 1991 Alternative temporal control systems for hypodermal cell differentiation in Caenorhabditis elegans. Nature 350: 162–165.

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., Koonin, E.V., 2006. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol. Direct 1, 7. https://doi.org/10.1186/1745-6150-1-7

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013. RNA-guided human genome engineering via Cas9. Science 339, 823–826. https://doi.org/10.1126/science.1232033

McAllister, B.F., McVean, G.A., 2000. Neutral evolution of the sex-determining gene transformer in Drosophila. Genetics 154, 1711–1720. https://doi.org/10.1093/genetics/154.4.1711

Meise, M., Hilfiker-Kleiner, D., Dübendorfer, A., Brunner, C., Nöthiger, R., Bopp, D., 1998. Sex-lethal, the master sex-determining gene in Drosophila, is not sex-specifically regulated in Musca domestica. Dev. Camb. Engl. 125, 1487–1494. https://doi.org/10.1242/dev.125.8.1487

Meister, G., Tuschl, T., 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431, 343–349. https://doi.org/10.1038/nature02873

Mello, C.C., Kramer, J.M., Stinchcomb, D., Ambros, V., 1991. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J. 10, 3959–3970.

Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J., Soria, E., 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. Evol. 60, 174–182. https://doi.org/10.1007/s00239-004-0046-3

Mok D. Z.., P. W. Sternberg, and T. Inoue, 2015 Morphologically defined sub-stages of C. elegans vulval development in the fourth larval stage. BMC Dev. Biol., 15: 26. https://doi.org/10.1186/s12861-015-0076-7

Moss, E.G., Lee, R.C., Ambros, V., 1997. The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. Cell 88, 637–646. https://doi.org/10.1016/s0092-8674(00)81906-6

Moss, E.G., Tang, L., 2003. Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. Dev. Biol. 258, 432–442. https://doi.org/10.1016/s0012-1606(03)00126-x

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

Olsen, P.H., Ambros, V., 1999. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 216, 671–680. https://doi.org/10.1006/dbio.1999.9523

O'Neil, M.T., Belote, J.M., 1992. Interspecific comparison of the transformer gene of Drosophila reveals an unusually high degree of evolutionary divergence. Genetics 131, 113–128. https://doi.org/10.1093/genetics/131.1.113

Paix, A., Folkmann, A., Rasoloson, D., Seydoux, G., 2015. High Efficiency, Homology-Directed Genome Editing in Caenorhabditis elegans Using CRISPR-Cas9 Ribonucleoprotein Complexes. Genetics 201, 47–54. https://doi.org/10.1534/genetics.115.179382

Paix A., A. Folkmann, and G. Seydoux, 2017 Precision genome editing using CRISPR-Cas9 and linear repair templates in C. elegans. Methods 121: 86–93.

Pan, L., Gong, Z., Zhong, Z., Dong, Z., Liu, Q., Le, Y., Guo, J., 2011. Lin-28 reactivation is required for let-7 repression and proliferation in human small cell lung cancer cells. Mol. Cell. Biochem. 355, 257–263. https://doi.org/10.1007/s11010-011-0862-x

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E., Ruvkun, G., 2000. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408, 86–89. https://doi.org/10.1038/35040556

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

Praitis, V., Casey, E., Collar, D., Austin, J., 2001. Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. Genetics 157, 1217–1226.

Reilly, M.B., Cros, C., Varol, E., Yemini, E., Hobert, O., 2020. Unique homeobox codes delineate all the neuron classes of C. elegans. Nature 584, 595–601. https://doi.org/10.1038/s41586-020-2618-9

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

Robert, V., Bessereau, J.-L., 2007. Targeted engineering of the Caenorhabditis elegans genome following Mos1-triggered chromosomal breaks. EMBO J. 26, 170–183. https://doi.org/10.1038/sj.emboj.7601463

Romer-Seibert J. S., N. W. Hartman, and E. G. Moss, 2019 The RNA-binding protein LIN28 controls progenitor and neuronal cell fate during postnatal neurogenesis. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 33: 3291–3303. https://doi.org/10.1096/fj.201801118R

Rougvie, A.E., Ambros, V., 1995. The heterochronic gene lin-29 encodes a zinc finger protein that controls a terminal differentiation event in Caenorhabditis elegans. Dev. Camb. Engl. 121, 2491–2500. https://doi.org/10.1242/dev.121.8.2491

Rougvie, A.E., Moss, E.G., 2013. Developmental transitions in C. elegans larval stages. Curr. Top. Dev. Biol. 105, 153–180. https://doi.org/10.1016/B978-0-12-396968-2.00006-3

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

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

Rybak, A., Fuchs, H., Hadian, K., Smirnova, L., Wulczyn, E.A., Michel, G., Nitsch, R., Krappmann, D., Wulczyn, F.G., 2009. The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. Nat. Cell Biol. 11, 1411–1420. https://doi.org/10.1038/ncb1987

Rybak, A., Fuchs, H., Smirnova, L., Brandt, C., Pohl, E.E., Nitsch, R., Wulczyn, F.G., 2008. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat. Cell Biol. 10, 987–993. https://doi.org/10.1038/ncb1759

Seggerson K., L. Tang, and E. G. Moss, 2002 Two genetic circuits repress the Caenorhabditis elegans heterochronic gene lin-28 after translation initiation. Dev. Biol. 243: 215–225. https://doi.org/10.1006/dbio.2001.0563

Slack, F.J., Basson, M., Liu, Z., Ambros, V., Horvitz, H.R., Ruvkun, G., 2000. The lin-41 RBCC gene acts in the C. elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Mol. Cell 5, 659–669. https://doi.org/10.1016/s1097-2765(00)80245-2

Sommer R. J., 2012 Evolution of regulatory networks: nematode vulva induction as an example of developmental systems drift, pp. 79–91 in *Evolutionary systems biology*, Springer.

Stein, L.D., Bao, Z., Blasiar, D., Blumenthal, T., Brent, M.R., Chen, N., Chinwalla, A., Clarke, L., Clee, C., Coghlan, A., Coulson, A., D'Eustachio, P., Fitch, D.H.A., Fulton, L.A., Fulton, R.E., Griffiths-Jones, S., Harris, T.W., Hillier, L.W., Kamath, R., Kuwabara, P.E., Mardis, E.R., Marra, M.A., Miner, T.L., Minx, P., Mullikin, J.C., Plumb, R.W., Rogers, J., Schein, J.E., Sohrmann, M., Spieth, J., Stajich, J.E., Wei, C., Willey, D., Wilson, R.K., Durbin, R., Waterston, R.H., 2003. The genome sequence of Caenorhabditis briggsae: a platform for comparative genomics. PLoS Biol. 1, E45. https://doi.org/10.1371/journal.pbio.0000045

Stinchcomb D. T., J. E. Shaw, S. H. Carr, and D. Hirsh, 1985 Extrachromosomal DNA transformation of Caenorhabditis elegans. Mol. Cell. Biol. 5: 3484–3496.Stothard, P., Pilgrim, D., 2003. Sex-determination gene and pathway evolution in nematodes. BioEssays News Rev. Mol. Cell. Dev. Biol. 25, 221–231. https://doi.org/10.1002/bies.10239

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

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

Tabara, H., Grishok, A., Mello, C.C., 1998. RNAi in C. elegans: soaking in the genome sequence. Science 282, 430–431. https://doi.org/10.1126/science.282.5388.430

Timmons, L., Fire, A., 1998. Specific interference by ingested dsRNA. Nature 395, 854. https://doi.org/10.1038/27579

Tocchini C., J. J. Keusch, S. B. Miller, S. Finger, H. Gut, *et al.*, 2014 The TRIM-NHL protein LIN-41 controls the onset of developmental plasticity in Caenorhabditis elegans. PLoS Genet. 10: e1004533.

True, J.R., Haag, E.S., 2001. Developmental system drift and flexibility in evolutionary trajectories. Evol. Dev. 3, 109–119. https://doi.org/10.1046/j.1525-142x.2001.003002109.x

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

Tucker, P.K., Lundrigan, B.L., 1993. Rapid evolution of the sex determining locus in Old World mice and rats. Nature 364, 715–717. https://doi.org/10.1038/364715a0

Vadla, B., Kemper, K., Alaimo, J., Heine, C., Moss, E.G., 2012. lin-28 controls the succession of cell fate choices via two distinct activities. PLoS Genet. 8, e1002588. https://doi.org/10.1371/journal.pgen.1002588

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

Vavouri, T., Semple, J. I., & Lehner, B. (2008). Widespread conservation of genetic redundancy during a billion years of eukaryotic evolution. *Trends in genetics : TIG*, *24*(10), 485–488. https://doi.org/10.1016/j.tig.2008.08.005

Verd B., N. A. Monk, and J. Jaeger, 2019 Modularity, criticality, and evolvability of a developmental gene regulatory network. Elife 8: e42832.

Verster, A.J., Ramani, A.K., McKay, S.J., Fraser, A.G., 2014. Comparative RNAi screens in C. elegans and C. briggsae reveal the impact of developmental system drift on gene function. PLoS Genet. 10, e1004077. https://doi.org/10.1371/journal.pgen.1004077

Waaijers, S., Portegijs, V., Kerver, J., Lemmens, B.B.L.G., Tijsterman, M., van den Heuvel, S., Boxem, M., 2013. CRISPR/Cas9-targeted mutagenesis in Caenorhabditis elegans. Genetics 195, 1187–1191. https://doi.org/10.1534/genetics.113.156299

Wang, S., Kimble, J., 2001. The TRA-1 transcription factor binds TRA-2 to regulate sexual fates in Caenorhabditis elegans. EMBO J. 20, 1363–1372. https://doi.org/10.1093/emboj/20.6.1363

Wei Q., Y. Shen, X. Chen, Y. Shifman, and R. E. Ellis, 2014 Rapid creation of forward-genetics tools for C. briggsae using TALENs: lessons for nonmodel organisms. Mol. Biol. Evol. 31: 468–473

Werren, J.H., Hatcher, M.J., 2000. Maternal-zygotic gene conflict over sex determination: effects of inbreeding. Genetics 155, 1469–1479. https://doi.org/10.1093/genetics/155.3.1469

Whitfield, L.S., Lovell-Badge, R., Goodfellow, P.N., 1993. Rapid sequence evolution of the mammalian sex-determining gene SRY. Nature 364, 713–715. https://doi.org/10.1038/364713a0

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

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

Zhang, L., Ward, J.D., Cheng, Z., Dernburg, A.F., 2015. The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in C. elegans. Dev. Camb. Engl. 142, 4374–4384. https://doi.org/10.1242/dev.129635

Zhao, Z., Boyle, T.J., Bao, Z., Murray, J.I., Mericle, B., Waterston, R.H., 2008. Comparative analysis of embryonic cell lineage between Caenorhabditis briggsae and Caenorhabditis elegans. Dev. Biol. 314, 93–99. https://doi.org/10.1016/j.ydbio.2007.11.015