Characterization of the function of the *C. elegans* heterochronic gene *lin-42/per* during larval development

A Dissertation

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BY

Katherine Ann McCulloch

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Ann E. Rougvie, PhD

Advisor

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There are many people who have made my time in graduate school such a great experience. I first must thank my advisor, Ann Rougvie, for her patience and direction. Ann's office door is always open for advice and discussion. Her advice and support over the years has been essential to my progress as a scientist.

I must also thank Rougvie lab members past and present for their friendship and assistance throughout the years. Past members Sarah Malmquist, Sara Maus, Aric Daul, Angela Barr, Dan Berg and Karla Opperman were wonderful colleagues and friends. I must particularly thank Jason Tennessen, from whom I inherited *lin-42*. His enthusiasm for this project and assistance early on were invaluable.

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I would also like to thank the extended worm community here at the University of Minnesota. Everyone in this community is always incredibly generous with reagents and advice.

Finally, I would like to thank my family and friends for their love and support.

ABSTRACT

The heterochronic pathway of *C. elegans* ensures the appropriate timing of post-embryonic development. Mutations in heterochronic genes cause skipping or reiteration of larval programs, resulting in severe developmental defects. Many *C. elegans* heterochronic genes are conserved in both sequence and function; therefore, study of developmental timing in *C. elegans* contributes to understanding of development in other organisms.

The heterochronic gene *lin-42* is the *C. elegans* homolog of *period*, a component of the circadian clock of *Drosophila* and mammals. *lin-42(lf)* results in precocious heterochronic phenotypes, in which later developmental events occur too early. *lin-42* also regulates molting, and *lin-42* mutants have delayed and prolonged larval molts compared to wild-type animals. In my thesis work, I show that *lin-42* confers robustness on developmental and molting pathways against environmental fluctuations, as *lin-42(lf)* heterochronic and molting phenotypes are very sensitive to changes in environment, which is not observed in wild-type animals. Also, I have found that *lin-42* regulates developmental timing by inhibiting expression of *let-7*-family miRNAs, likely at the level of transcription. Genetic analyses place *lin-42* upstream of this miRNAs, showing that regulation of let-7-family miRNAs is a key function of *lin-42* in the heterochronic pathway.

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LIST OF ABBREVIATIONS

DA	Dafachronic Acids
Daf-c	Dauer formation-constitutive
Daf-d	Dauer formation-defective
FASPS	Familial Advanced Sleep Phase Syndrome
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
Hid	High-temperature induced dauer
iHH	idiopathic hypogonadotropic hypogonadism
IP	immunoprecipitation
LH	Leutinizing hormone
miRISC	miRNA-induced silencing complex
NHR	Nuclear hormone receptor
NSAF	normalized spectral abundance factor
L1m	L1-molt
L2m	L2-molt
L3m	L3-molt
L4m	L4-molt
(lf)	loss-of-function mutation
pre-miRNA	precursor-miRNA
pri-miRNA	primary-miRNA
RISC	RNAi-induced silencing complex
(rf)	reduction of function mutation

SynDaf	Synthetic Dauer formation
(0)	null mutation

Clarification of contributions:

Chapter II

The primers and targeting plasmids to generate the *lin-42(0*) allele were designed and constructed by Angela Barr. Injections and isolation of *lin-42(0)* alleles were done in the laboratory of Erik Jorgensen by Christian Frøkjær-Jensen. Analysis of *lin-42(0)* heterochronic phenotypes was performed by Theresa Edelman. Analysis of *lin-42(0)* molting phenotypes was done in collaboration with Theresa Edelman and Ann Rougvie. Rescue experiments were done by Theresa Edelman and Chrisana Pokorny.

Chapter III

Analysis of *lin-42(mg152); lin-42(n1089); lin-42(ve11)* strains and construction and analysis of double mutants with these alleles were performed by Jason Tennessen except for *lin-42(ve11) daf-5; daf-16. RNAi* experiments were also undertaken by Jason Tennessen.

Appendix I

MudPIT mass spectrometry analysis was performed in the laboratory of James Wohlschlegl at UCLA as described (Duchaine et al., 2006).

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CHAPTER I. INTRODUCTION

Introduction

Precise regulation of temporal development is necessary to ensure that developmental programs occur at the appropriate stage and are executed in the correct sequence, and disruption of developmental timing mechanisms can result in catastrophic birth defects (Wilson, 1988). Temporal cues act in concert with spatial and sexual information to ensure that events in diverse tissue are coordinated appropriately. In humans, an example of a developmental event that is under strict temporal control is puberty, the series of physiological changes marking the juvenile-to-adult transition. The secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which initiates release of sex hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH), has long been known to be both necessary and sufficient to initiate puberty (Tena-Sempere, M. & Huhtaniemi, I., 2003;Wildt et al., 1980). How the timing of GnRH secretion is regulated has long been unclear, but the completion of the human genome project coupled with multiple genome-wide association studies have started to unravel some of this mystery. Many single-gene mutations have been identified that alter the timing of puberty (reviewed in (Tena-Sempere, 2012)). A major breakthrough in understanding how the timing of puberty is regulated was the discovery of kisspeptins and their receptor, GPR54, and their role in promoting GnRH secretion (reviewed in (Pinilla et al., 2012)). Mutations of *Gpr54* in humans were found to be associated with idiopathic

hypogonadotropic hypogonadism (iHH), which results in failure to undergo puberty and infertility (de Roux et al., 2003;Seminara et al., 2003). Studies in mice further validated the importance of kisspeptins in the timing of puberty. Loss of either *Kiss1* or *Gpr54* phenocopies iHH, and these mice are infertile and fail to undergo estrous (d'Anglemont de Tassigny et al., 2007;Lapatto et al., 2007). Since these initial findings, a wealth of research has established that activation of GPR54 by kisspeptins is critical for stimulating the release of GnRH and initiation of puberty in mammals (reviewed in (Pinilla et al., 2012)).

Another gene associated with the timing of puberty is *Lin-28b* (Elks et al., 2010;Sulem et al., 2009;Wildt et al., 1980). *Lin-28b* encodes an RNA-binding protein with roles in miRNA processing (Hagan et al., 2009;Moss et al., 1997;Moss and Tang, 2003;Piskounova et al., 2011). In humans, polymorphisms within *Lin-28b* are associated with precocious puberty, or puberty occurring too early, suggesting that *Lin-28b* may regulate the timing of puberty. Subsequent studies found that mouse models over-expressing *Lin-28a* exhibited delayed puberty, as well as health problems that are associated with aberrant timing of puberty in humans, including obesity and high incidence of cancers (Shaw et al., 2007). Further evidence for a role of *Lin-28* in the timing of puberty is that *Lin-28A* and *Lin-28B* mRNAs are developmentally expressed in the rat hypothalamus, the site of kisspeptide and GnRH-secreting neurons, with mRNA levels decreasing from neonatal to pubertal periods (Tena-Sempere, 2012). This indicates that *Lin-28* may play a role in regulating the timing of kisspeptide secretions. Further studies are necessary to elucidate the precise role of *Lin-28*

in the timing of puberty, and whether its function in regulating miRNA biogenesis is involved in this process.

It is becoming increasingly clear that environmental factors also contribute to developmental timing in humans. The average age of puberty onset in girls has been declining over time (Euling et al., 2008a). This change is thought to be caused in part by the rising rates of obesity in the population, as well as endocrine disrupting chemicals in the environment (Ahmed et al., 2009;Buck Louis et al., 2008;Euling et al., 2008a;Euling et al., 2008b). A critical link between body weight and the timing of puberty is the hormone leptin (Fernandez-Fernandez et al., 2006). Leptin is secreted by adipocytes that signal to the brain on the status of fat stores to regulate appetite and metabolism. Mutant mice unable to produce leptin fail to undergo puberty, and this defect is rescued when these animals are supplied exogenous leptin (Batt et al., 1982;Chehab et al., 1996). On the other hand, young wild-type rats given leptin do not enter precocious puberty, suggesting that leptin plays a permissive role in the timing of puberty (Cheung et al., 1997).

Endocrine-disrupting chemicals in the environment may also affect the timing of puberty. Many of these chemicals compete for binding to hormone receptors, such as estrogen or androgen receptors, and disrupt their activity. Several animal and human studies have linked exposure to such chemicals to altered timing of puberty (reviewed in (Buck Louis et al., 2008). For example, exposure of mice to bisphenol-A (BPA), an estrogen receptor agonist commonly

found in plastics, has been shown to cause precocious puberty in mice (Howdeshell et al., 1999).

Although recent work is starting to elucidate some developmental timing pathways in humans, studies from model organisms have long provided clues for identifying conserved timing mechanisms. Indeed, *lin-28* was first discovered in the model organism *C. elegans*, where it plays a critical role in developmental timing.

C. elegans as a model to study developmental timing

C. elegans has proven to be an incredibly useful model system in which to identify and characterize conserved developmental timing mechanisms (For review, see (Resnick et al., 2010)). *C. elegans* are small, transparent, non-parasitic nematodes that develop rapidly over just 3 days. These animals are extremely genetically tractable. They exist as hermaphrodites capable of self-fertilization, and as males, which allows for generating complex mutant strains. Additionally, knockdown of genes by RNAi can be achieved easily by feeding animals bacteria expressing double-stranded RNA. Recently, gene targeting has also become possible in *C. elegans* by techniques such as CRISPR-Cas and mosSCI/mosDEL (Chen et al., 2013;Dickinson et al., 2013;Friedland et al., 2013;Frokjaer-Jensen et al., 2010;Katic and Grosshans, 2013;Lo et al., 2013;Tzur et al., 2013;Waaijers et al., 2013;Zeiser et al., 2011)

Upon hatching, *C. elegans* progresses through four precisely timed larval stages (L1-L4), each ending in a molt. The completely mapped and virtually invariant cell lineage of the worm allows for the study of the timing of specific cell divisions in relation to these developmental stages (Sulston and Horvitz, 1977). Work begun by Victor Ambros and HR Horvitz utilized the post-embryonic development of hypodermal seam cells to identify genes that regulate developmental timing in *C. elegans* (Ambros and Horvitz, 1984). The seam cells are arranged in two lateral rows of cells in the hypodermis that extend along the length of the animal and contribute to cuticle formation (Figure 1A). At the beginning of each larval stage, these cells undergo an asymmetric division, in which the anterior daughter exits the seam and joins the surrounding hypodermal syncytium, while the posterior daughter retains the seam cell identity. At the beginning of the L2-stage, a sub-set of seam cells execute an additional symmetric division prior to the asymmetric division, increasing the number of seam cells per side from 10 to 16. At the larval-to-adult transition, the seam cells exit the cell cycle and terminally differentiate, fusing to form a syncytium. The seam cell syncytium contributes to the synthesis of an adult cuticle that contains easily observed ridges called alae (Figure 1B). Ambros and Horvitz identified mutations that caused specific events in the seam cell lineage to be skipped or reiterated (Ambros and Horvitz, 1984). Animals in which certain programs are skipped and later ones occur too early are termed “precocious mutants” (Figure 1C). Conversely, animals in which developmental programs are reiterated are called “retarded” mutants. In these mutants, later developmental events are

delayed or fail to occur (Figure 1D). Genetic analyses organized these genes into a heterochronic gene regulatory pathway that controls temporal development in the seam (Ambros, 1989). In addition to their function in the hypodermis, many heterochronic genes also regulate timing in other tissues, such as the vulva, nervous system, and gonad (Antebi et al., 1998;Antebi et al., 2000;Bettinger et al., 1997;Johnson et al., 2009;Thompson-Peer et al., 2012). Since the pioneering studies of Ambros and Horvitz, many more genes have been identified that function in the heterochronic pathway. These studies have revealed highly conserved regulators of developmental timing and novel mechanisms of gene regulation. Some important themes and players in the pathway are described in more detail below (Figure 2, Table 1).

miRNAs promote developmental transitions in *C. elegans*

miRNAs were first discovered as members of the heterochronic gene pathway of *C. elegans*. *lin-4* and *let-7* were initially identified by mutations that caused retarded heterochronic phenotypes (Ambros and Horvitz, 1984;Chalfie et al., 1981) (Figure 1D). Surprisingly, when these genes were cloned, the mutations were not found to affect protein-coding genes. Further work revealed a surprising finding: *lin-4* and *let-7* encode small (~23nt) non-coding RNAs, and were the first miRNAs identified in any organism (Lee et al., 1993;Reinhart et al., 2000). Since their discovery in the heterochronic pathway of the worm, miRNAs have been found to be ubiquitous, extant in plant and animal species, where they function in a broad array of biological pathways including development,

metabolism, and cancer. (Lagos-Quintana et al., 2001;Lau et al., 2001;Lee et al., 1993). let-7 miRNA, in particular, exhibits a remarkable degree of conservation: it is completely identical from worms to humans. In humans, let-7 miRNA has a well-characterized role as a tumor suppressor, and its targets include the oncogenes Ras, MYC, and HMGA2 (reviewed in, (Boyerinas et al., 2010)). These genes are all up-regulated in some tumors, and low levels of let-7 miRNA expression are diagnostic of cancerous cells (Bussing et al., 2008). Understanding the regulation and activity of let-7 miRNA, therefore, has been an important goal in cancer research.

Since the discovery of miRNAs in 1993, the process of miRNA biogenesis and function has been extensively interrogated. For reviews of miRNA processing, see (Bartel, 2004;Krol et al., 2010) (Figure 3A). Briefly, miRNAs are transcribed by RNA-polymerase II as long pri-miRNA transcripts which are capped and polyadenylated. pri-miRNAs are processed by a protein complex called the Microprocessor that includes the endoribonuclease Drosha/DRSH-1 and RNA-binding protein Pasha/PASH-1/DGCR8 (Denli et al., 2004;Lee et al., 2002;Lee et al., 2003). This step produces an approximately 70bp long (pre)cursor-miRNA with a hairpin structure. The pre-miRNA is exported from the nucleus and further processed by the RNAse III enzyme Dicer to generate a 22bp imperfect heteroduplex (Grishok et al., 2001;Hutvagner et al., 2001;Ketting et al., 2001). The duplex is then assembled into a pre-miRISC (miRNA Induced Silencing Complex), where the duplex is unwound, and the single strand mature miRNA is maintained in the complex (Hammond et al., 2000;Nykanen et al.,

2001). Once assembled, the miRISC, which includes ALG-1/2 argonautes and AIN-1/2 GW182 proteins, binds to target transcripts through imperfect base complementarity between the target 3'UTR and miRNA sequence (Zhang et al., 2007).

The mechanism by which miRNAs inhibit the expression of their targets is still under debate. Initial work suggested that base complementarity was critical. Perfect complementarity between the miRNA and its target would lead to transcript degradation, while imperfect complementarity caused inhibition of translation (Hutvagner and Zamore, 2002;Zeng et al., 2002;Zeng and Cullen, 2003;Zeng et al., 2003). In *C. elegans*, the predominant mechanism of miRNA-mediated regulation was thought to be through translational inhibition after initiation (Olsen and Ambros, 1999;Seggerson et al., 2002). Subsequent studies have added complexity to this simpler model, and many mechanisms have been proposed for miRNA action, including regulation of translation initiation and ribosome dropoff, RNA degradation, among others (reviewed in (Fabian and Sonenberg, 2012)). Recently, work from Andrew Fire's lab has suggested that miRNA mediated regulation of targets may be achieved by multiple mechanisms, and that regulation after initiation of translation is unlikely (Stadler et al., 2012). Clearly, a great deal of further study is needed to clarify how miRNAs down-regulate their targets.

Many miRNAs can be classified into families based on identity in the 5' "seed" sequence, which is thought to be critical for targeting (Figure 3B). Although family members have identical seed sequences, the degree to which

they share targets is unclear. In *C. elegans*, let-7 miRNA is the founding member of a miRNA family that includes miR-48, miR-241, and miR-84. Interestingly, these *let-7* paralogs also function in the heterochronic pathway (Abbott et al., 2005;Li et al., 2005). Although they have identical seed sequences to let-7 miRNA, there is some evidence to suggest that they do not functionally overlap. miR-48, miR-241, and miR-84 are genetically redundant and are up-regulated earlier than let-7 miRNA, suggesting they function in early stages of the pathway (Abbott et al., 2005). On the other hand, let-7 miRNA accumulates later than its paralogs and *let-7(0)* mutants have a different heterochronic phenotype than *mir-48(0) mir-241(0); mir-84(0)* mutants, implicating *let-7* in regulating later events (Abbott et al., 2005;Reinhart et al., 2000;Vadla et al., 2012). This suggests that miRNAs within the same family do not necessarily share functions or targets. Recently, protein factors have been identified that potentiate the activities of specific miRNAs. For example, *nhl-2* has been found to promote the activities of several miRNAs, including let-7-family miRNAs (Hammell et al., 2009b). *nhl-2(0)* enhanced the phenotypes of these miRNAs without affecting their levels. However, *nhl-2(0)* did not enhance all miRNA mutants tested, indicating that *nhl-2* activity is restricted to specific miRNAs. The identity of other protein factors that regulate miRNA activity and how their specificity is achieved is another active area of study.

There are at least five miRNAs with well-characterized roles in timing: *lin-4, let-7, mir-241, mir-48*, and *mir-84*. All of these miRNAs act to promote developmental transitions during larval development by regulating the expression

of specific transcription factors. LIN-14 transcription factor is necessary for L1 fates; HBL-1 promotes L2, while LIN-29 is required for the larval-to-adult transition. The precise temporal expression of these proteins is largely regulated via the stage-specific expression of miRNAs (Figure 3). lin-4 miRNA down-regulates *lin-14*, while miR-48, miR-241, and mir-84 function together to down-regulated HBL-1. LIN-29 is indirectly regulated via the activity of let-7 miRNA (Abbott et al., 2005;Arasu et al., 1991;Lee et al., 1993;Lin et al., 2003;Slack et al., 2000;Wightman et al., 1991). The actions of these miRNAs, in addition to other members of the heterochronic pathway, ensure that developmental transitions occur in the correct sequence.

lin-14 and *lin-4* regulate L1 programs

The heterochronic pathway is initiated by feeding, resulting in the up-regulation of lin-4 miRNA during the mid-L1 stage (Feinbaum and Ambros, 1999). Deletion of *lin-4* [*lin-4(0)*] causes the animal to repeat L1 programs, resulting in a retarded heterochronic phenotype (Ambros and Horvitz, 1984;Chalfie et al., 1981) (Figure 1D). These mutants never undergo the proliferative L2 division or form adult alae, and this phenotype indicates that lin-4 miRNA is necessary for the transition from L1 to L2. lin-4 miRNA primarily targets two transcripts: *lin-14* and *lin-28*. The LIN-14 transcription factor promotes L1 programs, and in *lin-14(lf)* animals, this event is skipped, causing subsequent events to occur one stage too early (Ambros and Horvitz, 1984;Ruvkun et al., 1989;Wightman et al., 1991) (Figure 1C). Conversely, a *lin-*

14(gf) mutation results in a retarded heterochronic phenotype, similar to *lin-4(0)* animals, and further analysis indicated that the *lin-14(gf)* mutations was in the 3'UTR of *lin-14*. (Ambros and Horvitz, 1984;Ruvkun et al., 1989;Wightman et al., 1991). In these mutants, LIN-14 protein is not down-regulated and can be detected by immunofluorescence and western blot analyses past the L1-stage (Ruvkun and Giusto, 1989;Ruvkun et al., 1989;Wightman et al., 1991;Wightman et al., 1993). In contrast, in both wild-type and *lin-14(gf)* animals, the levels of *lin-14* transcript remain relatively constant, indicating that *lin-14* expression is regulated post-transcriptionally (Wightman et al., 1993). Fusion of the *lin-14* 3'UTR to a lacZ reporter showed that this sequence was sufficient to induce temporal down-regulation. Importantly, this reporter was not repressed in a *lin-4* mutant background. Taken together, these data showed that LIN-14 is necessary to promote L1 programs, and its levels must decline for development to advance to the L2 stage. Furthermore, the decline in LIN-14 levels requires *lin-4* activity, which acts through the *lin-14* 3'UTR.

Complex regulation of the L2 to L3 transition

hbl-1 is a critical component of the heterochronic pathway, and is the *C. elegans* homolog of *Drosophila hunchback(hb),* which in flies is important for embryonic patterning (Fay et al., 1999). *hbl-1* encodes a zinc-finger transcription factor which is thought to regulate the expression of genes involved in L2-specific programs (Abrahante et al., 2003;Lin et al., 2003). *hbl-1(RNAi*) results in embryonic lethality, indicating that a null allele would likely be lethal (Abrahante

et al., 2003;Fay et al., 1999). However, post-embryonic knockdown of *hbl-1* and hypomorphic alleles result in precocious phenotypes, in which the proliferative division is often skipped and animals form alae one stage too early (Figure 1C). *Drosophila hb* has been implicated in the timing of neuronal development, where it is expressed in early-born neurons of the fly and is necessary to specify early neuronal fates (Isshiki et al., 2001). These analyses indicate that the developmental timing function of *hbl-1* is conserved, at least in *Drosophila*.

Many genes have been identified that regulate the timing of *hbl-1* expression. One of these is the second lin-4 miRNA target: *lin-28* (Ambros and Horvitz, 1984;Moss et al., 1997). *lin-28* encodes a RNA-binding protein with a cold-shock domain and CCHC zinc finger motif, and is expressed in the hypodermis and a variety of other tissues, predominantly in the cytoplasm. *lin-28(0)* mutants have a precocious heterochronic phenotype. In these animals, the L2-stage proliferative and asymmetrical divisions are skipped and alae are formed one or two stages too early (Figure 1C). *lin-28* appears to play dual roles in the heterochronic pathway (Moss et al., 1997;Seggerson et al., 2002;Vadla et al., 2012). Its first role is to promote L1 and L2 programs through regulation of *lin-14* and *hbl-1*. Whole-mount immunostaining experiments found that LIN-14 protein is down-regulated in *lin-28(0*) mutants compared to wild-type, indicating that LIN-28 promotes *lin-14* accumulation (Arasu et al., 1991;Wightman et al., 1993). Additionally, *hbl-1::gfp* reporters are also down-regulated in *lin-28* mutants compared to wild-type (Vadla et al., 2012). These data as well as many genetic experiments show that *lin-28* promotes the expression of both *lin-14* and

hbl-1 (Figure 2). Secondly, *lin-28* plays a critical role inhibiting the accumulation of let-7 miRNA (Vadla et al., 2012). Both northern blots and qRT-PCR experiments detect mature *let-7* early in *lin-28(0)* animals compared to wild-type, and biochemical analyses show that LIN-28 can bind to primary and precursor *let-7* molecules (Vadla et al., 2012;Van Wynsberghe et al., 2011). This indicates that LIN-28 functions to inhibit *let-7* processing. Therefore, *lin-28* promotes the expression of early acting genes, such as *lin-14* and *hbl-1* and inhibits the expression of the later-acting gene *let-7*. Through these activities, *lin-28* both directs early developmental programs while preventing later programs from occurring too early.

Interestingly, multiple studies have found that the role of *lin-28* inhibiting *let-7* processing is conserved to humans. Humans have two *lin-28* homologs, *Lin-28a* and *Lin-28b* that regulate let-7 miRNA biogenesis via distinct mechanisms (Guo et al., 2006;Moss and Tang, 2003). *Lin-28a* is predominantly localized to the cytoplasm, where it blocks the Dicer step of let-7 miRNA biogenesis (Hagan et al., 2009;Heo et al., 2008;Piskounova et al., 2011) (Figure 3A). In contrast, *Lin-28b* localizes to the nucleus, where it sequesters pri-*let-7* and prevents processing by the Microprocessor (Piskounova et al., 2011). In *C. elegans*, which has only one *lin-28* gene, there is some debate as to how LIN-28 regulates let-7 miRNA biogenesis. A study by Lehrbach *et al* 2009, indicated that LIN-28 blocks the Dicer step by binding to pre-*let-7*, recruiting the poly(U) polymerase PUP-2, and targeting let-7 miRNA for uridylation and degradation (Lehrbach et al., 2009). On the other hand, Van Whysberghe *et al* 2011, found

that *lin-28*-mediates inhibition of the Drosha step by binding to pri-*let-7* and inhibiting processing (Van Wynsberghe et al., 2011). The fact that pri-*let-7* levels decrease while pre-*let-7* levels increase in *lin-28* mutants by northern blot argues that *lin-28* acts at the primary step, and normally blocks *pri-let-7* processing. However, further work is needed to clarify the exact mechanism by which *lin-28* regulates let-7 miRNA biogenesis in *C. elegans*.

Although let-7 miRNA does not act until late in *C. elegans* larval development, its paralogs, miR-48, miR-241, and miR-84 function redundantly to down-regulate *hbl-1* expression during the L2 in the hypodermis (Abbott et al., 2005;Abrahante et al., 2003;Lin et al., 2003;Vadla et al., 2012). Mutation of any of these three miRNAs individually does cause a clear heterochronic phenotype, however, when all three are deleted, the result is a strong retarded heterochronic defect (Figure 1D). These triple mutants repeat the L2 division and delay forming adult alae. miR-48, miR-241, and miR-84 are detectable at low levels by northern blot in the late L1 and increase rapidly at the start of the L2-stage, remaining high through the rest of development (Abbott et al., 2005;Li et al., 2005). Several lines of evidence suggest that the primary target of these miRNAs is *hbl-1*(Abbott et al., 2005;Abrahante et al., 2003;Lin et al., 2003). First, *hbl-1* activity is necessary for the retarded phenotype of *mir-48(0) mir-241(0); mir-84(0)* triple mutants. Secondly, there are several let-7-family miRNA binding sites in the *hbl-1* 3'UTR and these sites are necessary for the down-regulation of a *Phbl-1::gfp* reporter. Also, the repression of this reporter requires the activity of these miRNAs. In *mir-48(0) mir-241(0); mir-84(0)* mutants, the expression of

Phbl-1::gfp persists past the L2-stage. In summary, the accumulation of miR-48,miR-241, and miR-84 and down-regulation of *lin-28* in the L2 causes *hbl-1* levels to decrease in the hypodermis allowing the L2-to-L3 transition to occur (Figure 2).

The heterochronic genes *daf-12* also plays a critical role in the L2-stage. In highly stressful environments, *C. elegans* can interrupt larval development and enter a highly stress resistant alternative L3-stage called a dauer (Cassada and Russell, 1975). *daf-12* is required for this response and acts as a developmental switch, which directs the worm through continuous development or larval arrest, depending on environmental conditions (Antebi et al., 1998;Antebi et al., 2000) (see below). *daf-12* encodes a nuclear hormone receptor (NHR) that, like most NHRs, contains a N-terminal DNA-binding domain and a C-terminal ligand binding domain (Antebi et al., 1998;Antebi et al., 2000). Interactions at the ligand-binding domain regulate DAF-12 activity (Ludewig et al., 2004). In favorable environments, DAF-12 promotes reproductive development and acts as a transcriptional activator. In unfavorable environments, DAF-12 interacts with a co-repressor, DIN-1, and this complex promotes larval arrest and inhibits reproductive development. Interestingly, *daf-12(0)* mutants do not have a strong heterochronic phenotype (Antebi et al., 1998;Antebi et al., 2000). However, point mutations within the ligand binding domain do confer a strong retarded defect, in which L2-programs are reiterated (Figure 1D). It is thought that these mutants lock DAF-12 into a constitutive repressor mode, causing the heterochronic

defects observed. Therefore, *daf-12* is a critical gene regulating larval development in *C. elegans,* integrating developmental timing and larval arrest.

daf-12 regulates the expression of several genes in the heterochronic pathway. In *daf-12(lf)* mutants, LIN-28 protein levels are elevated and stabilized and can be detected in the L3-stage (Morita and Han, 2006;Seggerson et al., 2002). Using translational reporters, it was found that *daf-12*-mediated repression of *lin-28* requires its 3'UTR. Additionally, *lin-28* is epistatic to *daf-12*, indicating the *daf-12* regulates developmental timing primarily through control of *lin-28* (Antebi et al., 1998). However, *daf-12* also directly regulates the transcription of some *let-7*-family miRNAs (Bethke et al., 2009;Hammell et al., 2009a). Using a heterologous transcriptional reporter system, it was found that DAF-12 can bind to and activate promoter elements upstream of *mir-241* and *mir-84* sequences, and the levels of miR-241 and miR-84 by qRT-PCR are reduced in *daf-12* mutants. Therefore, *daf-12* promotes the L2-to-L3 transition by down-regulating LIN-28 and promoting miRNA expression.

Genomic analyses, including *in vitro* selection and ChIP-chip experiments, have also identified other putative *daf-12* transcriptional targets (Hochbaum et al., 2011;Shostak et al., 2004). Interestingly, these include genes that encode components of the RISC. However, many of the *daf-12*-mediated effects on expression of these genes are mild, and more investigation is needed to further understand and validate these relationships.

Studies to date have shown that there are multiple mechanisms in place to ensure correct timing of the L2-to-L3 transition in *C. elegans*. Post-transcriptional

regulation of *hbl-1* expression by several miRNAs and proteins such as LIN-28 and DAF-12 are important in ensuring that *hbl-1* expression is appropriately regulated. Next, components of the late timer, such as *let-7* and *lin-41*, function to ensure proper timing of the larval-to-adult transition.

let-7 and the larval to adult transition

The accumulation of let-7 miRNA in the L3 is required for the appropriate timing of the larval to adult transition in *C. elegans* (Reinhart et al., 2000). The L2 proliferative seam-cell division occurs normally in *let-7(0)* mutants; however, the L4-to-adult transition fails to occur in the seam, indicating that let-7 miRNA activity is necessary for this process (Figure 1D). *let-7(0)* mutants undergo an extra larval stage and the seam terminally differentiates at the next, supernumerary molt. The primary target of let-7 miRNA is another conserved gene, *lin-41*(Reinhart et al., 2000;Slack et al., 2000;Vadla et al., 2012). LIN-41 is a TRIM-NHL family member protein (containing a tri-partite motif including a RING, B-box, and coiled-coil domains; and the NHL domain, found in NCL-1, HT2A, and LIN-41). Regulation of *lin-41* is a conserved function of let-7 miRNA, as it contributes to repression of *lin-41* in differentiating human cells (Worringer et al., 2013). Although the molecular mechanism of *lin-41* in *C. elegans* is still unknown, studies in mammalian systems have described multiple functions for *lin-41*. For example, mouse *lin-41* (*mlin-41*) can bind to Dicer and Argonaute proteins, functioning as an E3 ubiquitin ligase (Rybak et al., 2009). These results

raise the possibility that *lin-41* may destabilize RISC components to inhibit miRNA activity. Whether *lin-41* regulates miRNA activity in the worm, and how it ultimately regulates *lin-29*, is still unclear.

lin-41 is required to prevent precocious accumulation of the transcription factor *lin-29* (Bettinger et al., 1996;Rougvie and Ambros, 1995;Slack et al., 2000). *lin-29(0)* is epistatic to *lin-41(0),* indicating that *lin-29* acts downstream of *lin-41*. *lin-41* likely regulates *lin-29* indirectly, perhaps by regulating miRNA activity. *lin-29* mRNAs co-purify with RISC components, indicating that it may be directly regulated by miRNAs (Zhang et al., 2007). *lin-29* is required for the formation of the adult cuticle, and when LIN-29 is up-regulated as LIN-41 levels decrease, the larval-to-adult transition can occur (Figure 2).

Although the many core heterochronic genes described here have been identified and ordered into a regulatory pathway, there are also many genes that have been identified as heterochronic based on mutant phenotypes whose function in the pathway remains unclear. One such gene is *lin-42.*

lin-42 encodes proteins with homology to *period,* a core component of the circadian clock of flies and mammals

The heterochronic gene *lin-42* has been the focus of my thesis work. *lin-42* loss-of-function *(lf),* results in precocious heterochronic phenotypes, in which adult alae are formed at the L3m (Figure 1C). *lin-42* is the *C. elegans* homolog of *period* genes of flies and mammals (Jeon et al., 1999). *period* genes are

highly conserved and play a critical role in the circadian clock, which synchronizes gene expression, physiology and behavior to the 24-hour cycle in many organisms, including humans (Yu and Hardin, 2006). The region of LIN-42 with highest similarity to PERIOD is the N-terminal PAS domain. PAS (Per-Arnt-Sim) domains are found in many circadian clock proteins, including PERIOD, and act as protein-protein interaction domains. This domain is critical for the proper activity of PERIOD and its cellular localization (Huang et al., 1993;Vosshall et al., 1994). Although *lin-42* may not function in a canonical circadian timekeeper in *C. elegans*, its function in developmental timing indicates that PAS-domain containing proteins are key regulators of biological timekeepers across species.

The *lin-42* genomic locus is complex and encodes at least three isoforms that share homology with *period,* two of which are non-overlapping (Tennessen et al., 2006) (Figure 4, www.wormbase.org). Only Lin-42b and Lin-42c encode PAS domains. However, Lin-42a shares homology with *period* as well. Interestingly, over-expression of Lin-42a is sufficient to rescue an upstream deletion *n1089* as well as premature stop downstream *ve11* (Tennessen et al., 2006) (Figure 4). In contrast, over-expressing Lin-42c rescues *n1089* but not *ve11.* These analyses indicate that the downstream sequence of *lin-42* is most critical for its function. There are two protein domains encoded by this sequence with similarity to PERIOD, noted as SYQ and LT domains (Figure 4). The functions of the SYQ and LT domains are not as well characterized as the PAS domain, however, in *Drosophila*, there is evidence to suggest that these

sequences are involved in the interaction with other circadian clock proteins (Chang and Reppert, 2003;Sun et al., 2010).

Studies of circadian timekeeping in animals were pioneered in *Drosophila*, where mutations in the *period* gene alter circadian behaviors including locomotion and olfaction (Konopka and Benzer, 1971). In humans, circadian rhythms have been studied extensively, and roles for circadian timekeeping have been implicated in everything from energy homeostasis to depression (Reviewed in, (Bass and Takahashi, 2010;Ciarleglio et al., 2011)). Mutations in hPER2, a human homolog of *period*, have been linked to the sleep disorder Familial Advanced Sleep Phase Syndrome (FASPS), where those affected have a 4-hour advance in their circadian rhythm (Toh et al., 2001). There is also a clear role for *period* in cancer (reviewed in (Savvidis and Koutsilieris, 2012)). For example, human *Per1* can function as a tumor suppressor, and it regulates the expression of, and interacts with, many checkpoint proteins that regulate cell-cycle progression (Gery and Koeffler, 2010).

The components and mechanism of the circadian clock are highly conserved throughout evolution, but in animals have been most extensively investigated in *Drosophila* (Reviewed in (Yu and Hardin, 2006)) (Figure 5). *period* function is important in regulating the transcriptional feedback loop that ensures periodic expression of circadian genes. In the fly clock, starting from mid-day, the transcription factors clock (CLK) and cycle (CYC) bind to e-box promoter elements to up-regulate the expression of circadian-regulated genes, including *period* and its binding partner *timeless* (Darlington et al., 1998;Hao et

al., 1997;McDonald et al., 2001;Wang et al., 2001). *period* and *timeless* mRNAs begin to accumulate and reach their peak levels during the early evening. However, PER and TIM proteins are both negatively regulated post-translationally, and are not abundant until later in the evening. PER is phosphorylated by double-time (DBT) kinase and casein kinase 2 (CK2), and this directs PER for degradation by the ubiquitin/proteosome pathway (Akten et al., 2003;Kloss et al., 1998;Nawathean and Rosbash, 2004;Price et al., 1998) (Figure 5). TIM levels are regulated by light. The photoreceptor cryptochrome (CRY) undergoes conformational changes in response to light, binds to TIM, and promotes its degradation by the proteasome (Busza et al., 2004;Ceriani et al., 1999;Dissel et al., 2004;Rosato et al., 2001). During the night, TIM levels stabilize and it binds to PER-DBT complexes, and the complex is transported into the nucleus. Once in the nucleus, PER-DBT-TIM complexes bind to CLK-CYK to inhibit their activity (Lee et al., 1999) (Figure 5). Finally, at dawn, TIM and PER are degraded, relieving repression of CLK-CYC, and the cycle begins again.

The *C. elegans* LIN-42 protein is homologous to PERIOD and this implies that LIN-42 has a similar molecular function, as a transcriptional repressor. LIN-42 also contains many putative phosphorylation domains; however whether LIN-42 is regulated via post-translational modifications is still unknown. Other circadian clock genes have homologs in the worm, including *kin-20/doubletime* and *tim-1/timeless*. RNAi experiments indicated that single depletion of these genes can cause a mild precocious heterochronic phenotype and also enhance the precocious phenotype of *lin-42* mutants, suggesting that they may also

function in the heterochronic pathway (Banerjee et al., 2005). A *clock/cycle* homolog, *aha-1*, does not interact with the heterochronic pathway genetically or have a developmental timing phenotype on its own. Although *tim-1(RNAi)* enhanced the *lin-42(lf)* phenotype, LIN-42 and TIM-1 do not interact in a yeast-two hybrid system, suggesting that these proteins may not interact *in vivo* (Gardner, 2005). Therefore, the degree to which *tim-1* and *kin-20* function in developmental timing is still unknown.

Interestingly, miRNAs have recently been shown to function in the circadian clock. Circadian regulation of miRNAs and, conversely, miRNA regulation of clock gene expression have been observed in several organisms, including humans (Hansen et al., 2011;Kojima et al., 2011). For example, many miRNAs have been found to be rhythmically expressed in *Drosophila,* and their circadian expression pattern is abolished in *clock* mutants (Yang et al., 2008). Additionally, many circadian clock components may be regulated in part by miRNAs. For example, miR-141 was shown to regulate the circadian gene *Clock* (Meng et al., 2006). Many clock genes also have potential miRNA binding sites in their 3'UTRS, including *Per1/2.* The Lin-42b and Lin-42a 3'UTR does contain some predicted miRNA binding sites. However, expression of *lin-42* with a miRNA-resistant 3'UTR can rescue the heterochronic phenotype of *lin-42* mutants, so whether miRNA mediated regulation of *lin-42* is necessary for its function in developmental timing is unclear (Jeon et al., 1999;Tennessen et al., 2006). Studies have shown than miRNA-mediated regulation of *lin-42* may important in inhibiting up-regulation of *lin-42* during starvation induced L1-arrest;

therefore, miRNAs may regulate *lin-42* in specialized developmental contexts (Kasuga et al., 2013;Zhang et al., 2011).

There have also been some studies characterizing potential circadian behaviors and transcriptional cycling in *C. elegans* (Simonetta and Golombek, 2007;Simonetta et al., 2009;van der Linden et al., 2010). Similar to studies in *Drosophila*, automated locomotory tracking systems have quantified circadian behavior in *C. elegans*. In these systems, interruption of an infrared beam is used to detect movement of the animals in culture. Using such automated systems, circadian locomotory behavior has been reported in adult worms (Simonetta and Golombek, 2007;Simonetta et al., 2009). These animals showed 24-hr patterns of activity and could be entrained by light and temperature. For example, after 5 days of exposure to a light-dark (LD) cycle, worms were transferred to continuous darkness (DD), but maintained their previous locomotory pattern. This type of entrainment is a hallmark of circadian regulation (Simonetta et al., 2009). Mutations that disrupt the PAS-domain encoding region of *lin-42* (*mg152* and *n1089*) reportedly cause prolonged locomotory periods, but this work requires additional study, particularly to resolve the fact that LIN-42 levels markedly decrease in adults (Tennessen et al., 2006). Possible effects of mutations in other circadian clock genes have not been reported.

Genome-wide studies have been undertaken in *C. elegans* to identify potential circadian cycling of gene expression (van der Linden et al., 2010). Using micro-arrays, many transcripts were identified that exhibit 24-hr periodicity. Interestingly, none of the circadian clock homologs in the worm, including *lin-42,*

kin-20, and *tim-1*, had a circadian expression pattern following entrainment by light or temperature. Although circadian-like behaviors can be observed in *C. elegans*, the requirement of circadian clock homologs for these behaviors is unclear and many of these genes do not oscillate in a circadian manner. Whether *C. elegans* has a *bona fide* circadian pacemaker is still being investigated.

lin-42 regulates developmental timing in *C. elegans*

lin-42(lf) causes precocious heterochronic phenotypes. In these animals, the seam cells terminally differentiate one stage too early at the L3m, and the vulva precursor cells also divide early at low penetrance (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006) (Figure 1C). *lin-42(RNAi)* revealed that *lin-42* also has a critical role in timing the development of the gonad. *lin-42(RNAi*) causes the gonad arms to turn precociously, by the L2m rather than the L3m in 32% of animals. Immunolocalization for endogenous LIN-42 also revealed that LIN-42 is expressed in all of these tissues, as well as in muscle and the nervous system. Therefore, *lin-42* regulates developmental timing in multiple tissues, including the seam, vulva, and gonad.

The temporal expression pattern of *lin-42* is also intriguing. Due to its sequence homology to *period*, one key question was whether *lin-42* mRNA levels cycled. RT-PCR analyses found that *lin-42* mRNA levels cycle over developmental time, with peak expression observed in mid-to-late larval stages

(Jeon et al., 1999). In contrast, *tim-1* levels did not oscillate during larval development. Not only do *lin-42* mRNA levels cycle, but an antibody raised against C-terminal LIN-42 protein revealed that protein levels oscillate in a similar manner (Tennessen et al., 2006). This dynamic expression pattern is rare in the heterochronic pathway. Most heterochronic genes are expressed either early or late in development, which correlates with their temporal requirement. In contrast, *lin-42* is expressed throughout larval development, and its expression oscillates with the molting cycle. This expression pattern indicates that *lin-42* may have multiple or reiterative roles in the heterochronic pathway, rather than acting in a single stage.

lin-42 and the heterochronic pathway

Genetic analyses have been performed to position *lin-42* within the broader heterochronic pathway. Until recently, a null allele of *lin-42* did not exist, making genetic analyses using extant hypomorphic alleles difficult to interpret. Generation of null allele is described in Chapter II. Studies using RNAi have, however, revealed some interesting interactions in the pathway (Tennessen et al., 2006).

Deletion of *lin-4* results in a retarded phenotype, in which the L1 program is repeated in the hypodermis (Ambros and Horvitz, 1984;Chalfie et al., 1981) (Figure 1C). Therefore, *lin-4* mutants have fewer seam cells than wild-type and fail to form alae at the L4-to-adult transition. Interestingly, *lin-42(RNAi)* suppressed the alae formation defect in *lin-4* mutants but not the reduced seam

cell number (Tennessen et al., 2006). This result indicates that the restoration of alae formation does not require suppression of the seam cell defect of *lin-4*. This also provides strong evidence that, although *lin-42* is expressed in the L1 and L2, it may not regulate the L2 proliferative division.

An interesting genetic interaction was also observed with *lin-46*, which is homologous to the mammalian protein gephyrin, and likely acts as a scaffolding protein (Pepper et al., 2004). Similar to *lin-42, lin-46* has a dynamic expression pattern, but in the opposite phase: *lin-46* is expressed for a brief period during each molt. *lin-46(lf)* mutants have a retarded heterochronic phenotype in which they repeat the L2 proliferative division and fail to form complete alae at the L4-molt. Interestingly, *lin-42(RNAi); lin-46(lf)* double mutant animals have a *lin-42(RNAi)* phenotype (Tennessen et al., 2006). This suggests the *lin-42* is downstream of *lin-46* in the heterochronic pathway. It is important to note, however, that the alae of *lin-46; lin-42(RNAi)* animals are less robust than *lin-42(RNAi)* alone, indicating that *lin-46* likely acts through other factors besides *lin-42*.

Epistasis experiments were also performed with *daf-12* mutants. *lin-42* was knocked down by RNAi in three different *daf-12* mutant backgrounds and animals were analyzed for heterochronic phenotypes in the seam. *rh61rh411* and *rh61rh412* are likely null alleles, while *rh62rh157* is a loss-of-function allele that, as noted above, causes a stronger heterochronic phenotype than null alleles, and is thought to be a constitutive repressor that does not cause larval arrest (Antebi et al., 2000). In all cases, mutual suppression was observed,

indicating that these genes act in opposition to regulate timing, rather than functioning in a simple linear pathway (Tennessen et al., 2006).

let-7 miRNA is required for proper execution of the larval to adult transition. *let-7(mn112)* is a null allele of *let-7* which results in a retarded heterochronic phenotype (Meneely and Herman, 1979;Reinhart et al., 2000) (Figure 1D). *lin-42(RNAi); let-7(mn112)* animals mutually suppress their phenotypes, indicating that, similar to *daf-12*, *lin-42* and *let-7* do not act in a linear pathway to regulate timing (Tennessen et al., 2006).

Many of the genetic interactions tested between *lin-42* and heterochronic pathway members resulted in mutual suppression; therefore, placement of *lin-42* in the pathway is unclear. *lin-42* in general may act in opposition to *daf-12* and miRNAs to inhibit later developmental pathways from occurring too early in development. However, the specific role for *lin-42* in heterochronic pathway is still unknown.

lin-42 is necessary for the timing and completion of larval molts

The cycling expression pattern of *lin-42* also indicates that it may be involved in molting. Indeed, initial analyses of *lin-42(lf)* mutants found that these animals fail to complete the L4-molt, and become stuck in their cuticles. (Abrahante et al., 1998). Recent work from Allison Frand's lab has established that *lin-42* regulates the timing of the molting cycle. Delays in the timing of onset and exit from each molt were observed in *lin-42(ok2385)* mutants, which deletes the entire downstream region of *lin-42,* leaving just the Lin-42c isoform intact

(Monsalve et al., 2011) (Figure 4). For example, while the first molt occurs at approximately 16hrs after release from L1-arrest in wild-type at 25°C, this molt is delayed by 2 to 4 hours in *lin-42(lf)*. Additionally, this defect becomes more severe as animals progress through development. Strikingly, the L4-stage can last on average 22 hrs, while this stage is only about 10 hrs long in wild-type animals. RT-PCR and transcriptional reporters suggest that Lin-42a is expressed during the molts, and may be necessary to regulate molting during larval development. Like the alae formation defect, over-expression of this isoform rescues the molting phenotype of *lin-42(lf)* animals. Genetic analyses revealed that *lin-42* may act in parallel or upstream of *nhr-25*, a nuclear hormone receptor which has been known to regulate molting (Gissendanner and Sluder, 2000;Hada et al., 2010;Hayes et al., 2006;Monsalve et al., 2011). Further study is needed to clarify how *lin-42* and *nhr-25* act to regulate the timing of the molts. Interestingly, other heterochronic pathway components, let-7 miRNA and miR-84 had previously been shown to be required to exit the molting cycle at the larval-to-adult transition (Hayes et al., 2006). However, unlike *lin-42*, these miRNAs are not required for the timing or execution of larval molts.

lin-42 and *daf-12* integrate the developmental timer and stress response pathways

Outside the laboratory, an organism may encounter a variety of environmental stressors, and it is important to understand how environmental signals impact developmental timing programs. Nutritional status and chemicals

in the environment can clearly influence developmental timing in mammals. How does an organism respond to such environmental changes during development? *C. elegans* has proven to be a remarkably powerful system for studying how environment affects development. The worm has a well-described stress response pathway, wherein larvae can enter a stress-resistant diapause stage in response to highly adverse conditions (For review, see (Fielenbach and Antebi, 2008)). Conditions of stress such as high temperature, crowding, and starvation in early larval development will result in arrest of the developmental timer and formation of a highly stress-resistant Dauer Larva (Cassada and Russell, 1975).

Genetic screens have identified many genes that regulate dauer formation. Many of these mutants have a dauer formation defective (Daf-d) or dauer formation constitutive (Daf-c) phenotype. One of these genes is *daf-12*, where, interestingly, both Daf-d and Daf-c mutants have been identified (Antebi et al., 1998;Antebi et al., 2000). *daf-12* acts at the convergence of developmental timing and dauer formation. In favorable environments, *daf-12* promotes continuous development and is a key player in the heterochronic pathway. *daf-12(0)* mutants are unable to form dauers, showing that *daf-12* is necessary to enter dauer.

The signaling pathways that regulate DAF-12 activity are highly complex, involving inputs from insulin and TGFβ signaling pathways, which in turn regulate the synthesis of hormones that regulate DAF-12 (For review, see (Fielenbach and Antebi, 2008)) (Figure 6). In favorable environmental conditions, insulin and

TGFβ signaling are high, allowing the expression of genes involved in steroid hormone biosynthesis, one of which is the P450 enzyme encoded by *daf-9* (Gerisch and Antebi, 2004;Motola et al., 2006) DAF-9 activity is necessary to synthesize dafachronic acids (DA), steroid hormones that bind to DAF-12 to promote reproductive development (Motola et al., 2006). In stressful environmental conditions, signaling from TGFβ and insulin pathways is reduced, relieving repression of *daf-5* and *daf-16/FOXO* transcription factors, respectively (da Graca et al., 2004;Ogg et al., 1997). These proteins are thought to inhibit expression of hormone biosynthesis enzymes, causing DA levels to decrease. In the absence of hormone, DAF-12 binds to its co-repressor called DIN-1, and this repressor complex inhibits continuous development and promotes dauer formation (Ludewig et al., 2004;Motola et al., 2006).

lin-42 also regulates dauer formation (Tennessen et al., 2010) (Figure 6). *lin-42(lf)* animals have a high-temperature induced dauer (Hid) phenotype at 27°C. While only 2% of wild-type animals form dauers at this temperature, 71% of *lin-42(lf)* animals form dauers at 27°C. This finding suggests that *lin-42* functions to prevent inappropriate dauer formation in mildly stressful conditions. Detailed genetic analysis showed that *lin-42* acts to modulate ligand-free DAF-12 signaling. While the *lin-42* phenotype is not affected by Daf-d mutations of *daf-16* or *daf-3*, it is suppressed by *daf-12(0)*. These results demonstrate that *lin-42* does not act in the insulin or TGFβ signaling pathways. Moreover, *lin-42(lf)* has a synthetic dauer formation (SynDaf) phenotype with loss-of-function mutations of *daf-9*. At 25°C, neither of these genes are Daf-c. However, 33% of *lin-42(lf);*

daf-9(lf) double mutants form dauers at this temperature, suggesting that *lin-42* does not act in the dafachronic acid synthesis pathway, but downstream in opposition to *daf-12*. Further evidence for this conclusion is the interaction between *lin-42(lf*) and *daf-12(rh61),* a *daf-12(lf)* allele*. daf-12(rh61)* mutants contain a premature stop in the ligand-binding domain of *daf-12*, which renders the protein insensitive to ligand (Antebi et al., 2000;Motola et al., 2006). Interestingly, *lin-42(lf); daf-12(rh61)* mutants are partially SynDaf (Tennessen et al., 2010). Taken together, these data reveal that *lin-42* is necessary to prevent inappropriate dauer formation, and acts in opposition to *daf-12* activity in the dauer formation pathway. Therefore, *daf-12* and *lin-42* are important for integrating the developmental timing and stress response pathways in *C. elegans.*

Impact of studying *C. elegans lin-42/period* and developmental timing

PERIOD and the circadian clock in general regulate many processes in flies and mammals, including cell cycle, metabolism, and behavior, synchronizing these processes to the 24-hr cycle (Bass and Takahashi, 2010;Ciarleglio et al., 2011;Savvidis and Koutsilieris, 2012). Circadian clocks are also highly sensitive to environment, and can be modulated by light, temperature, and nutritional status. Although not clearly involved in a *bona fide* circadian clock, in *C. elegans, lin-42/period* is critical to the timing of larval development and molting. Studies of LIN-42/PER in *C. elegans* provide a new system in which to study the function and activity of such proteins in biological timekeepers, and how these

pathways respond to environmental conditions. The goal of my thesis work has been to understand its roles in responding to environmental stress and in the heterochronic pathway.

In Chapter II, I discuss the generation of a null allele of *lin-42* and our preliminary characterization of its phenotypes. This is important because previously analyzed alleles left one *lin-42* isoform intact, complicating genetic and molecular analyses. The null mutants are viable, but have more severe defects than previously studied loss-of-function alleles, including a severe molting defect which results in a highly penetrant larval arrest phenotype. This new *lin-42* allele will be instrumental for understanding the role of *lin-42* in developmental timing and molting.

In Chapter III, I demonstrate that the phenotype of *lin-42* mutants can be suppressed by mild temperature stress as well as decreased signaling output from the TGFβ and insulin signaling pathways. Developmental timing mechanisms are extremely robust and are not generally affected by mild environmental stress. The effect of stress on *lin-42* mutant phenotypes is intriguing and reveals modulation of developmental timing by the environment. We also show that the temperature suppression requires *daf-12* but, unlike in dauer formation, it is *daf-16*/FOXO-dependent. These studies illustrate that even mild environmental fluctuations affect developmental timing in *C. elegans*, which can only be appreciated in a sensitized context.

In Chapter IV, I demonstrate that one role for *lin-42* in regulating developmental timing is to prevent precocious expression of *let-7*-family miRNAs.

In *lin-42(lf)* animals, mature *let-7*-family miRNAs are over-expressed compared to wild-type. *lin-42* likely acts at the transcriptional level, as pri-miRNA levels for these miRNAs are up-regulated in *lin-42* mutants. The regulation of miRNAs by circadian clock genes has been reported in flies, mice, and human cells; therefore, understanding how *lin-42* regulates miRNA expression will provide insight into the regulatory relationship between circadian clock-related genes and miRNAs.

Figure 1: Heterochronic genes regulate the timing of seam cell development. **A**. Lateral view of the organization of hypodermal seam cells. (Adapted from (Daul, 2008)) **B.** Cuticular ridges termed alae are formed along the length of the animals at the L4-molt in wild-type. Hypodermal seam cells are required for alae synthesis. **C.** Seam lineages of various precocious heterochronic mutants. *lin-14(0)* mutants skip the L1, execute L2-stage programs in the L1 stage, and form alae too early . *lin-28(0)* mutants skip the L2 proliferative division and form alae too early (Ambros and Horvitz, 1984). *hbl-1(lf*) and *lin-42(lf)* results in precocious alae formation at the L3-molt (Abrahante et al., 1998;Abrahante et al., 2003;Lin et al., 2003). * *hbl-1(RNAi)* animals often skip the L2 proliferative division, though this is not observed in *hbl-1(lf)* mutants **D.** Seam lineages of various retarded heterochronic mutants. *lin-4(0)* animals repeat the L1 seam program through larval development and undergo extra molts (Ambros, 1989). *daf-12(rh61)* mutants repeat L2-seam programs (Antebi et al., 1998). *let-7(0)* mutants repeat L3-programs, while a *mir-48 (0) mir-241(0); mir-84(0*) triple mutant results in the repeat of L2 seam-programs (Abbott et al., 2005;Reinhart et al., 2000). *lin-29* is necessary to execute the larval-to-adult switch in the seam, and *lin-29(0)* mutants fail to form alae (Rougvie and Ambros, 1995).

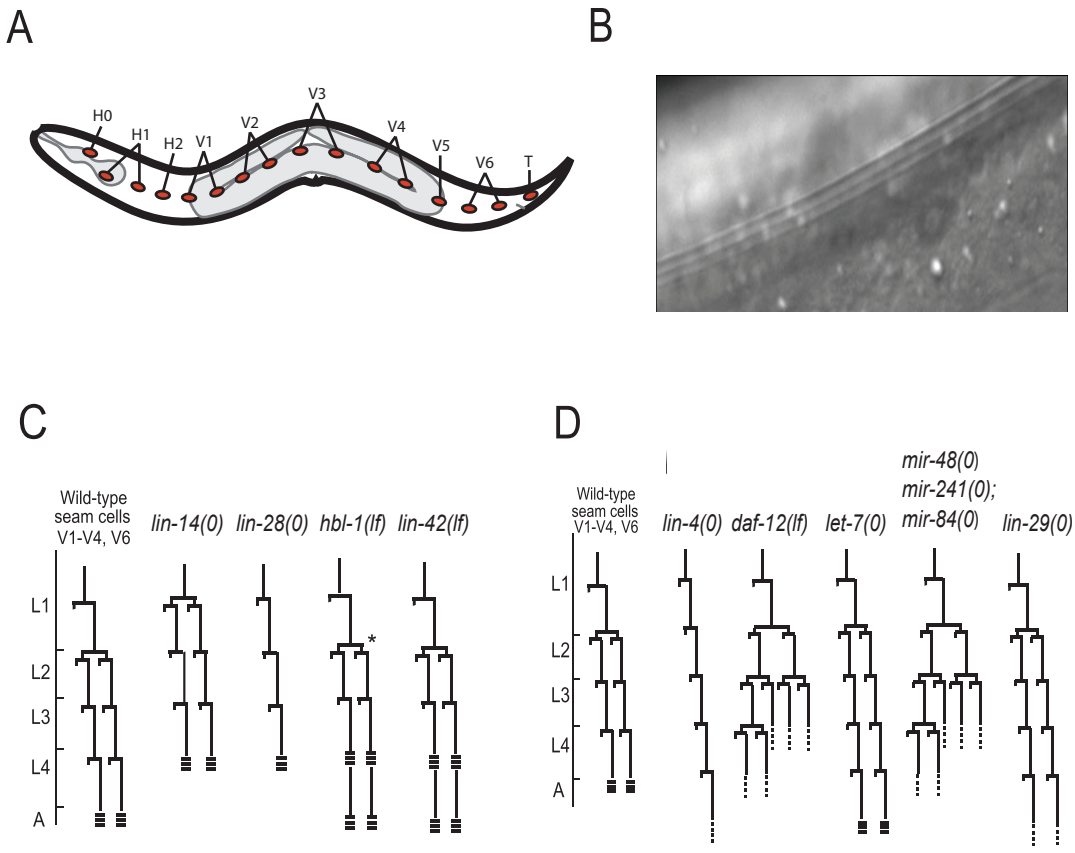

A
B
H0
H1
H2
V1
V2
V3
V4
V5
V6
T
C
D
Wild-type
seam cells
V1-V4, V6
lin-14(0)
lin-28(0)
hbl-1(lf)
lin-42(lf)
L1
L2
L3
L4
A
*
Wild-type
seam cells
V1-V4, V6
lin-4(0)
daf-12(lf)
let-7(0)
mir-48(0)
mir-241(0);
mir-84(0)
lin-29(0)
L1
L2
L3
L4
A

Figure 1

Figure 2: The heterochronic pathway of *C. elegans.* Shown is a simplified version of the heterochronic pathway. This model is supported by both genetic and biochemical data. Not all characterized members are shown, and not all indicated interactions are direct. Arrows indicate activation and blunt ends indicate repression. It should be noted that *daf-12* here represents its DA bound form, which promotes reproductive development. When ligand-free DAF-12 is bound to DIN-1, the heterochronic pathway is inhibited and the animals enter dauer.

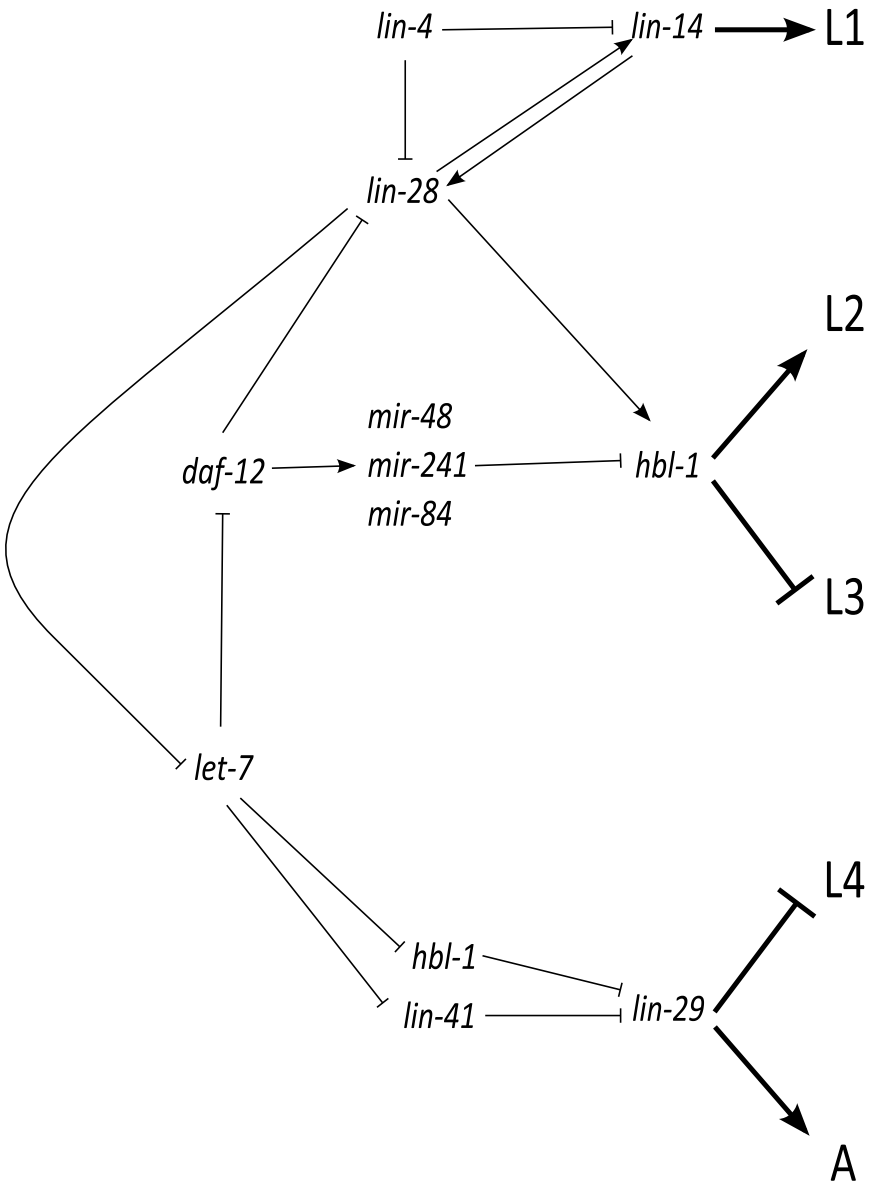
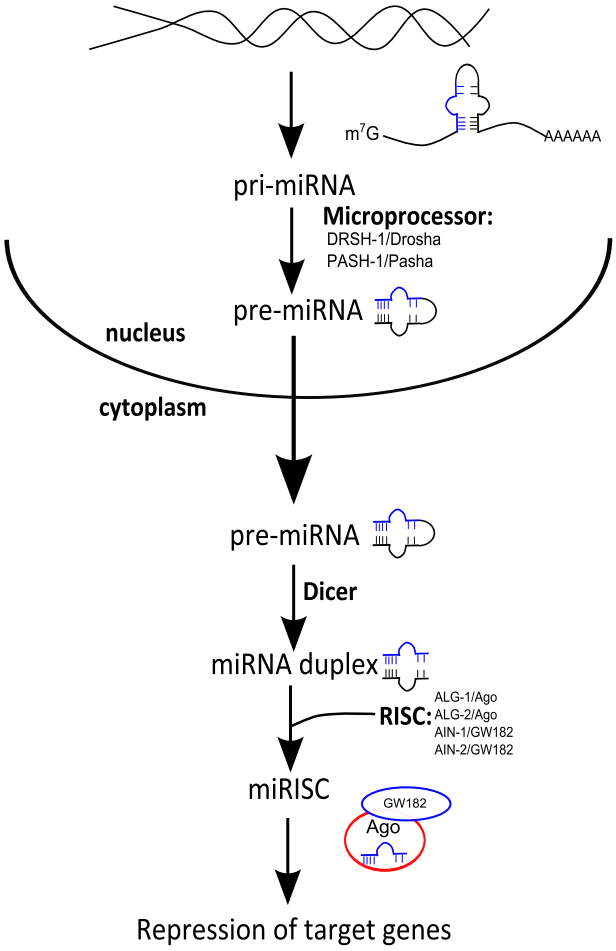

lin-4
lin-14
L1
lin-28
L2
mir-48
daf-12
mir-241
hbl-1
mir-84
L3
let-7
L4
hbl-1
lin-41
lin-29
A

Figure 2

Figure 3: miRNAs are highly processed molecules that regulate gene expression post-transcriptionally. A. Simplified diagram of miRNA processing. miRNAs are first transcribed by RNA polymerase II generating a long primary-miRNA transcript. These pri-miRNAs are further processed in the nucleus by a large protein complex called the Microprocessor, which produces a pre-miRNA hairpin that is exported to the cytoplasm where it is further processed into the short 22nt miRNA. This molecule is then incorporated into the RISC. The miRNA-RISC complex (miRISC), can bind to target mRNAs through imperfect complementarity between the miRNA and 3'UTR of its target. See text for further details. **B.** Some miRNAs, including let-7 miRNA and lin-4 miRNA, are part of families that share identity in their 5' end. The region of identity is highlighted in yellow.

A



B

let-7 family

let-7 miRNA	UGAGGUAGUAGGUUGUAUAGUU
miR-48	UGAGGUAGGCUCAGUAGAUGCGA
miR-241	UGAGGUAGUAUGUAAUAUUGUA
miR-84	UGAGGUAGUAUGUAAUAUUGUA
miR-793	UGAGGUAUCUUAGUUAGACAGA
miR-794	UGAGGUAAUCAUCGUUGUCACU
miR-795	UGAGGUAGAUUGAUCAGCGAGCUU

lin-4 family

lin-4 miRNA	UCCCUGAGACCUCAAGUGUGA
miR-237	UCCCUGAGAAUUCUCGAACAGCU

Figure 3

Figure 4: *lin-42* is a complex locus encoding multiple isoforms with homology to *period.* Reverse complement of the genomic locus of *lin-42* as annotated in wormbase. The *lin-42* locus produces three major isoforms. Lin-42b and Lin-42c both encode proteins with a PERIOD-like PAS domain. Over-expression of Lin-42a is sufficient to rescue both upstream (*n1089, mg152*) and downstream mutations (*ve11, ok2385*), while over-expression of Lin-42c cannot rescue downstream lesions. Note that in all characterized alleles shown here, at least on isoform of *lin-42* remains intact.

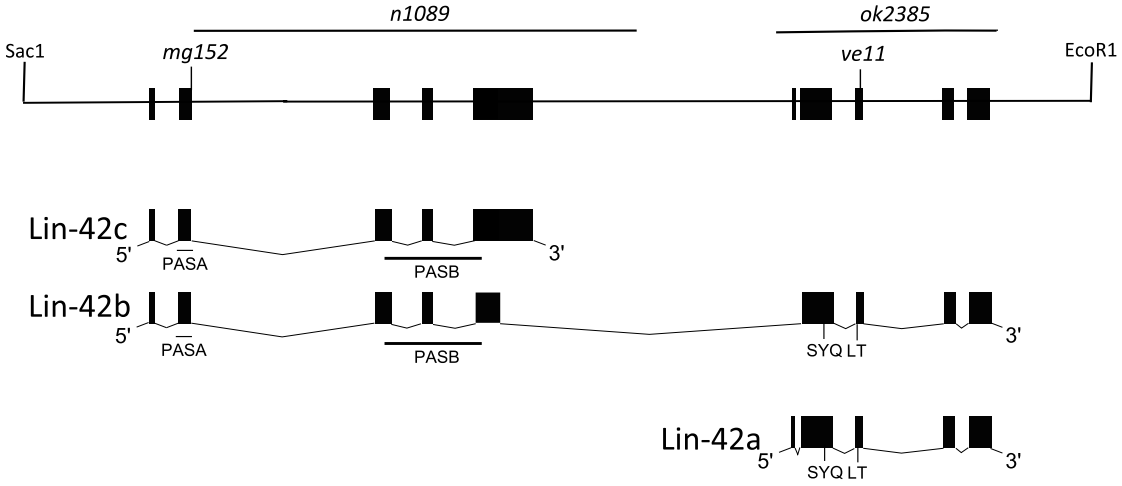

n1089
ok2385
Sac1
mg152
ve11
EcoR1
Lin-42c
5'
PASA
PASB
3'
Lin-42b
5'
PASA
PASB
SYQ LT
3'
Lin-42a
5'
SYQ LT
3'

Figure 4

Figure 5: The *PERIOD* feedback loop of *Drosophila melanogaster.* Shown is a simplified version of the *period/timeless* feedback loop of the Drosophila circadian clock. *period* and *timeless* transcription is promoted by CLOCK (CLK) and CYCLE (CYC) heterodimers. PERIOD (PER) and TIMELESS(TIM) proteins are both regulated post-translationally. Phosphorylation of PERIOD by casein kinase 2 (CK2) and DOUBLETIME (DBT) causes PER degradation by the proteosome. TIM levels are regulated by CRYPTOCHROME (CRY), which during the day promotes TIM degradation. At night, CRY undergoes a conformational change which relieves repression of TIM, and this allows TIM to bind to phosphorylated PER-DBT complexes, stabilizing PER. This entire complex is transported into the nucleus, where PER-DBT-TIM binds to CLK-CYC and inhibits the activity of this complex. See text for further details.

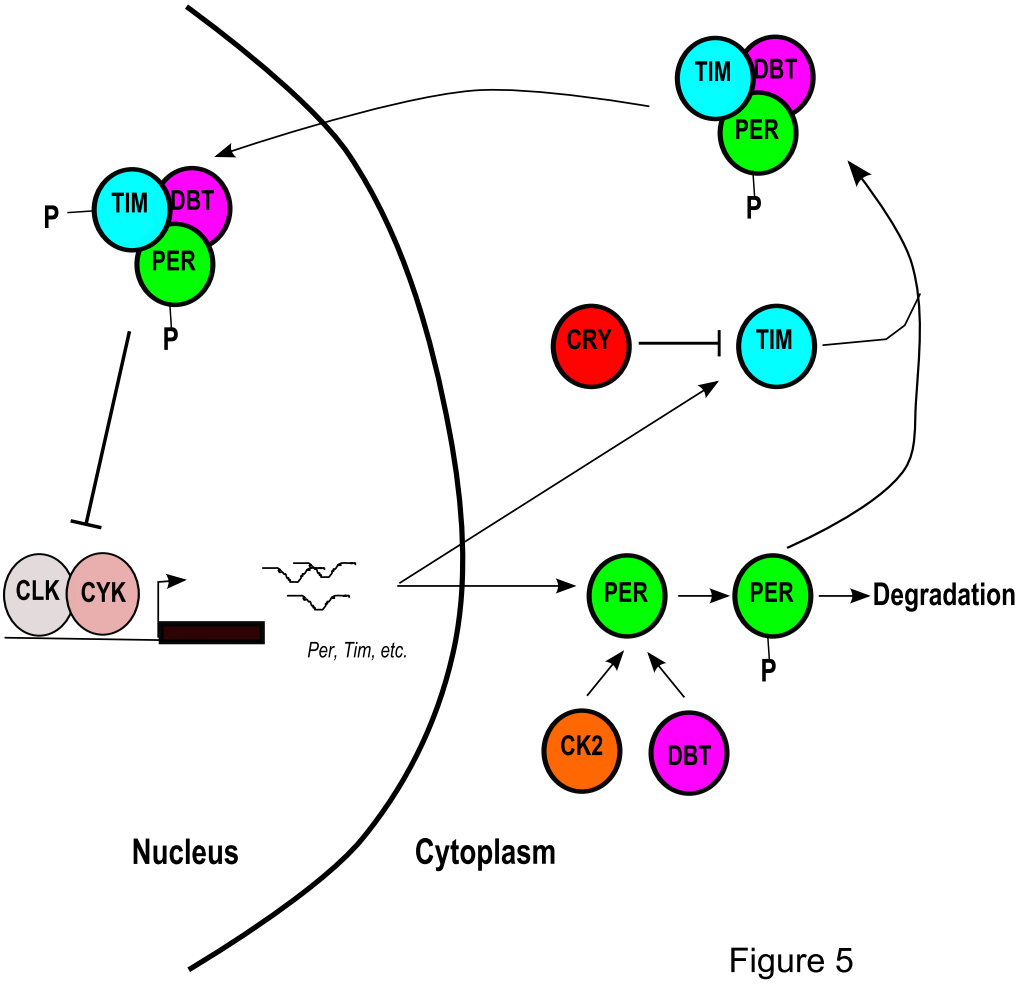

TIM
DBT
PER
P
P
TIM
DBT
PER
P
CRY
TIM
CLK
CYK
Per, Tim, etc.
PER
PER
Degradation
P
CK2
DBT
Nucleus
Cytoplasm

Figure 5

Figure 6: DAF-12 and LIN-42 regulate dauer formation in *C. elegans.* Dauer formation is regulated by multiple signaling pathways. *daf-12* is the most downstream player in dauer formation that has been identified. *daf-12* encodes a nuclear hormone receptor, and its activity is regulated via interactions at the ligand-binding domain. In favorable environments, several signaling pathways, including insulin and TGFβ pathways, regulate the synthesis of hormone biosynthesis genes, which promote the synthesis of dafachronic acids from cholesterol. Dafachronic acid binds to DAF-12, and this form of DAF-12 promotes reproductive development. In highly stressful environmental conditions, the output from these signaling pathways decreases. This causes dafachronic acid levels to decline, allowing DAF-12 to bind to the co-repressor DIN-1, and promotes dauer formation. The PERIOD homolog LIN-42 opposes ligand-free DAF-12 activity. See text for further details.

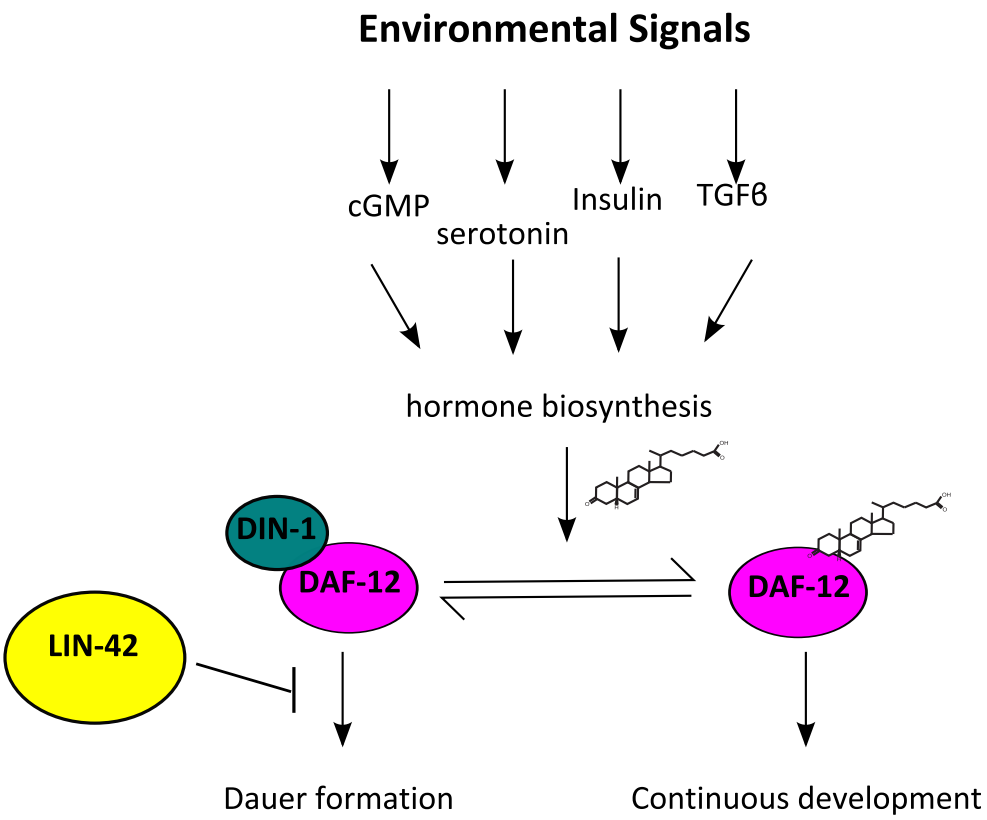

Environmental Signals
cGMP
serotonin
Insulin
TGFβ
hormone biosynthesis
DIN-1
DAF-12
LIN-42
DAF-12
Dauer formation
Continuous development

Figure 6

Table 1: Heterochronic genes that regulate developmental timing in the seam

Gene	Molecular Identity	Loss-of-function phenotype
lin-4	miRNA	Retarded
lin-14	transcription factor	Precocious
lin-28	zinc-finger RNA-binding protein	Precocious
lin-46	gephryn-like protein	Retarded
hbl-1	hunchback-like transcription factor	Precocious
mir-48/241/84	miRNA	Retarded
lin-42	period-like protein	Precocious
daf-12	nuclear hormone receptor	Retarded
let-7	miRNA	Retarded
lin-41	TRIM/NHL RNA-binding protein	Precocious
lin-29	transcription factor	Retarded

CHAPTER II. Generation and characterization of a *lin-42* null allele

Summary

The *C. elegans* heterochronic pathway regulates the timing of post-embryonic development, and *lin-42* is a critical element of this pathway. *lin-42* encodes the *C. elegans* homolog of *period* genes of *Drosophila* and mammals, an important player in circadian clock. *lin-42(lf)* results in precocious phenotypes, in which later developmental events occur too early. In addition to its role in timing, *lin-42* also regulates molting, and larval molts are delayed in *lin-42(lf)* animals. The *lin-42* locus is complex, encoding three isoforms, two of which do not overlap, and are transcriptionally controlled by distinct promoters. All previously characterized *lin-42* alleles left one of these isoforms intact, complicating analyses of *lin-42*. To address this problem, MosDel technology was used to completely delete the *lin-42* coding region. This null mutation results in more severe molting and heterochronic defects than previously characterized hypomorphic alleles, indicating that the individual *lin-42* isoforms contribute to heterochronic and molting pathways. Further genetic and molecular experiments using this allele will contribute to understanding the function of *lin-42* in *C. elegans* larval development.

Introduction

The heterochronic pathway of *C. elegans* ensures that specific larval developmental programs are executed in the correct sequence (For review, see (Resnick et al., 2010)). The worm progresses rapidly through four larval stages prior to becoming a reproductive adult, and each of these stages end in a molt. Mutations in heterochronic genes alter the timing of developmental programs relative to these molts. Many genes have been identified that regulate temporal development, and genetic analyses have organized them into a regulatory pathway. However, there are some heterochronic genes that, although when mutant produce strong developmental timing defects, do not fit neatly into this pathway. One of these genes is *lin-42* (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006). *lin-42* is particularly interesting as it encodes the *C. elegans* homolog of *Drosophila* and mammalian *period*, a core member of the circadian clock. Therefore, *lin-42* provides a link between two biological timekeepers: the heterochronic pathway and circadian clock. In circadian biology, *period* function is critical for cyclical expression of circadian-regulated genes and is thought to inhibit gene expression via direct interactions with transcription factors (Lee et al., 1999).

All identified *lin-42* mutations are hypomorphic alleles, and this has complicated genetic analysis of *lin-42* and its role in timing. Genetic epistasis is one of the most powerful methods available to organize genes into a regulatory

pathway. In this type of analysis, double mutants are constructed and to test if one mutants phenotype is masked by the other. Classic epistasis testing relies on the use of null mutations. If residual activity of a gene remains, genetic interactions can be challenging to interpret, as any affects could also be the result in modulation of activity or levels of the genes of interest. Therefore, isolation of a *lin-42* null allele would aide in placement of *lin-42* within the heterochronic pathway.

Mutation of *lin-42* results in precocious heterochronic phenotypes. For example, in these animals, the hypodermal seam cells terminally differentiate one stage too early, at the L3 molt (L3m) instead of the L4 molt (L4m) (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006). In addition to heterochronic phenotypes, some *lin-42* mutants have also been reported to exhibit a molting defect (Monsalve et al., 2011). In wild-type animals, larval molts occur with remarkable synchrony, and each molt is initiated at approximately the same time amongst individuals. However, in *lin-42(lf)* mutants, the timing and duration of larval molts is delayed, resulting in lengthened larval development and some larval arrest.

Initial analysis identified *lin-42* as the predicted gene F47F6.2, which encodes an approximately 49.8kD protein containing PERIOD-like PAS domains, LIN-42C (Jeon et al., 1999) (Figure 1A). Subsequent 3'RACE experiments revealed that the final exon in F47F6.2 could be alternatively spliced to downstream exons that were predicted to be a separate gene, F47F6.1, producing another *lin-42* isoform, Lin-42b (Tennessen et al., 2006). Genetic

analyses confirmed that F47F6.1 and F47F6.2 were the same gene, as a mutation within F47F6.1, *ve11*, fails to complement *mg152*, a mutation in F47F6.2 (Tennessen et al., 2006) (Figure 1A). In the rest of this chapter, I will refer to F47F6.2 as “upstream” and F47F6.1 as “downstream” for simplicity.

Interestingly, the downstream region of *lin-42* also encodes protein sequence with PERIOD homology, the SYQ and LT domains, and in flies there is some evidence to suggest that these homology domains are important for interactions with transcription factors in the circadian clock (Chang and Reppert, 2003;Sun et al., 2010). The downstream isoform, Lin-42a, can be independently expressed from a distinct promoter (Tennessen et al., 2006). Therefore, the *lin-42* locus encodes at least 3 isoforms, two of which are non-overlapping (Figure 1A). All characterized *lin-42* alleles leave one of these isoforms, either Lin-42a or Lin-42c, completely intact, which makes analysis of *lin-42* phenotypes and genetic relationships challenging to interpret.

Structure-function analysis of *lin-42* has also been difficult due to the lack of a null mutation. Rescue experiments indicate that Lin-42a, when over-expressed, can rescue mutations in Lin-42b and Lin-42a, such as *ve11* and *ok2395* (Monsalve et al., 2011;Tennessen et al., 2006) (Figure 1A). On the other hand, over-expression of Lin-42c can rescue upstream mutations, such as *mg152*, but cannot rescue downstream mutations. These results suggest that the downstream sequences are necessary for *lin-42* activity, while the upstream sequences encoding PAS domains may play a more regulatory role. It is unclear what function Lin-42b may play in development, as rescue experiments with this

isoform would be challenging to interpret if endogenous sequence of this isoform is still present, as is true for all extant *lin-42* alleles.

To aide in investigation of *lin-42* and its roles in both developmental timing and molting, we generated a *lin-42* null allele using MosDel technology (Frokjaer-Jensen et al., 2010). With this method, an approximately 10.2kb deletion was generated that removes the entire *lin-42* coding region. Analysis of this mutant revealed that animals lacking *lin-42* activity are viable, but have more severe molting and developmental timing defects than do previously characterized mutants in which one isoform is left intact.

MATERIALS & METHODS

MosDel deletion of *lin-42*

Mos1 mediated deletion of the *lin-42* locus was performed essentially as described (Frokjaer-Jensen et al., 2010). There are several *Mos1* insertion sites near the *lin-42* locus. The ttTi42556 *Mos1* site 400bp upstream of the first *lin-42* exon was used.

To generate the deletion template, we used an ~1.5kb “right” homology region adjacent to the *Mos1* insertion site at the 5’ end of the gene, and a “left homology region in the Lin-42a/b 3’UTR (Figure 1B). Primers were designed to amplify these regions with the addition of attB sites for Gateway Cloning (Invitrogen). The primers used for these reactions were:

Left: AB27 5’-ggggacaactttgtatagaaaagttggactgaaaattggtgtatgaaca-3’/ AB28 5’-ggggactgctttttttgtacaaacttgccgtcttcccgaaaactt-3’

Right: AB25 5’-ggggacagcagctttcttgtacaaagtggaacctaaaactcctcggt-3’/AB26 5’-ggggacaactttgtataataaagttgacgaatcatgttccctgt-3’

A Gateway LR recombination reaction was used insert the left and right homology regions into pCFJ66, flanking *C. briggsae unc-119* to yield the deletion template, pALB8 (Figure 1B).

Injection mixes contained: pALB8 (deletion template 50 ng/µl), pJL43.1(Pglh-2::transposase 50 ng/µl), pGH8(Prab-3::mCherry 10 ng/µl), Pmyo-2::tdtomato (2.5 ng/µl), and pCFJ104(Pmyo-3::mCherry 5 ng/µl). This mix was injected into strain RG1514 *ttTi42556; unc-119(ed3)*. Animals in which the *lin-42* locus is deleted and *unc-119* rescuing fragment inserted in its place should not carry the fluorescently labeled extra-chromosomal array (RFP-), but have wild-type movement (*unc-119*+). Candidate strains were genotyped by PCR to confirm deletion of *lin-42*. One primer inside the *unc-119* insertion and one in the genomic sequence outside of the homology domains were used for genotyping. Only DNA from animals where *lin-42* was deleted and replaced by (*unc-119+)* resulted in a PCR product. To genotype the *lin-42(0)* allele, the following primer set was used:

AB35 5’-cgaaaatttcaaaaagctcgt-3’/ AB37 5’-caattcatcccggtttctgt-3’

Two *lin-42(0)* strains were isolated: EG15911 *lin-42(ox461 [unc-119(+)]); unc-119(ed3)* and EG15910 *lin-42(ox460 [unc-119(+)]); unc-119(ed3).* The genotypes were confirmed by sequencing. Strains were outcrossed 3X to generate RG1590 *lin-42(ox461)* and RG1591 *lin-42(ox460)*, respectively.

Cloning

The Lin-42b/c promoter was cloned from a genomic *lin-42* plasmid clone, pHG82, and subcloned into pSCA to generate pTBE17 (Stratagene) (Gardner, 2005). pTBE17 was digested with XmaI and HindIII to excise the promoter. The *dpy7* promoter of pJT97 was replaced with the Lin-42b/c promoter to generate pCP2 (Tennessen et al., 2010). This construct encodes the LIN-42B isoform tagged with GFP under control of the Lin-42b/c promoter.

Genetics and Strains

C. elegans were grown and maintained as described (Brenner, 1974). Strains used in this study were:

EG15911 *lin-42(ox461); unc-119(ed3)*, EG15910 *lin-42(ox460); unc-119(ed3),* RG1590 *lin-42(ox461),* MT2257 *lin-42(n1089),* ARF224 *lin-42(ok2385),* RG1665 *lin-42(ox461); veEx593* (Lin-42a*, sur-5::gfp*), RG1741 *lin-42(ox461); veIs26* (*lin-42c::gfp*), RG1739 *lin-42(ox461), veEx651* (*lin-42b::gfp*).

To generate Lin-42b over-expression lines, pCP2(5 ng/µl) and *str-1::gfp* (100 ng/µl) was injected into wild-type animals. Arrays were then crossed *into lin-42(ox461)* animals.

Analysis of molting and heterochronic phenotypes

To establish molting behavior of *lin-42(0)* animals, eggs were picked to plates seeded with OP50 and hatched at 20°C overnight. The next day, animals were monitored on a Kramer FBS10 dissecting microscope until they stopped pumping, entering the first molt. These animals were singly-picked to individual plates and monitored every 2 hrs for pumping for 5 days. Wild-type animals were monitored every hour until they reached adulthood.

For quantifying the proportion of larval arrest in *lin-42* mutant populations, eggs were picked to new plates, and newly hatched L1 larvae were single-plated and raised at 20°C. Animals that did not reach adulthood by seven days were classified as arrested.

To analyze heterochronic phenotypes, eggs were plated and analyzed at the appropriate developmental stage. For animals containing *lin-42(ox461)*, eggs were isolated by hypochlorite treatment and then dropped onto NGM plates.

RESULTS

Generation of *lin-42(0)* alleles by MosDel technology

A breakthrough in *C. elegans* reverse genetics occurred in 2010, when work from the Jorgensen lab at the University of Utah demonstrated use of the *Drosophila Mos1*-mediated transposon system to generate targeted deletions in the *C. elegans* genome (Frokjaer-Jensen et al., 2010). In this system, a *Mos1*

insertion site near the gene of interest is excised by *Mos1* transposase provided by injection of a helper plasmid encoding this enzyme. The resulting double strand break (DSB) can be repaired from another co-injected "template" plasmid containing *C. briggsae unc-119* bordered by homology regions flanking the desired deletion (Figure 1B, See Materials and Methods). Deletions are generated in an *unc-119* mutant background that contains a *Mos1* insertion near the gene of interest. Successful deletions can be isolated by screening for animals that have wild-type movement but do not carry the extra-chromosomal with the repair plasmid, which is followed by co-transformed fluorescent reporters.

We utilized the *Mos1* insertion ttTi42556, 420bp upstream of the *lin-42* start site (Figure 1B). Using this strategy, two *lin-42(0)* alleles were isolated and confirmed by PCR and sequencing: *lin-42(ox460)* and *lin-42(ox461).* The two deletions appear to be identical, removing ~10.2kb of sequence and deleting the entire *lin-42* coding region and replacing it with *C. briggsae unc-119* (Figure 1B). The rest of this study will describe analysis of *lin-42(ox461),* abbreviated *lin-42(0)* (Figure 1A).

Heterochronic phenotypes of *lin-42(ox461)*

Similar to previously described mutants, *lin-42(0)* animals have a precocious heterochronic phenotype in which the seam cells terminally differentiate and form an adult cuticle with alae ridges one stage too early at the L3m (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006) (Figure

2A). In contrast, wild-type animals do not form alae until the L4m. The developmental timing defect of *lin-42(0)* animals is stronger than that of either *lin-42(n1089)* or *lin-42(ok2385)* mutants, with a larger proportion of animals forming complete alae at the L3m (Figure 2B,C). In our analyses, the vulval cell migrations that occur at the L3 to L4 transition in wild-type are also observed in *lin-42(0)* animals at the third molt, therefore, the vulva was used to stage these animals (Figure 2C).

Many precocious heterochronic mutants, such as *lin-28*, also show reductions in seam-cell number, due to skipping of the proliferative division in the L2-stage (Ambros and Horvitz, 1984;Moss et al., 1997). To determine whether *lin-42(0)* animals omit the L2-proliferative division, a hypodermal specific *Pelt-5::mcherry* integrated transgene was used to count seam cells in these mutants at the young-adult stage (Liu et al., 2009). As for previously described hypomorphic alleles, no difference in seam-cell number was observed in *lin-42(0)* animals compared to wild-type, and therefore these mutants do not skip the proliferative division in the seam (Tennessen et al., 2006) (Table 1). Taken together, *lin-42(0)* animals had a similar but more severe heterochronic defect than those with mutations in Lin-42a or Lin-42c, indicating that both regions contribute to regulating developmental timing in *C. elegans.*

lin-42(ox461) causes highly penetrant molting defects

The *lin-42(ok2385)* allele, which deletes the downstream region of *lin-42* has been reported to result in delayed and prolonged larval molts (Monsalve et al., 2011). This molting defect lengthens larval development compared to wild-type, and can make staging animals challenging. To understand the temporal dynamics of the molting cycle in *lin-42(0)* mutants, individual animals were monitored every 2 hours from hatching until adulthood for lethargus (Figure 3A). Lethargus is a characteristic behavior lasting approximate 2hrs at the end of each larval stage in which the worms cease pharyngeal pumping and movement as a new cuticle is synthesized prior to ecdysis.

Similar to *lin-42(ok2385)* animals, the first larval molt was delayed and lengthened in *lin-42(0)* mutants compared to wild type, but most animals completed this first ecdysis (Monsalve et al., 2011) (Figure 3A). However, the molting defect became more severe as animals progressed through larval development. Indeed, many animals failed to complete the second larval molt, and arrested development as small larvae (Figure 3B, C). Some animals failed to initiate lethargus, while others that did stop pumping failed to shed the L2-cuticle (Figure 3A). Additionally, the length of molts and larval stages of animals that did progress through development varied dramatically, resulting in marked asynchrony in the population.

Most *lin-42(0)* animals that bypass early larval arrest had executed the third larval molt after approximately 2.5 days of development. The fourth larval stage was the most delayed and asynchronous in these mutants (Data not shown). At least another full day following the third molt was required for these

animals to initiate the final molt, and those animals that do enter the fourth molt often fail to ecdyse, causing lethality due to internal hatching of progeny (Table 1).

To compare the molting defect of *lin-42(0)* animals to hypomorphic mutants, *lin-42* mutant animals were monitored over several days for development and scored for larval arrest (Figure 3C). Two *lin-42(lf)* mutants were analyzed in parallel with *lin-42(0)* animals: *lin-42(n1089),* which disrupts Lin-42b and Lin-42c, and *lin-42(ok2385*), which affects the Lin-42a and Lin-42b isoforms (Figure 1B). Consistent with previous analyses, all *lin-42(n1089)* larvae progressed to adulthood (Monsalve et al., 2011). In contrast, only 47% of *lin-42(ok2385)* animals became adults. These analyses suggested that the sequence deleted in *lin-42(n1089)* is not critical for promoting the molting cycle, as its deletion does not produce a strong phenotype. However, we found that the arrest phenotype of the null was more severe than that of *lin-42(ok2385)* mutants, where only ~25% of *lin-42(0)* animals become adults (p=0.003, Fisher's Exact Test). This result indicates that, while on its own, the upstream sequence of *lin-42* is not essential for regulating the molting cycle; Lin-42c and/or Lin-42b may contribute to regulation of this process.

The time necessary to reach adulthood was also assessed in *lin-42* mutant animals that escaped arrest. Here too, *lin-42(0)* mutants had the most severe delay, with the majority of animals requiring five days to become adults, compared to three days for wild type (Figure 3D). *lin-42(ok2385)* mutants had a less severe defect, and most of these animals became adults by four days.

Therefore, similar to *lin-42(ok2385), lin-42(0)* mutants have a molting defect where entry and exit from larval molts are delayed. However, *lin-42(0)* mutants have a more severe phenotype than *lin-42(ok2385)* animals, suggesting that Lin-42c and/or Lin-42b sequence contributes to the regulation of molting in *C. elegans.*

Structure-function analysis of *lin-42*

Previous studies revealed that over-expression of Lin-42a can rescue both *lin-42(n1089)* and *lin-42(ve11)* mutants, whereas over-expression of Lin-42c rescues only *lin-42(n1089)* animals (Tennessen et al., 2006) (Figure 2B). This result suggests that Lin-42a encodes the functional region of the protein, while Lin-42c, which encodes the PAS homology domains, may be more regulatory. Therefore, when LIN-42A is over-expressed, the other isoforms are not required for full function. We tested the abilities of these isoforms to rescue *lin-42(0)* heterochronic and molting phenotypes. The same over-expression arrays used in previous studies were crossed into *lin-42(0)* mutants and analyzed for heterochronic and molting phenotypes (Jeon et al., 1999;Tennessen et al., 2006). Transgenic expression of Lin-42a rescued both the heterochronic and molting defects of *lin-42(0)* animals; however over-expression of Lin-42c rescued neither phenotype (Table 1).

The *lin-42(0)* mutant provided the opportunity to investigate the function of Lin-42b in the absence of all endogenous isoforms (Figure 1A). A plasmid encoding the Lin-42b isoform with its native promoter was generated using cDNA

sequence to prevent independent expression of the Lin-42a and Lin-42c isoforms. A caveat to this approach is the potential loss of intergenic enhancers; however, this transgene rescued both the heterochronic and molting defects of *lin-42(0)* animals (Table 1). Taken together, these data indicate that both Lin-42a and Lin-42b can function in these pathways when over-expressed.

DISCUSSION

lin-42/per is a key member of the *C. elegans* heterochronic pathway, and mutations in this gene cause precocious defects, in which certain developmental programs are executed too early in development. However, study of *lin-42* function in the context of timing has been challenging due to the absence of a null mutation (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006). To aide in analysis of *lin-42* and its role in development, we generated a null allele which deleted the entire *lin-42* coding region using mosDEL technology (Frokjaer-Jensen et al., 2010).

Investigation of the heterochronic and molting phenotypes of *lin-42(0)* mutants revealed that they are similar, but more severe, than previously analyzed hypomorphs. Altogether, these data suggest that in the complete absence of *lin-42* activity, heterochronic and molting phenotypes are more compromised than when smaller regions of the locus are deleted. Indeed, that fact that *lin-42(0)* animals have more severe defects than mutations in either

upstream or downstream regions of the gene suggests that both of these sequences are important in the heterochronic and molting pathways.

Though this genetic analysis suggested that both upstream and downstream regions are critical for *lin-42* function, we observed that over-expression of Lin-42b or Lin-42a rescues the heterochronic and molting defects of *lin-42(0)* animals, while over-expression of Lin-42c did not. This result is similar to rescue analyses of mutants in which only the Lin-42a/b isoforms, such as *ve11* and *ok2385,* are disrupted (Monsalve et al., 2011;Tennessen et al., 2006). Although these data indicated that Lin-42a is the functional region of the gene, it is possible these results are due to over-expression. Mutations within the downstream sequence, such as *mg152,* do result in heterochronic defects, therefore, it likely that this region has some role in regulation of timing. One possibility is that PAS domains encoded by Lin-42c function in the localization of LIN-42 via protein interactions. However, if LIN-42A is present in excess, this localization function may not be required. Future experiments using single-copy insertions of individual isoforms in the null mutant will be informative in further understanding the contribution of specific isoforms to *lin-42* function. A key question is whether single-copy transformation of *lin-42(0)* animals with Lin-42a will strongly rescue this mutation or result in a similar phenotype to *lin-42(n1089)*.

Also of interest is determining in which tissues *lin-42* function is important for heterochronic and molting pathways. By immunofluorescence, LIN-42 protein appears to be widely expressed (Tennessen et al., 2006). The heterochronic function of *lin-42* is likely required in the hypodermis, as the *lin-42* timing defect

primarily affects this small sub-set of cells. However, the molting phenotypes of *lin-42* involve several aspects of this event, including behavior and ecdysis, therefore, it is possible *lin-42* is regulates molting from multiple tissues. Use of tissue-specific rescue constructs in the *lin-42(0)* background will be informative to understand from which tissues *lin-42* regulates developmental timing and molting.

With the heterochronic and molting phenotypes of *lin-42(0)* largely characterized, future work will also involve performing epistasis analyses with other members of the heterochronic pathway. In previous genetic analyses, only *lin-29*, the most terminal member of the heterochronic pathway, was epistatic to *lin-42* (Abrahante et al., 1998;Tennessen et al., 2006). A majority of *lin-42* genetic analyses with retarded heterochronic mutants resulted in mutual suppression; therefore, we were unable to conclusively place *lin-42* in the heterochronic pathway. However, in all of these analyses, we could not rule out the possibility that residual *lin-42* activity and/or expression could be altered. The use of an allele that completely removes *lin-42* activity will allow clearer interpretation of genetic interactions.

Figure 1: *lin-42(ox461)* deletes the *lin-42* coding region

A. Shown is the *lin-42* genomic locus and transcripts. The *lin-42* locus generates at least 3 transcripts: Lin-42a, Lin-42b, and Lin-42c. Lin-42a and Lin-42c are non-overlapping and expressed from distinct promoters. In all characterized *lin-42(lf)* alleles, one of these isoforms is left intact. *lin-42(ox461)* deletes the entire coding region and removes all *lin-42* isoforms. **B.** Generation of a *lin-42* null allele with MosDEL technology. The deletion template, pALB8, contains the *C. briggsae unc-119* rescuing fragment flanked by homology regions that border *lin-42*. Co-injection of pALB8 with a helper plasmid encoding Mos1 transposase causes excision of the *Mos1* element resulting in a double-strand break that is repaired by homologous recombination using the deletion template. The resulting mutation results in deletion of *lin-42* sequences replaced by *C. briggsae unc-119*. See Materials and Methods for details.

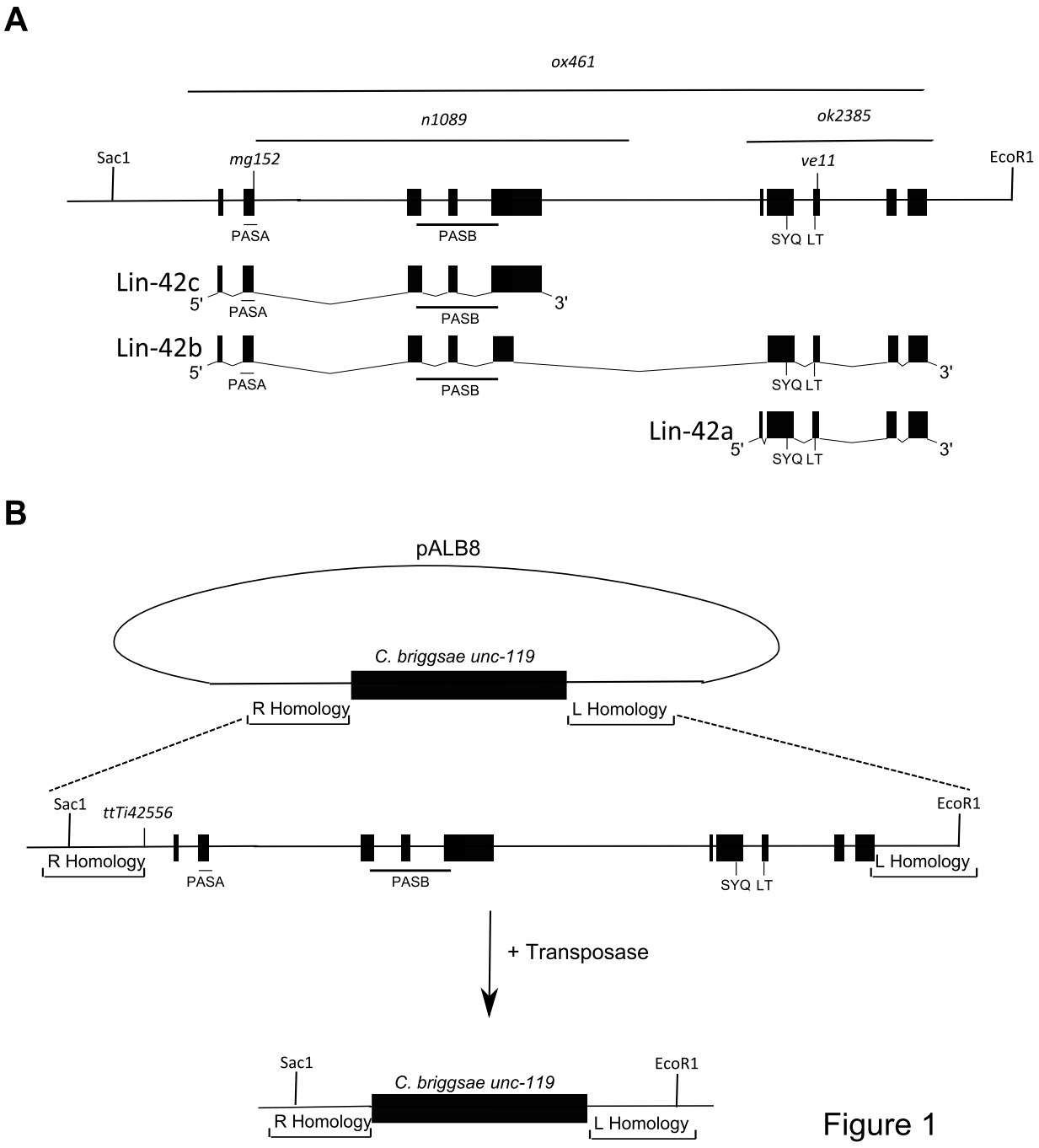

A
ox461
n1089
ok2385
Sac1
mg152
ve11
EcoR1
PASA
PASB
SYQ LT
Lin-42c
5'
3'
Lin-42b
Lin-42a
B
pALB8
C. briggsae unc-119
R Homology
L Homology
ttTi42556
+ Transposase

Figure 1

Figure 2: *lin-42(0)* mutants have a precocious heterochronic phenotype

A. *lin-42* mutations cause precocious seam cell development. Shown is a representative lineage diagram of a single seam cell in wild-type and *lin-42(0)* mutants. A horizontal line indicates a cell division, and triple horizontal bars indicate alae formation. Stages are relative, and the actual intermolt lengths of wild-type and *lin-42(0)* animals (in hours) are different. **B.** *lin-42(0)* mutants have a more severe heterochronic defect than *lin-42(lf)* animals**.** Wild-type, *lin-42(n1089), lin-42(ok2385),* and *lin-42(ox461)* animals were analyzed at the L3-molt for alae formation. Animals were score for either complete, partial (alae with gaps), or no alae **C.** *lin-42(0)* animals form precocious alae at the L3-molt stage. Shown are images of L3-molt cuticle of wild-type and *lin-42(0)* animals at the third molt taken at 60X magnification using DIC Normarski microscopy. An image of vulva is shown in the bottom panel to denote staging, indicated by an arrow. Alae are indicated by arrowheads.

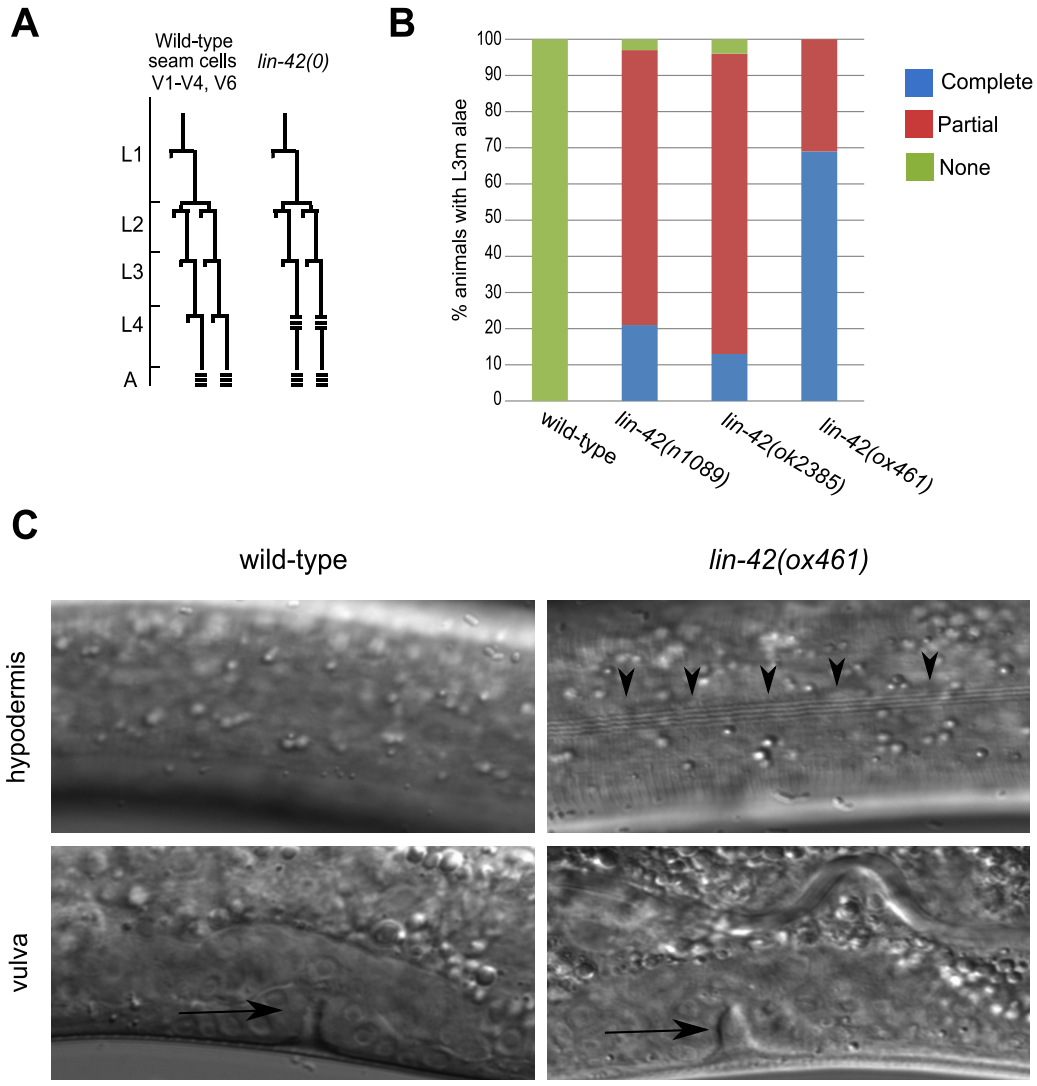

A
Wild-type
seam cells
V1-V4, V6
lin-42(0)
L1
L2
L3
L4
A
B
% animals with L3m alae
100
90
80
70
60
50
40
30
20
10
0
wild-type
lin-42(n1089)
lin-42(ok2385)
lin-42(ox461)
Complete
Partial
None
C
wild-type
lin-42(ox461)
hypodermis
vulva

Figure 2

Figure 3: *lin-42(0)* animals have delayed and lengthened larval molts

A. Initiation and duration of larval molts are delayed in *lin-42(0*) mutants. Individual animals were followed and monitored for pharyngeal pumping. The molts can be defined by a period of lethargus, in which they cease pharyngeal pumping and do not move. A vertical line indicates a shed cuticle was observed on the plate. Shown are representative animals. n=68 for *lin-42(ox461).* n=10 for wild-type. **B.** Larval arrest of *lin-42(0)* mutants. Micrographs of 4-day old wild-type and *lin-42(ox461)* animals are shown. A majority *of lin-42(0)* mutants become stuck in cuticles in the second larval molt. While wild-type animals and some *lin-42(0)* mutants have progressed to adulthood, these arrested animals remain small larvae (bottom right panel). Scale bar=5μm **C.** *lin-42(0)* mutants have a more severe larval arrest phenotype than *lin-42(ok2385)* animals. Wild-type, *lin-42(n1089), lin-42(ok2385),* and *lin-42(ox461)* mutants were individually plated and monitored for developmental progression. Shown are the percent animals arrested 7 days post-hatching. n≥ 100 **p≤0.01 Fisher's Exact Test **D.** The time of the larval-to-adult transition is delayed in *lin-42* mutants. Shown are the percent of animals from (C) that bypassed larval arrest and reached adulthood on a particular day post-hatching.

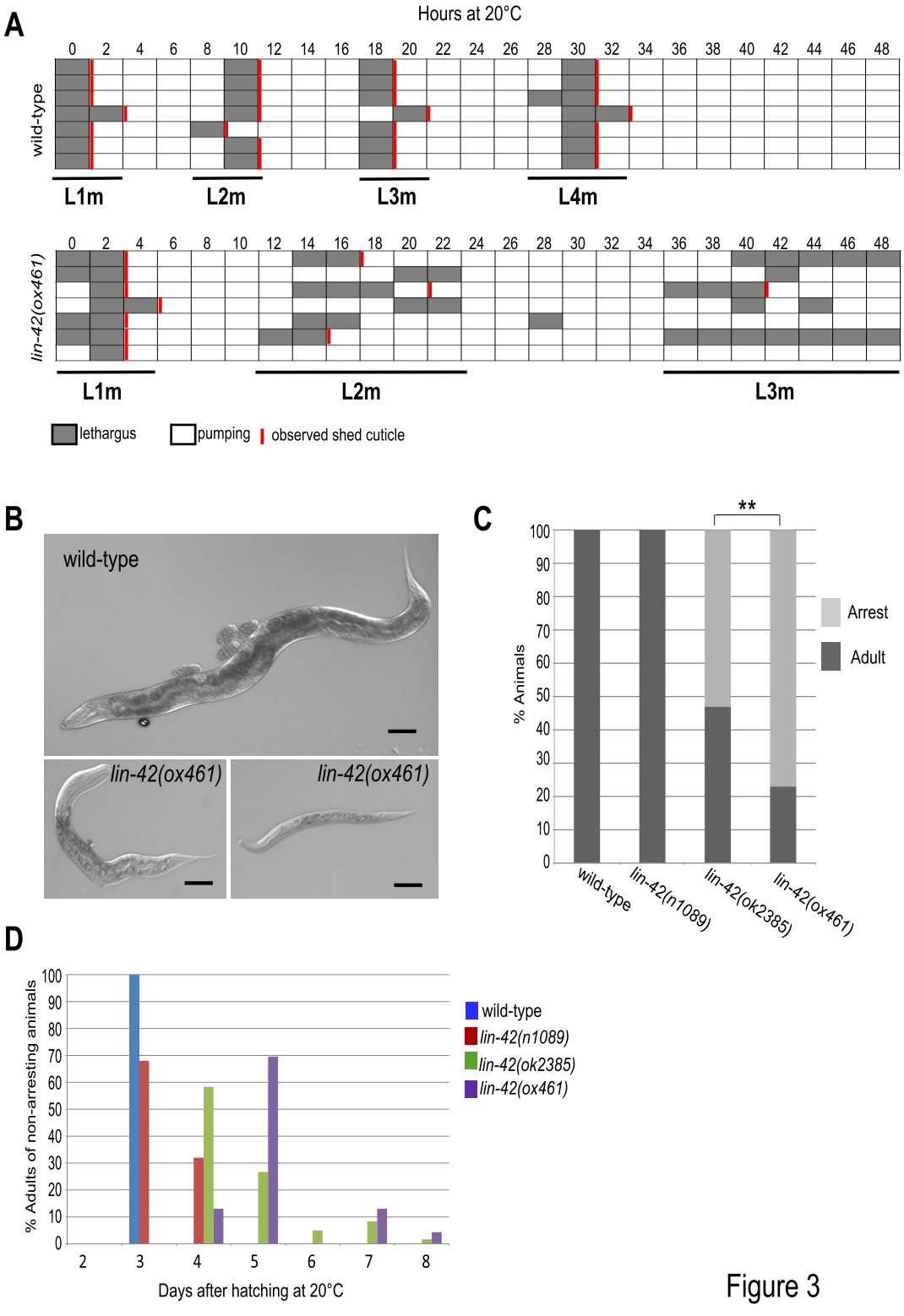

A
Hours at 20°C
wild-type
L1m
L2m
L3m
L4m
lin-42(ox461)
L1m
L2m
L3m
lethargus
pumping
observed shed cuticle
B
wild-type
lin-42(ox461)
lin-42(ox461)
C
**
% Animals
Arrest
Adult
wild-type
lin-42(n1089)
lin-42(ok2385)
lin-42(ox461)
D
% Adults of non-arresting animals
Days after hatching at 20°C
wild-type
lin-42(n1089)
lin-42(ok2385)
lin-42(ox461)

Figure 3

Table 1: Rescue of *lin-42* heterochronic and molting defects

Genotype	Animals with L3m alae (%)			Arrest (%) [c]	Adult Phenotypes	
	Complete	Partial	None		seam # [d]	Egl (%)
wild-type	0	0	100	0	15.7 ±0.75	0
lin-42(ox461)	69	31	0	77	16 ± 0.56 [a]	100
ox461; Ex[lin-42a]	0	28	72	1	nd	3
ox461; Ex[lin-42b]	0	4	96	16	nd	10
ox461; In[lin-42c] [b]	57	43	0	78	nd	100

n≥20

[a] p=0.1414 Welch's t-test

[b] n=14 for heterochronic defects

[c] % of animals raised from hatched eggs that arrested as young larvae

[d] Average # of seam cells in young adults as determined by *pelt-5::mcherry* expression

Chapter III: Modulation of developmental timing by environmental stress in *C. elegans*

SUMMARY

Under standard laboratory conditions, *C. elegans* progresses rapidly through four larval stages each separated by a molt prior to becoming a reproductively competent adult. The heterochronic genes are critical in regulating the temporal progression of development relative to each molt. However, *C. elegans* larval development is plastic, and both the molting and heterochronic pathways are responsive to environmental signals. Under extreme stress, such as severe crowding or high temperature (>30°C), the temporal and molting programs are suspended, and the worm enters a specialized dauer larval stage that is optimized for survival. Whereas dauer formation is a response to extreme environmental insults, the developmental and molting timers are remarkably robust under less severe stress. We show here that mutations in the heterochronic gene *lin-42* cause the heterochronic and molting programs to become sensitive to environmental conditions. Under mild temperature stress (25°C), both the heterochronic and molting defects of *lin-42* mutants raised at lower temperatures are suppressed. Strikingly, detailed genetic analysis shows that suppression of the heterochronic and molting phenotypes by high temperature is achieved through separate pathways. Additionally, we find that

genetic manipulation of stress response pathways, such and insulin and TGFβ signaling can also affect *lin-42* phenotypes, via a distinct pathway from temperature-induced suppression. Taken together, these results show that *lin-42* function is necessary to confer robustness on heterochronic and molting timers, and in its absence, these pathways are more sensitive to environmental conditions through several parallel pathways.

INTRODUCTION

Developmental programs are in general thought to be canalized, or very robust against mild environmental or genotypic variation (Waddington, 1942). Some traits, however, can differ between individuals in response to variable environmental conditions. This is known as phenotypic plasticity, or the ability to manifest two or more phenotypes in response to different environmental conditions (Schlichting and Pigliucci, 1993;West-Eberhard, 2003). A dramatic example of phenotypic plasticity is developmental diapause, in which animals can enter a highly stress resistant state in the face of adverse environmental conditions (Schiesari and O'Connor, 2013). Understanding the molecular basis of these events, or how environmental conditions regulate genetically encoded developmental programs, is a key question of ecology, evolution, and developmental biology. Also important is determining how the canalization of developmental programs under milder stress is achieved.

The nematode *C. elegans* has proven to be an exceptional model to study the impact of environment on development. Under favorable environmental conditions, the worm progresses through four precisely timed larval stages (L1-L4) to the adult in less than three days at 20°C. The developmental program of *C. elegans* is incredibly robust, and the timing of cell divisions relative to each molt are invariably executed over a broad range of temperatures, even though the length of intermolt periods lengthen as growth temperatures decrease (Byerly

et al., 1976). This reproducibility suggests that the pathways that regulate these programs are highly buffered against weak environmental insults.

One regulatory system that could contribute to this buffering is the heterochronic gene pathway, which controls the timing and sequence of stage specific cell divisions patterns relative to larval stages in *C. elegans* (for review, see (Resnick et al., 2010)). The temporal regulation of hypodermal seam cell divisions has been most extensively studied, and these cells undergo stage specific developmental events that serve as markers of timing (Figure 1A). At the final larval molt, the seam cells terminally differentiate and contribute to the formation of adult cuticle, which is characterized by the formation of lateral ridges called alae. Mutations in this pathway cause defects in the timing of seam cell development. In precocious heterochronic mutants, these cells skip earlier programs, causing terminal differentiation to occur too soon. Conversely, in retarded heterochronic mutants, early programs are reiterated, causing a delay of differentiation (Figure 1A).

Although in general the heterochronic pathway is fairly robust, when the worm is exposed to extreme environmental stress, i.e. severe crowding or starvation, in which further development would be disadvantageous*, C. elegans* can halt the developmental timer and enter dauer diapause, an example of phenotypic plasticity (Cassada and Russell, 1975). The molecular mechanisms that regulate dauer formation have been intensely studied (for review, see (Fielenbach and Antebi, 2008)). Mutants have been isolated that are either dauer formation constitutive (Daf-c) or defective (Daf-d), and investigation of

these mutants has identified many pathways that regulate dauer formation (Figure 1B). Briefly, environmental signals are primarily transduced by insulin and TGFβ signaling pathways. Insulin signaling regulates the activity of the transcription factor *daf-16/Foxo* (Ogg et al., 1997). The TGFβ pathway acts through *daf-5/Sno* and *daf-3/SMAD* (da Graca et al., 2004;Patterson et al., 1997). These downstream transcription factors are thought to regulate the expression of hormone biosynthesis genes, which control in the synthesis of dafachronic acids (DA) from cholesterol (Liu et al., 2004;McElwee et al., 2003;Murphy et al., 2003). DA regulate the activity of DAF-12, a nuclear hormone receptor (NHR) (Antebi et al., 1998;Antebi et al., 2000;Darlington et al., 1998;Nawathean and Rosbash, 2004;Phillips et al., 2007;Schackwitz et al., 1996).

daf-12 is required for dauer formation, and functions essentially as a molecular switch, promoting either dauer formation or continuous development, and the activity of DAF-12 is regulated through interactions in its ligand-binding domain (Antebi et al., 1998;Antebi et al., 2000) (Figure 1B). In favorable environments, DA are produced, and ligand-bound DAF-12 promotes continuous development. In unfavorable environments, insulin and TGFβ signaling decrease, resulting in reduced production of DA (Motola et al., 2006). Under these conditions, ligand-free DAF-12 to bind to a co-repressor, DIN-1, and this complex inhibits developmental progression and promotes dauer formation (Ludewig et al., 2004). In addition to regulating stress response, *daf-12* is also a member of the heterochronic pathway. Certain mutations in *daf-12* cause a

retarded heterochronic phenotype, and the strongest defects are observed in animals with mutations in the ligand-binding domain (Figure 1A). Null alleles of *daf-12* are Daf-d and have a weak heterochronic phenotype, due to the absence of both activator and repressor activity (Antebi et al., 2000;Motola et al., 2006). Therefore, *daf-12* is a key node that integrates stress response and developmental timing pathways.

lin-42 also functions in both the dauer formation and heterochronic pathways. *lin-42* encodes the *C. elegans* homolog of *period,* a component of the circadian timekeeper of *Drosophila* and mammals (Jeon et al., 1999). Circadian clocks are important for integrating environmental signals with development and cell cycle, and *lin-42* plays a similar role in *C. elegans* developmental timing (Bass and Takahashi, 2010;Gery and Koeffler, 2010). *lin-42(lf)* animals are Daf-c at 27°C, indicating that *lin-42* functions to prevent inappropriate dauer entry (Tennessen et al., 2010). Genetic analysis shows that *lin-42* acts in opposition to *daf-12* activity to regulate dauer formation, downstream of the insulin and TGFβ pathways. In addition to being Daf-c, *lin-42(lf)* animals also exhibit precocious heterochronic phenotypes. In these mutants the seam terminally differentiates one-stage too early, at the L3 molt (L3m) (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006) (Figure 1A). Finally, *lin-42* regulates also the molting cycle (Monsalve et al., 2011). The timing and duration of larval molts is delayed and lengthened in certain *lin-42(lf*) mutants compared to wild-type animals. These data indicate that *lin-42* is involved in multiple larval developmental pathways: stress response, developmental timing, and molting.

The *C. elegans* heterochronic pathway is very robust against environmental fluctuations. While extreme stress (i.e. crowding, temperatures > 30°C) causes arrest of the timer, moderate crowding was found not to affect developmental timing, even in several mutants where the pathway is compromised, such as *lin-4, lin-14*, and *lin-28* mutants (Liu and Ambros, 1989). Here we provide evidence that the heterochronic and molting phenotypes of *lin-42* mutants can be influenced by temperature. Reduced signaling from insulin and TGFβ pathways also suppress the *lin-42* heterochronic phenotypes by an independent pathway from temperature-induced effects. These data show that *lin-42* is a key factor that confers robustness on developmental timing and molting pathways.

MATERIALS AND METHODS

Nematode Maintenance and Strains

C. elegans were maintained as described previously (Shingleton et al., 2005). All strains were maintained at 20°C except for those containing *daf-2(e1370)* or *daf-7(e1372*), which were maintained at 15°C.

Other alleles used in this study are: *lin-42(n1089)*, *lin-42(mg152)*, *lin-42(ve11)*, *lin-42(ok2385), lin-42(ox461)*, *lin-28(ga54), daf-16(mu86), daf-3(mgDf90), daf-5(e1386), daf-12(rh61rh411), daf-2(e1370),* and *daf-7(e1372)*.

To construct strains containing *daf-16(mu86*) or *daf-5(e1386),* mutant alleles were followed by PCR as described (Tennessen et al., 2010). The small

deficiency *mgDf90* deletes the entire *daf-3* locus (Patterson et al., 1997). PCR was used to follow the *daf-3(mgDf90)* deletion. *lin-42(ve11); lin-28(ga54)* double mutants were made by crossing *lin-28(ga54)* hermaphrodites with *lin-42(ve11)* heterozygous males carrying a rescuing extrachromosomal array (*veEx293*) that rescues the egg-laying defects of *lin-42.* The array was selected against when double mutants were isolated. *lin-28(ga54)* was isolated by following the protruding vulva phenotype, and *lin-42(ve11)* disrupts a DraIII site in the locus. Primers used to amplify this region were: DB28/DB30(5'GTTTCGCCAAAAATAAGCG3'/5'CCAGATCAGGAAAGTTGCCAGC3').

RNAi knockdown of *lin-42* was performed as described (Tennessen et al., 2006).

Analysis of heterochronic phenotypes

Animals were raised from hatched L1s or eggs on NGM plates seeded with OP50 bacteria at the indicated temperature. Animals were visualized on a Zeiss Axiovision Microscope using Nomarski DIC optics. *lin-42(ox461)* animals are difficult to stage due to the timing defects in multiple tissues as well as molting defects (Monsalve et al., 2011;Tennessen et al., 2006) (Chapter II). However, these mutants typically enter the third molt after 56 hrs post hatching and can be staged using vulval development as a marker (unpublished observations). It should be noted that *lin-42* mutations are Synthetic for Dauer formation (SynDaf) with TGFβ and insulin signaling pathway mutants (Tennessen

et al., 2010). Only animals that did not arrest were scored for heterochronic phenotypes. *lin-42(0); daf-7(lf)* double mutants either formed dauers or entered *lin-42* larval arrest at 20°C, therefore, these animals were analyzed at 15°C. Statistical analyses were performed using R (ver. 2.10.1).

Analysis of larval arrest

To quantify larval arrest, eggs were picked onto medium plates and allowed to hatch at 20°C or 25°C as appropriate. Hatched L1s were then individually plated and monitored for developmental progression. At 20°C, *lin-42(ox461)* animals can take 4-6 days to become adults. At 25°C, they takes about 3-5 days. Animals that did not reach adulthood by the correct time were classified as arrested.

Western Blot Analysis

Quantitative western blotting was performed as follows. Eggs were obtained by hypochlorite treatment and allowed to hatch overnight in M9 starvation buffer. Arrested L1s were transferred to 10cm NGM plates seeded with OP50 bacteria. For collection, worms were washed off plates with M9, washed three times, and worm pellets were then transferred to a 1.5ml eppendorf tube. A volume of lysis buffer (50mM HEPES pH7.6, 140mM KCl, 1.0% Triton, 10% glycerol, 5mM DTT, 1.5X protease inhibitors) equal to that of the compact worm pellet was added, and tubes were flash frozen in liquid nitrogen and stored at -80°C overnight.

To prepare samples for SDS-PAGE, an equal volume of 2X SDS-sample buffer was added to the worm pellet. The sample was then boiled for five minutes, vortexed for three minutes, and then boiled for an additional five minutes. The samples were then spun in a table-top centrifuge for 10 minutes at maximum speed to clear out debris.

SDS-PAGE and transfer were performed using BioRad Mini-PROTEAN electrophoresis and transfer system (Bio-Rad Laboratories). Blots were simultaneously probed with rabbit anti-LIN-28 (a gift of E. Moss) at 1:2000 and mouse anti-actin (MP Biomedicals) at 1:10,000. Fluorescently conjugated secondary antibodies were used for quantitative blotting. A 1:2500 dilution each of anti-rabbit 680 (Life Technologies) and anti-mouse 800 (Rockland) were used against the LIN-28 and actin antibodies respectively. Blots were analyzed on an Odyssey Li-Cor system and Odyssey software was used to obtain integrated fluorescent intensity values for the LIN-28 bands and actin bands. Further analysis was performed using Microsoft Excel and R.

RESULTS

lin-42 prevents temperature from affecting seam cell developmental programs

One hallmark defect of *lin-42* mutants is the formation of an adult cuticle at the L3m, rather than at the larval-to-adult transition (Figure 1A). This phenotype is partially suppressed when animals are raised in mildly stressful, but not dauer-

inducing, environmental conditions, such as moderate crowding or temperatures ≥ 25°C (Abrahante et al., 1998). To characterize this effect in more detail, heterochronic phenotypes were analyzed in several *lin-42* reduction-of-function (*rf)* mutants when raised at 20°C or 25°C: *mg152*, *n1089*, *ok2385, ve11*, and also in animals treated with *lin-42* RNAi. The *lin-42* locus produces multiple isoforms, two of which are non-overlapping (Figure 1C). The *n1089* and *mg152* alleles disrupt Lin-42b and Lin-42c, while *ve11* and *ok2385* disrupt Lin-42a and Lin-42b. In addition to these *rf* backgrounds, we also analyzed a recently generated *lin-42* null [*lin-42(0)*] mutant, *lin-42(ox461),* that was generated using mosDEL technology (Frokjaer-Jensen et al., 2010) (Figure 1C, Chapter II). *ox461* deletes the entire *lin-42* coding region and all isoforms.

All *lin-42(rf)* mutants analyzed had significantly less severe heterochronic defects at 25°C compared to 20°C, indicating there is reduced requirement for *lin-42* at 25°C ($p<0.0001$, Fisher's Exact Test, Figure 2). In contrast to *lin-42(rf)* mutants, the heterochronic phenotypes of *lin-42(0)* and *lin-42(RNAi)* animals were not affected by temperature (Figure 2). Therefore, the temperature suppression of the *lin-42* precocious alae phenotype requires residual *lin-42* activity. The fact that multiple *lin-42(rf)* alleles, including point mutations and large deletions that disrupt different isoforms, are affected by temperature indicates that this is not a classical temperature sensitive effect, where protein structure and activity are altered. Rather, temperature and residual *lin-42* activity function in concert to achieve this suppression. Also striking is the observation that alleles that leave either Lin-42a (*mg152* and *n1089*) or Lin-42c (*ve11* and

ok2385) intact are affected by temperature. The proteins encoded by these isoforms are unrelated in sequence, and yet each is sufficient to suppress *lin-42(rf)* phenotypes at 25°C.

We also tested whether this temperature effect was a feature of other precocious mutants or specific to *lin-42.* We examined the precocious phenotypes of *hbl-1(lf)* and *lin-41(lf)* mutants raised at 20°C or 25°C (Abrahante et al., 2003;Slack et al., 2000). No significant difference in heterochronic phenotypes was observed in these mutants when raised at different temperatures ($p>0.05$, Table S1).

Neither insulin nor TGFβ signaling are required for temperature-induced changes in developmental timing

One possible mechanism through which temperature may regulate developmental timing is via the signaling pathways that regulate stress response. *C. elegans* form stress-resistant dauer larvae in response to harsh environmental conditions, and insulin and TGFβ signaling are required to transduce the environmental signals that regulate this process (Fielenbach and Antebi, 2008). Harsh environments, such as starvation, extreme crowding, or high temperature, cause the signaling from these pathways to decrease, which relieves repression of downstream transcription factors that promote stress response (Figure 1B). Daf-d mutations in the downstream transcription factors of the insulin and TGFβ pathways were used to

determine whether either of these pathways is involved in transducing the temperature signal that affects *lin-42(rf)* phenotypes.

daf-16/Foxo is the downstream output of the insulin signaling pathway (Ogg et al., 1997). Animals that are mutant for *daf-16* cannot respond to changes in insulin signaling, and are therefore Daf-d. Double mutant analysis revealed that *daf-16* is not required for the temperature suppression of *lin-42*, as the heterochronic phenotype of *lin-42(rf); daf-16(0)* double mutants was suppressed by high temperature, similar to *lin-42(rf)* on its own (p=0.006, Fisher's Exact Test, Figure 3A).

daf-3/Smad and *daf-5/*Sno/Ski are downstream transcription factors of the TGFβ signaling pathway, and animals with mutations in these genes are Daf-d because they cannot respond to changes in TGFβ signaling (da Graca et al., 2004;Patterson et al., 1997). We observed that the heterochronic defects of both *lin-42(rf) daf-5* and *lin-42(rf); daf-3* mutants were suppressed at 25°, indicating that neither *daf-3* nor *daf-5* are required for this response (p≤0.0001, Table S1).

Although TGFβ and insulin signaling are not individually required for the temperature suppression of *lin-42(rf)* mutants*,* there is cross-talk between these pathways, and it is possible they may function redundantly in this process (Lee et al., 2001;Liu et al., 2004;Shaw et al., 2007). However, the precocious alae defect of *lin-42(rf) daf-5(lf); daf-16(0)* triple mutants was significantly suppressed at 25°C compared to 20°C (p=0.0006, Figure 3A).

These genetic data show that the insulin and TGFβ pathways are not required to transduce the temperature stress signals that suppress *lin-42(rf)* heterochronic defects.

daf-12 is required for temperature suppression of *lin-42(rf)*

Insulin and TGFβ signaling pathways regulate dauer formation by modulating the activity of DAF-12/NHR via DA (Motola et al., 2006) (Figure 1B). In favorable environments, insulin and TGFβ signaling promote dafachronic acid synthesis, and these molecules bind to DAF-12, directing developmental progression. In stressful environments, signaling output from insulin and TGFβ pathways decreases, resulting in reduced levels of hormones. In the absence of dafachronic acid, DAF-12 binds to the co-repressor DIN-1, and the repressor complex promotes dauer formation (Ludewig et al., 2004). Although insulin and TGFβ signaling are not required for the temperature suppression of *lin-42(rf),* it remained possible that *daf-12* activity was involved. As previously reported, a *daf-12(0)* mutation partially suppresses *lin-42* heterochronic phenotypes at 20°C (Tennessen et al., 2006) (Table S1). If *daf-12* is not necessary for temperature sensitivity of *lin-42*, *lin-42(rf); daf-12(0)* double mutants should be further suppressed for precocious heterochronic defects by growth at 25°C. However, we found that the percent of *lin-42(rf); daf-12(0)* animals with L3m alae was similar at both 20°C and

25°C. (p=0.86, Figure 3B). This result indicates that *daf-12* activity is required to transduce the temperature signal that modulates *lin-42(rf)* phenotypes.

Together, these analyses indicate that temperature suppression of *lin-42(rf)* precocious phenotypes is independent from the insulin and TGFβ signaling pathways, but is dependent upon *daf-12* activity. Interestingly, this regulatory relationship contrasts with the dauer formation pathway, where *daf-16, daf-5/3*, and *daf-12* activities are all required for dauer entry (da Graca et al., 2004;Ogg et al., 1997;Patterson et al., 1997).

lin-42 antagonizes *daf-12*-dependent changes in LIN-28 levels in response to temperature

The requirement for *daf-12* in the temperature sensitivity of *lin-42(rf)* hypodermal phenotypes prompted us to look to *daf-12* and its functions in the heterochronic pathway for other genes involved in temperature suppression of *lin-42(rf)* (Antebi et al., 1998;Antebi et al., 2000). *daf-12* mutations result in retarded heterochronic phenotypes, in which the proliferative L2 division is repeated (Figure 1A). Given that DAF-12 activity is modulated by environmental signals, it is possible that changing temperatures may alter DAF-12 activity, affecting the expression of its targets in the heterochronic pathway, and resulting in suppression of *lin-42* precocious alae formation. One gene in the heterochronic pathway known to be regulated by *daf-12* is *lin-28* (Moss et al., 1997). Similar to *lin-42* mutants, mutation of *lin-28* results

in precocious alae formation, and it is possible that they could play parallel roles in the pathway (Figure 1A). In *daf-12* mutants, LIN-28 protein levels are elevated and are not down-regulated appropriately in mid-larval stages (Morita and Han, 2006;Seggerson et al., 2002). Additionally, *lin-28(0)* mutants are epistatic to *daf-12(lf)*, suggesting that *lin-28* acts downstream of *daf-12* (Antebi et al., 1998). Taken together, one model for the temperature suppression of *lin-42(rf)* is that LIN-28 levels are up-regulated at high temperature by modulation of DAF-12 activity. If *lin-28* acts in parallel with *lin-42*, then this change in LIN-28 levels may compensate for reduced *lin-42* function.

We first tested genetically whether *lin-28* and *lin-42* act in parallel to regulate developmental timing. *lin-28(0)* animals form precocious alae at the L3m, but also at the L2 molt (L2m) at low frequency, however *lin-42(rf)* mutants do not (Abrahante et al., 1998;Ambros and Horvitz, 1984). Analysis of *lin-28(0); lin-42(rf)* double mutants revealed enhancement of the *lin-28* precocious heterochronic defect ($p=0.003$, Fisher's Exact Test, Figure 4). Therefore, *lin-42* and *lin-28* function in parallel to regulate developmental timing in the hypodermis.

daf-12 activity is necessary for suppression of *lin-42* precocious phenotypes at 25°C. If the requirement for *daf-12* is achieved through up-regulation of *lin-28*, then *lin-28* should also be necessary for suppression of *lin-42* at high temperatures. Indeed, *lin-28(0); lin-42(rf)* double mutants were

not significantly different in the extent of alae formation at 20°C vs. 25°C (p=0.2, Figure 4). A similar result was observed for *lin-28(0)* animals, showing that, like *hbl-1* and *lin-41*, *lin-28* phenotypes alone are not affected by temperature (p=0.65, Figure 4). These results demonstrate that, similar to *daf-12, lin-28* is necessary for the temperature suppression of *lin-42*.

A prediction of our model is that LIN-28 levels increase at high temperatures in a *daf-12*-dependent manner. To test this, quantitative western blotting was performed to measure LIN-28 levels in animals raised at 20°C compared to 25°C (see Materials and Methods). First, a timecourse was performed extending from mid-L1 to the late-L2 stage to confirm previously published results that LIN-28 protein is highly expressed in the L1-stage and decreases during the L2 stage (Seggerson et al., 2002) (Figure 6A). Samples were then collected from wild-type animals raised at 20°C and 25°C to the mid-L2 stage and LIN-28 protein levels were measured. *C. elegans* develop faster when raised at 25°C than at 20°C (Byerly et al., 1976). However, approximate developmental stage can be estimated using the timing of molts to mark the beginning and end of each stage. The mid-larval stage is estimated by calculating the median time point between molts. Using this method, the wild-type mid-L2 stage occurs at approximately 24 hrs at 20°C and 16hrs at 25°C starting from synchronized L1 larvae.

A small, but reproducible (over 6 biological replicates) and statistically significant increase in LIN-28 protein levels was measured in animals raised

at 25°C (Figure 6B,C). To test whether this increase was *daf-12* dependent, this experiment was repeated with *daf-12(0)* mutants. No significant difference in LIN-28 protein levels was observed between the two temperatures in these samples (Figure 6D).

Taken together, these results indicate that *lin-42* and *lin-28* act in parallel to regulate developmental timing, and that *lin-28* is necessary for the temperature suppression of *lin-42(rf)* phenotypes. We also show that LIN-28 levels increase at 25°C in a *daf-12* dependent manner. These data are consistent with a model wherein reduced *lin-42* function is compensated for by increased LIN-28 levels at elevated temperatures.

Insulin and TGFβ signaling can affect developmental timing pathways

Changing ambient temperature by just 5°C has a strong effect on the heterochronic phenotype of *lin-42(rf)* mutants. However, starvation and crowding are also potent environmental stressors and induce dauer formation regardless of temperature (Cassada and Russell, 1975). As previously described, insulin and TGFβ signaling pathways are important transducers of environmental signals and are critical in regulating dauer formation. Although we found that these pathways are dispensable for modulation of developmental timing by temperature stress, we wanted to determine if other

types of stress (i.e. crowding), which may be transduced by these pathways, can suppress *lin-42(lf)* heterochronic phenotypes.

Genetic disruption of upstream insulin and TGFβ signaling pathway components was used to mimic environmental stress. Raising Daf-c mutants of *daf-2*/InR and *daf-7*/TGFβ at semi-permissive temperature (20°C) results in reduction of the signaling output of these pathways and activation of stress response without inducing dauer formation (Kimura et al., 1997;Ren et al., 1996). Double mutants between *daf-2(lf)* or *daf-7(lf)* and *lin-42* alleles were generated to test whether these mutations would affect the *lin-42* phenotype at 20°C.

We found that all *lin-42* mutants analyzed, including the null, were suppressed by reduced insulin signaling ($p<0.0001$, Fisher's Exact Test, Figure 6). Additionally, *daf-16,* which is necessary to transduce changes in insulin signaling, is required for this suppression as *daf-2(lf); lin-42(rf); daf-16(0)* animals were not suppressed for heterochronic defects (Figure 6).

Decreased TGFβ signaling caused by a *daf-7(lf)* mutation also suppressed the heterochronic defects of all *lin-42* mutants tested ($p<0.0001$, Figure 6). The downstream effector of TGFβ signaling, *daf-5*, is required for the suppression of *lin-42* by *daf-7*, as *lin-42(ve11) daf-5; daf-7* mutants had a similar phenotype to *lin-42(ve11)* on its own (Figure 6).

In dauer formation, *daf-12* is required to promote the dauer decision when insulin and TGFβ signaling decrease (Thomas et al., 1993;Vowels and

Thomas, 1992). *daf-12* is downstream of these signaling pathways, as Daf-d *daf-12* mutations are epistatic to *daf-2* and *daf-7* mutants, which are Daf-c. To determine whether *daf-12* contributed to regulation of developmental timing by insulin and TGFβ pathways, *lin-42* activity was knocked down by RNAi in both *daf-7(lf); daf-12(0)* and *daf-2(lf); daf-12(0)* mutants and these animals were scored for heterochronic phenotypes at the L3m. Surprisingly, these animals were suppressed compared to *daf-12(0); lin-42(RNAi)* mutants (Figure 6). Therefore, unlike in the dauer formation pathways, *daf-12* activity is not required for suppression of *lin-42(lf)* heterochronic defects via the insulin and TGFβ signaling pathways.

In summary, our data show that temperature affects the heterochronic pathway through steroid hormone signaling, independently of insulin and TGFβ signaling. However, other types of stress, mimicked by genetic reduction of the output from insulin and TGFβ signaling, also suppress *lin-42* phenotypes, and they do so independently of *daf-12*. These findings indicate that there are multiple independent environmental signaling pathways that can suppress *lin-42(lf)* phenotypes. Another intriguing observation is that, unlike for temperature suppression, *lin-42(0)* mutants can be strongly suppressed by reduced insulin and TGFβ signaling. These results show that suppression of *lin-42* heterochronic defects by reduced insulin or TGFβ signaling does not require any residual *lin-42* function, further suggesting a distinct mechanism of suppression than that for temperature.

The larval molting program is temperature sensitive in *lin-42* mutants

Another striking phenotype of some *lin-42* mutants is a severe molting defect often resulting in larval arrest (Monsalve et al., 2011). A majority of *lin-42(0)* mutants fail to complete larval development, often arresting at the L1m to L2 stage, and becoming trapped in their cuticles. These animals can survive for several weeks as small larvae, but never become reproductively competent adults (Chapter II). *lin-42(rf)* alleles that disrupt the Lin-42a isoform, such as *lin-42(ok2385),* also display molting defects, however, they are much less severe than in the null (Figure 1C, 7A). *lin-42(n1089)* mutants, in which Lin-42c is deleted, do not have a strong molting defect.

Temperature stress also affects the *lin-42* molting phenotype. Raising *lin-42(0)* animals at 25°C rather than 20°C strongly suppressed this larval arrest phenotype ($p<.0001$, Fisher's Exact Test, Figure 7A). Surprisingly, the molting phenotype of *lin-42(ok2385)* animals was not significantly affected by high temperature ($p=0.08$, Figure 7A). Taken together, these results suggest that mild temperature stress suppresses the heterochronic phenotype of *lin-42(rf)* mutants and the molting phenotype of *lin-42(0)* mutants. Furthermore, the suppression of these distinct phenotypes is achieved through independent pathways. Modulation of *lin-42* heterochronic phenotypes by temperature

requires residual *lin-42* activity; while conversely, suppression of molting defects of *lin-42* is only observed when all *lin-42* isoforms are absent.

Double mutants between *lin-42(0)* and Daf-d mutations in insulin, TGFβ, and steroid hormone signaling pathways were analyzed to determine which, if any of these pathways are required for the temperature suppression of the *lin-42* molting phenotype. Similar to suppression of heterochronic phenotypes, neither *daf-16* nor *daf-5* was necessary for temperature suppression of *lin-42(0)* molting defects (Figure 7B). Interestingly, although the suppression of *lin-42* heterochronic phenotypes at 25°C requires *daf-12*, the suppression of larval arrest does not. The molting defect of *lin-42(0); daf-12(0)* double mutants was less severe at 25°C compared to 20°C (Figure 7B). This result further supports the idea that the pathways mediating temperature sensitivity of the heterochronic defects and molting defects observed in *lin-42* animals are distinct.

The effect of Daf-c mutations in TGFβ and insulin pathway components on the molting defects at 20°C in *lin-42* mutants was also tested. *daf-7(lf); lin-42(0)* double mutant animals were analyzed to determine whether reduced TGFβ signaling alters the *lin-42* molting phenotype. The proportion of animals that arrested in these double mutants was similar to that of *lin-42(0)* alone (Figure 7C). It is likely, therefore, that reductions in TGFβ signaling have little effect on the molting defect of *lin-42(0)* mutants.

We next analyzed *daf-2(lf); lin-42(0)* double mutants to test whether decreased insulin signaling could affect *lin-42(0)* larval arrest. Interestingly, we found that reduction in insulin signaling mildly suppresses the larval arrest phenotype of *lin-42(0)* (p=0.005 Fisher's Exact Test, Figure 7C). This suppression acts through *daf-16/FOXO*, as *lin-42(0); daf-2(lf); daf-16(0)* triple mutants are not significantly different from *lin-42(0)* animals alone (p=0.27, Figure 7D). Therefore, although reductions in signaling from either insulin or TGFβ pathways can modulate the heterochronic defects of *lin-42* mutants, only insulin signaling seems to have any significant effect on the molting phenotype of the *lin-42(0)* mutation. This also suggests that insulin signaling may be involved in regulating the molting cycle of *C. elegans*.

DISCUSSION

lin-42(rf) reveals temperature regulation of developmental timing

In this study, we describe temperature sensitivity of developmental timing pathways in *C. elegans*. However, these effects are only observed at the phenotypic level in a sensitized genetic background in which *lin-42* function is compromised, suggesting that *lin-42* activity is important in buffering these pathways against variable environmental conditions. When raised at 25°C, rather than the standard laboratory condition of 20°C, *lin-42(rf)* precocious phenotypes in the hypodermis are much less penetrant, revealing environmental

modulation of these phenotypes. Amongst precocious mutants, this effect is specific to *lin-42*, since phenotypes of other precocious mutants, such as *hbl-1, lin-41* and *lin-28*, were not affected by temperature. Amongst the retarded mutants, only *lin-46* has been reported to be affected by temperature. *lin-46* encodes a gephyrin-like protein which may function as a scaffold to promote assembly of large multi-protein complexes (Pepper et al., 2004). *lin-46* is suppressed at 20°C compared to 15°C, and it is proposed that there is a greater requirement for *lin-46* in this condition to compensate for inefficient assembly of protein complexes at colder temperatures.

Of the key signaling pathways that are required for interpretation of environmental signals in the worm (i.e. TGFβ, Insulin, and hormone signaling, see Figure 1B), only hormone signaling appears required for temperature suppression of *lin-42(rf).* This result is intriguing, because both *daf-5/daf-3* and *daf-16* are necessary for dauer formation, and are thought to act upstream of *daf-12* in regulating stress response (Gerisch and Antebi, 2004;Motola et al., 2006;Thomas et al., 1993;Vowels and Thomas, 1992). Therefore, the pathways that mediate dauer formation are not the same as those that modulate developmental timing in response to environment. Moreover, TGFβ signaling in particular is important for transducing temperature signals via the ASI neuron in dauer formation, as DAF-7::GFP is down-regulated in this neuron at high temperatures (Ren et al., 1996;Schackwitz et al., 1996). However, this pathway appears to be dispensable for the temperature suppression of *lin-42*. It is possible that there is a separate signal generated by the ASI that is transduced

by *daf-12*, or that other neurons implicated in detecting temperature are involved in this response, such as AFD (Mori and Ohshima, 1995). Indeed, mutations in *tax-2* or *tax-4*, which encode a cGMP-gated ion channel critical for AFD function, are mildly Daf-c, indicating that this neuron does interface with dauer-formation (Coburn and Bargmann, 1996;Komatsu et al., 1996).

The nuclear hormone receptor *daf-12* is required for the temperature suppression of *lin-42(rf)* heterochronic phenotypes, and our data suggest it acts by up-regulating levels of LIN-28 at higher temperatures, compensating for reduced *lin-42* activity (Figure 8). How does *lin-28* compensate molecularly for loss of *lin-42*? Intriguingly, both of these genes function in miRNA regulation. *lin-28* inhibits late larval programs by blocking processing of let-7 miRNA, which is critical for promoting the larval to adult transition (Reinhart et al., 2000;Vadla et al., 2012;Van Wynsberghe et al., 2011). In *lin-28* mutants, let-7 miRNA accumulates too early, causing precocious phenotypes. *lin-42* also regulates *let-7* expression, however it likely acts at the level of transcription, as *pri-let-7* levels increase in *lin-42(lf)* animals (Chapter IV). It is possible, then, that elevated LIN-28 levels at high temperature block the processing of excess transcript caused by reduced *lin-42* activity, preventing over-expression of let-7 miRNA. The fact that the heterochronic defects of *lin-42(0)* mutants cannot be suppressed at high temperatures is surprising. One possible explanation for this result is that *let-7* transcript levels *in lin-42(0)* animals are more elevated than in *lin-42(rf)* animals, and may overwhelm LIN-28 activity. A second possibility is that some residual *lin-42* function is necessary regulation of miRNA processing by *lin-28.*

Our genetic data indicate that both LIN-42A and LIN-42C isoforms can function in the temperature suppression of *lin-42(rf)* phenotypes.

The Lin-42c isoform encodes the most highly conserved domain in the protein, PAS protein-protein interaction domains. In the circadian clock, protein interactions via PAS domains are critical for regulating the localization and stability of PERIOD (Huang et al., 1993;Vosshall et al., 1994). The function of the PAS domain in LIN-42 is not understood, however, LIN-42C and LIN-42B can dimerize *in vitro* (Gardner, 2005), Less is understood about the C-terminal sequence of PERIOD, which also contains small region of homology with LIN-42A. However, there is evidence to suggest that this domain is critical for interactions with the transcription factors CLOCK and CYCLE in *Drosophila* (Chang and Reppert, 2003;Sun et al., 2010). Although these data from analysis of *Drosophila* PERIOD suggest specific functions for these conserved domains, this may not be the case for LIN-42.

In summary, *lin-42/per* function is important for buffering the heterochronic pathway against variable temperatures that affect DAF-12/NHR activity. In other systems, including mammals, Circadian clocks are important for integrating environmental conditions, such as temperature and nutrition, with gene expression and physiology, and several NHRs are known to modulate circadian timekeeping (Reviewed in (Bass and Takahashi, 2010)). For example, the NHRs REV-ERBα and PPARα, which are involved in lipid metabolism, directly interact with PER2 in mice to integrate metabolic signaling with circadian clocks (Schmutz et al., 2010). Therefore, interactions between *period*-like genes and

nhrs may be a conserved mechanism in buffering developmental and cellular programs in variable environments.

Insulin and TGFβ signaling affect developmental timing pathways

Although insulin and TGFβ signaling are not involved in temperature suppression of *lin-42*, we found that reductions in output from either of these pathways also suppress the *lin-42* heterochronic phenotype at 20°C. Therefore, insulin and TGFβ signaling may transduce other environmental inputs that can affect developmental timing. Importantly, unlike temperature, effects on developmental timing via these pathways do not require residual *lin-42* or *daf-12* function. This indicates that suppression by reduced insulin or TGFβ signaling is independent of temperature induced suppression, and that, unlike in dauer formation; *daf-12* is not downstream of these pathways for stress suppression of *lin-42(lf).*

The heterochronic phenotype of *sea-2(lf)* has also been reported to be affected by decreased insulin signaling (Huang et al., 2011). *sea-2* encodes a zinc-finger RNA-binding protein that, when mutated, results in a weak retarded heterochronic phenotype. Genetic experiments indicate that, similar to *daf-12, sea-2* inhibits *lin-28* expression. Although temperature modulation of *sea-2* phenotypes was not reported, *daf-2(lf)* was found to enhance the retarded defect of *sea-2(lf)* mutants. Thus, both *lin-42* and *sea-2* mutants are sensitized to changes in insulin signaling.

How does insulin signaling affect developmental timing pathways? One candidate target is the miRNA lin-4. There is some evidence to suggest that *lin-4* expression is regulated by reduced insulin signaling during starvation-induced L1-arrest (Baugh and Sternberg, 2006). Expression of a *Plin-4::gfp* reporter is inhibited by L1-arrest, and this reporter was mildly de-repressed in *daf-16* mutants. One possibility is that reductions in insulin signaling during larval development may repress lin-4 miRNA expression in opposition to the precocious phenotype of *lin-42*. Arguing against this model, however, is that *lin-28* itself is down-regulated by lin-4 miRNA, but the *lin-28::gfp* reporter is not affected in *daf-2(lf)* mutants (Huang et al., 2011). Therefore, it is likely that insulin-signaling regulates a different member of the pathway.

lin-42(0) molting defect is suppressed by environmental stress

lin-42 mutants with disruptions in the Lin-42a isoform, in addition to heterochronic phenotypes, have a severe molting defect. Interestingly, although high temperature has no affect on *lin-42(0)* heterochronic phenotypes, it clearly suppresses the molting defect of these mutants. In contrast with the null, *lin-42(ok2385)* mutants were not suppressed for molting at high temperature. One possible explanation for this result is that the LIN-42C isoform may interfere with suppression of molting defects, and that *lin-42(lf)* molting phenotypes are only affected by temperature in its absence. This is supported by the fact that the percentage of *lin-42(ox461)* animals that arrest

at 25°C is similar to the proportion of *lin-42(ok2385)* animals that arrest at both temperatures.

We also found that *daf-16*, *daf-5,* and *daf-12*, are not required suppression of *lin-42(0)* molting defects by high temperature. This is in contrast to the modulation of heterochronic defects in which *daf-12* function is necessary. How temperature may regulate the molting cycle in the worm is unclear. *C. elegans* are ectotherms, and their developmental rate, including the timing of molts, can be affected by changing the environmental temperature. This is generally thought to be due to acceleration or deceleration of various biological processes. However, the strong affect on molting in *lin-42(0)* animals by temperature suggests that it may specifically impinge on molting pathways; therefore, similar to the heterochronic pathway, *lin-42* may be important in conferring robustness to molting regulatory pathways.

Many genes have been identified that are necessary for molting in *C. elegans* (Frand et al., 2005). Several of these encode collagens and other proteins that are involved in constructing or shedding cuticles. Only a few identified genes are transcription factors or other genes that may regulate the timing of these molts at a systemic level. Of particular interest are *nhr-25* and *nhr-23* (Gissendanner and Sluder, 2000;Kostrouchova et al., 1998). These genes encode nuclear hormone receptors, and mutations in either cause severe molting defects, similar to those observed in *lin-42(0)* mutants. Indeed, *nhr-25(RNAi)* enhances the molting defect of *lin-42(ok2385),* suggesting they act in

parallel to regulate molting (Monsalve et al., 2011). It is unknown how the activities of these NHRs are regulated, however, it possible that they may receive environmental signals via an endocrine system, similar to DA regulation of *daf-12*. It will be of interest in future to identify the signals that regulate the activity of these proteins, and how these NHRs function with LIN-42 to regulate molting.

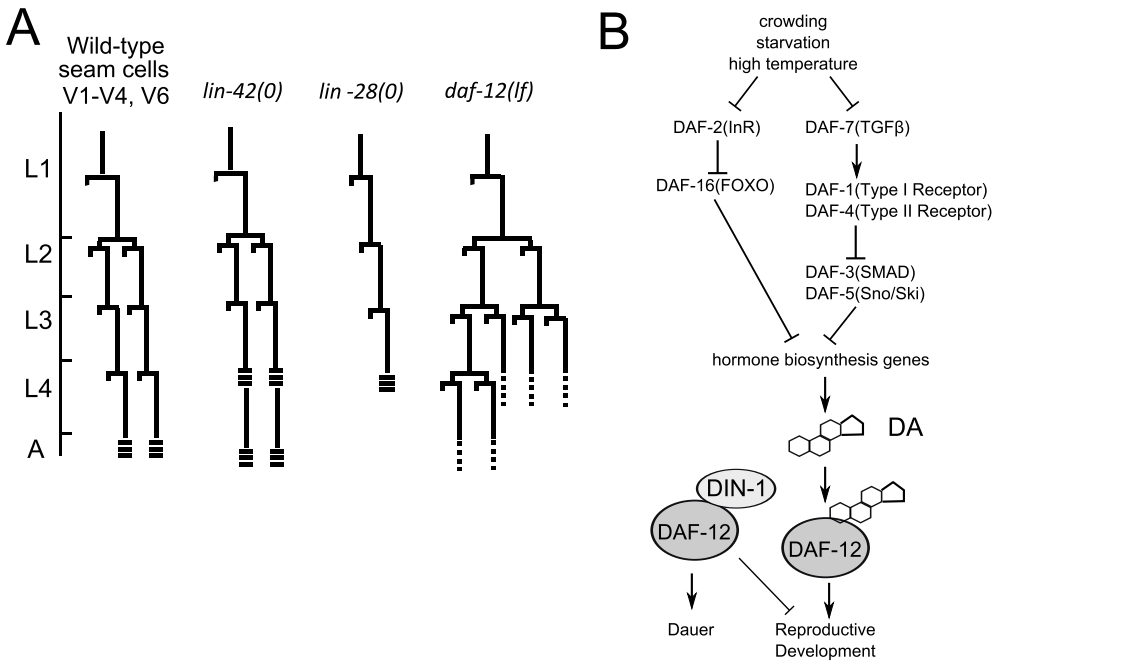
In addition to temperature, we found that *daf-2(lf)* had a slight, but statistically significant affect on the molting defect of *lin-42(0*) mutants. Although a role for the regulation of molting in *C. elegans* by insulin signaling has not been established, in *Drosophila,* insulin signaling is critical in regulating larval development and the molting cycle (Rewitz et al., 2013). After hatching, *Drosophila* progress through three larval instars prior to pupation and finally eclosion. Prior to pupariation, *Drosophila* larvae must reach the "critical weight" threshold, which is the minimum weight at which the larvae can stop feeding and commit to metamorphosis. Insulin signaling is a key pathway by which critical weight is assessed in these animals, and disruption of insulin signaling prior to the third larval instar in *Drosophila* results in the delay of pupariation (Shingleton et al., 2005). Our results suggest there is a role for insulin signaling in *C. elegans* molting during continuous development as well, and it is possible that insulin signaling may be involved in an endocrine network that regulates molting. Future work is needed to elucidate the role of this pathway in molting.

Several independent stress signaling pathways affecting developmental timing and molting

Our work shows that multiple independent stress signaling pathways can affect developmental and molting pathways in *C. elegans,* however, phenotypic effects are only observed in *lin-42(lf)* mutants. Previous work showed that loss of *lin-42* sensitizes animals to dauer formation (Tennessen et al., 2010). Interestingly, the stress signals that regulate developmental timing and molting are transduced through different pathways than those that regulate dauer formation. Thus, it appears that a key function for *lin-42* in *C. elegans* development is to confer robustness on developmental, molting, and dauer pathways against environmental fluctuations.

Figure 1: *lin-42* regulates developmental timing in *C. elegans*

A. Shown are representative lineages of hypodermal seam cells in wild-type animals and heterochronic mutants. Vertical lines denote time, and horizontal lines indicate cell divisions. In wild-type animals, the seam cells terminally differentiate at the L4 molt, and contribute to the formation of an adult cuticle, which has ridges called alae. In *lin-42(0)* and *lin-28(0)* mutations cause precocious phenotypes, in which the larval-to-adult transition occurs one stage too early, at the L3 molt. *daf-12* mutations result in retarded heterochronic defects, in which the seam cells fail to terminally differentiate at the L4 molt and repeat larval programs. **B.** Simplified diagram of pathways regulating dauer formation in *C. elegans*. See text for details. **C.** Shown is the reverse complement of the *lin-42* locus as annotated in Wormbase (www.wormbase.org). The *lin-42* locus encodes at least three isoforms, two of which are non-overlapping. In all reported *lin-42(rf*) alleles, either Lin-42a or Lin-42c remain intact. *lin-42(mg152)* and *lin-42(ve11)* are lesions resulting in premature stop codons. *lin-42(n1089)* and *lin-42(ok2385)* delete the upstream and downstream regions, respectively. *lin-42(ox461*) deletes the entire coding region and is a null allele.


A
Wild-type
seam cells
V1-V4, V6
lin-42(0)
lin -28(0)
daf-12(lf)
L1
L2
L3
L4
A
B
crowding
starvation
high temperature
DAF-2(InR)
DAF-7(TGFβ)
DAF-16(FOXO)
DAF-1(Type I Receptor)
DAF-4(Type II Receptor)
DAF-3(SMAD)
DAF-5(Sno/Ski)
hormone biosynthesis genes
DA
DIN-1
DAF-12
DAF-12
Dauer
Reproductive
Development

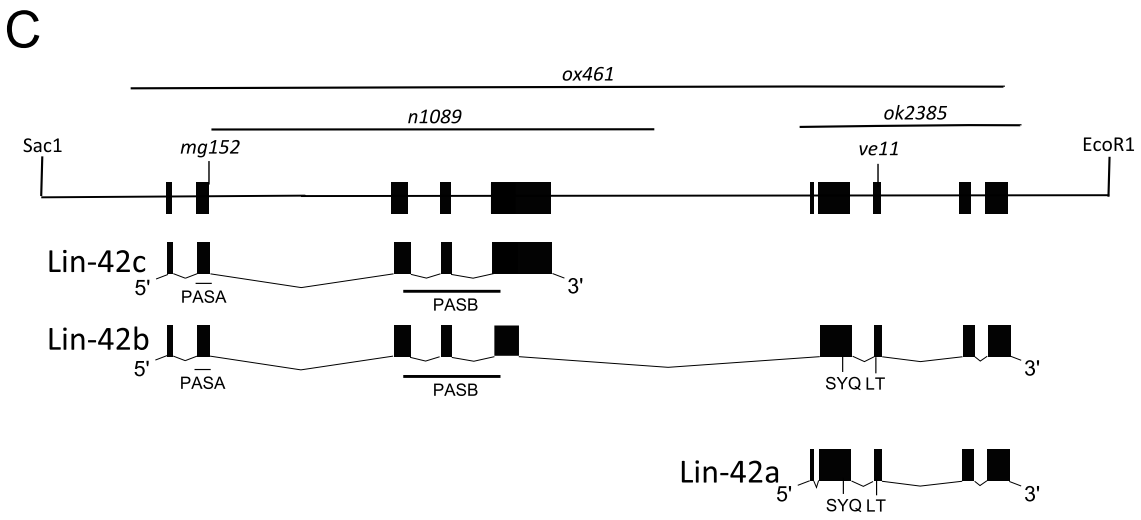

C
ox461
n1089
ok2385
Sac1
mg152
ve11
EcoR1
Lin-42c
5'
PASA
PASB
3'
Lin-42b
5'
PASA
PASB
SYQ LT
3'
Lin-42a
5'
SYQ LT
3'

Figure 1

Figure 2: Mildly stressful temperatures suppress the heterochronic phenotype of *lin-42(rf)* alleles, but not *lin-42(RNAi)* or *lin-42(0)* mutants

lin-42 mutant animals were scored for complete, partial (alae with gaps), or no alae at the L3 molt when raised at 20°C or 25°C. Wild-type animals do not form alae until the L4 molt. Also see Table S1 ($n \geq 41$, *** $p < 0.0001$, Fisher's Exact Test).

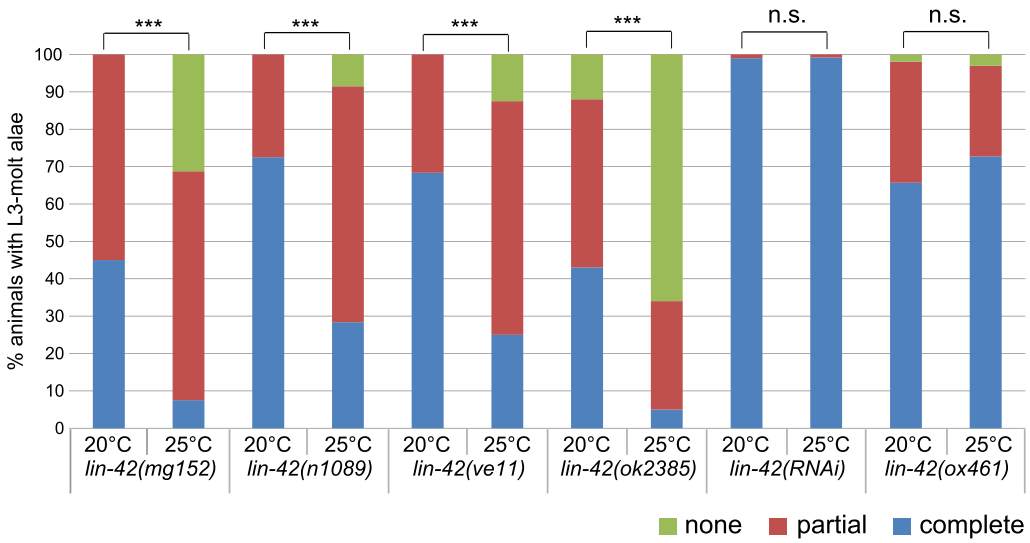

***
***
***
***
n.s.
n.s.
% animals with L3-molt alae
100
90
80
70
60
50
40
30
20
10
0
20°C
25°C
lin-42(mg152)
lin-42(n1089)
lin-42(ve11)
lin-42(ok2385)
lin-42(RNAi)
lin-42(ox461)
none
partial
complete

Figure 2

Figure 3: *daf-12* is required for the suppression of *lin-42* heterochronic phenotypes at high temperatures

A. *daf-16* and *daf-5* are not required for the temperature suppression of *lin-42(rf)* phenotypes. *daf-16(mu86)*; *lin-42(lf)* and *daf-16(mu86); daf-5 (e1386) lin-42(ve11)* mutants were analyzed for L3-molt alae when raised at either 20°C or 25°C. Also see Table S1 (n≥36,**p≤0.01, ***p≤0.001 ,Fisher's Exact Test). **B.** *daf-12* is required for the temperature suppression of *lin-42(rf)* phenotypes. *daf-12(rh61rh411); lin-42(lf)* double mutants were analyzed for L3-molt alae at either 20°C or 25°C. There was no statistical difference between any double mutants raised at these temperatures. Also see Table S1. (n≥35, n.s. not significant)

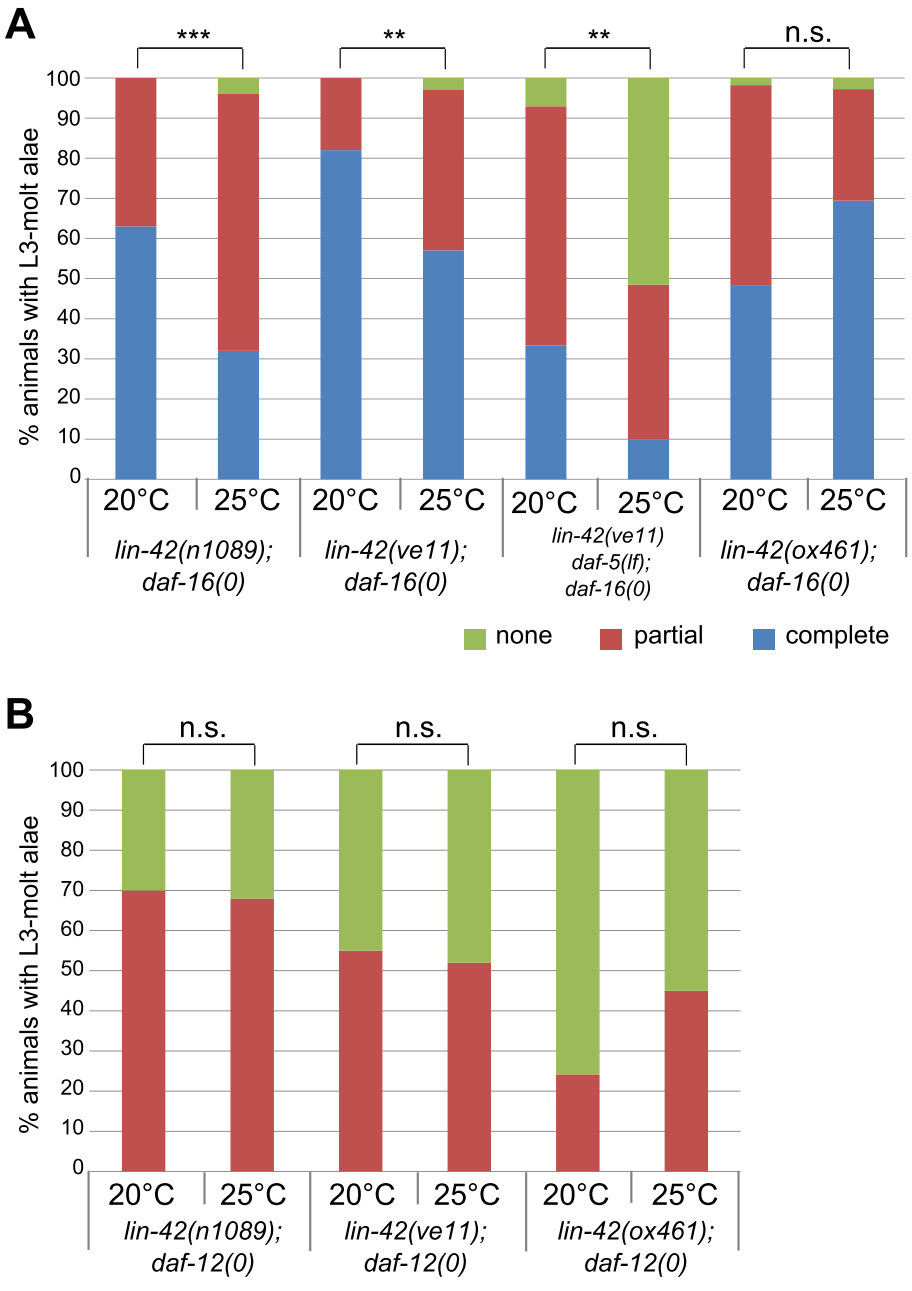

A
***
**
**
n.s.
% animals with L3-molt alae
100
90
80
70
60
50
40
30
20
10
0
20°C
25°C
lin-42(n1089); daf-16(0)
lin-42(ve11); daf-16(0)
lin-42(ve11) daf-5(lf); daf-16(0)
lin-42(ox461); daf-16(0)
none
partial
complete
B
n.s.
n.s.
n.s.
% animals with L3-molt alae
lin-42(n1089); daf-12(0)
lin-42(ve11); daf-12(0)
lin-42(ox461); daf-12(0)

Figure 3

Figure 4: *lin-28* acts in parallel with *lin-42* and is required for the temperature sensitivity of *lin-42* heterochronic phenotypes

lin-42(ve11), lin-28(ga54), and *lin-28(ga54); lin-42(ve11)* mutants were raised at 20°C or 25°C and scored for complete, partial, or no alae formation at the L2 molt. Also see Table (n≥24, **p≤0.01, Fisher's Exact Test).

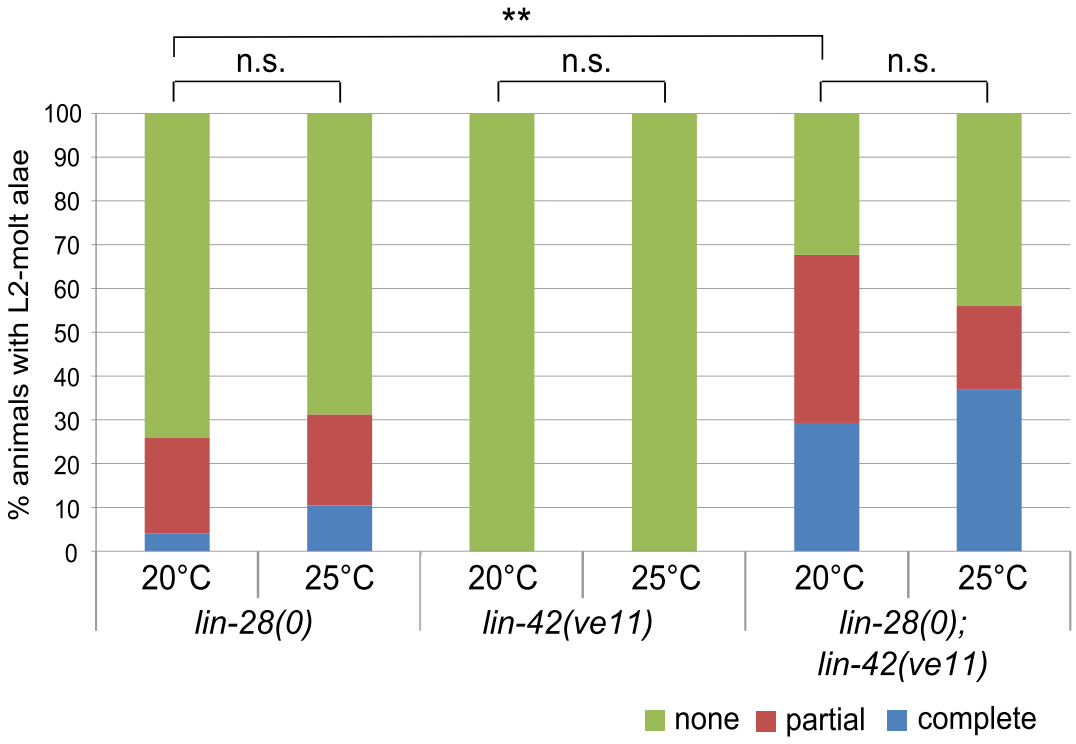

**
n.s.
n.s.
n.s.
% animals with L2-molt alae
100
90
80
70
60
50
40
30
20
10
0
20°C
25°C
20°C
25°C
20°C
25°C
lin-28(0)
lin-42(ve11)
lin-28(0);
lin-42(ve11)
none
partial
complete

Figure 4

Figure 5: LIN-28 is up-regulated at 25°C in a *daf-12*-dependent manner

A. LIN-28 protein is strongly expressed in the L1, and levels begin to decrease at the L2-stage. Western blot for LIN-28 protein in wild-type animals from the L1 to L3 stage is shown. Actin was used as a loading control. Average LIN-28 levels relative to actin are plotted showing decrease of LIN-28 beginning at the L2 stage (Seggerson et al., 2002). **B,C.** LIN-28 levels are significantly up-regulated at 25°C. Protein samples were isolated from wild-type animals raised at 20°C and 25°C in the mid-L2 stage and loaded in triplicate for western blot analysis. (B) Western blot analysis of LIN-28 normalized to actin (as fluorescence intensity). The average relative LIN-28 signal relative to actin is plotted (*$p<0.05$, two-sided, two sample students t-test). Error bars represent standard deviation. A representative biological replicate is shown (C) Average relative LIN-28 levels in animals raised at 25°C compared to 20°C. Shown is the average of 6 biological replicates (**$p<0.01$, one-sided one-sample student's t-test $\mu=1$). **D.** Up-regulation of LIN-28 by temperature requires *daf-12.* Western blot analysis of protein samples from *daf-12(rh61rh411)* mutants. Analysis was performed as with wild-type samples. Shown is a representative of 3 biological replicates. ($p=0.4$, two-sided, two sample student's t-test).

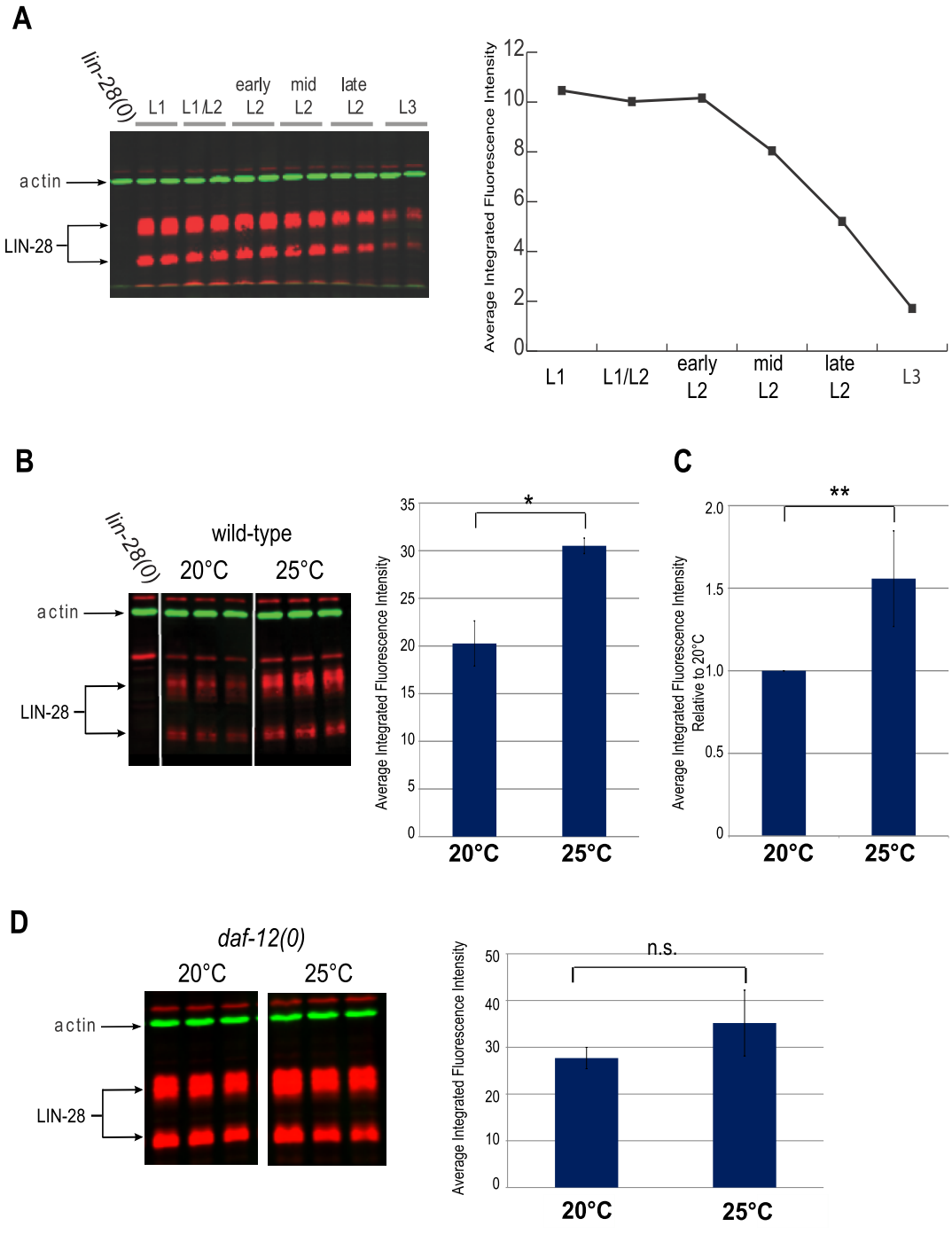

A
lin-28(0)
L1
L1/L2
early L2
mid L2
late L2
L3
actin
LIN-28
Average Integrated Fluorescence Intensity
B
lin-28(0)
wild-type
20°C
25°C
actin
LIN-28
Average Integrated Fluorescence Intensity
*
20°C
25°C
C
**
Average Integrated Fluorescence Intensity Relative to 20°C
20°C
25°C
D
daf-12(0)
20°C
25°C
actin
LIN-28
n.s.
Average Integrated Fluorescence Intensity
20°C
25°C

Figure 5

Figure 6: Disruption of stress-signaling pathways suppress *lin-42* heterochronic mutant phenotypes

lin-42(lf); daf-2(e1370) and *lin-42(lf);daf-7(e1372)* double mutants were analyzed for L3-molt alae at 20°C. *lin-42(ox461); daf-7* double mutants are strongly daf-c at 20°C, and were raised at 15°C. Also see Table S1 (n≥ 38, ***p≤0.0001, n.s. not significant, Fisher's Exact Test, double and triple mutants are compared to *lin-42* mutant alone).

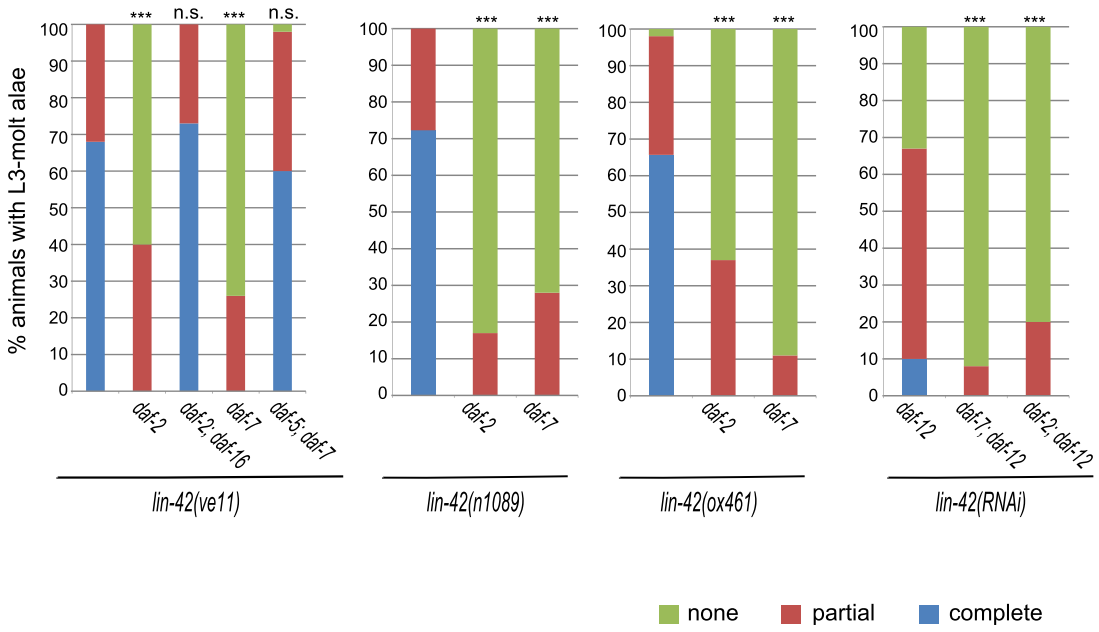

% animals with L3-molt alae
100
90
80
70
60
50
40
30
20
10
0
***
n.s.
***
n.s.
daf-2
daf-2; daf-16
daf-7
daf-5; daf-7
lin-42(ve11)
***
***
daf-2
daf-7
lin-42(n1089)
***
***
daf-2
daf-7
lin-42(ox461)
***
***
daf-12
daf-7; daf-12
daf-2; daf-12
lin-42(RNAi)
none
partial
complete

Figure 6

Figure 7: The larval arrest phenotype of *lin-42(0)* mutants is suppressed by mild temperature stress and *daf-2(lf)* A. The larval arrest of *lin-42(0)* is suppressed by high temperature. *lin-42* mutants were raised from hatched L1s at 20°C or 25°C and monitored for developmental progression (n≥38, ***p<0.0001 Fisher's Exact Test). **B.** *daf-12*, *daf-16*, and *daf-5* are not required for temperature suppression of *lin-42(0)* larval arrest. *lin-42(0); daf-12(rh61rh411), lin-42(0); daf-16(mu86),* and *lin-42(0) daf-5(e1386)* animals were analyzed from hatched L1 and monitored for developmental progression at either 20°C or 25°C (n≥19,*p<0.05, **p<0.01 , ***p<0.001 Fisher's Exact Test) **C.** *daf-2*, but not *daf-7*, mildly suppressed the *lin-42(0)* molting phenotype. *lin-42(0)* and *lin-42(0); daf-2(e1370)*animals were raised at 20°C and monitored for developmental progression. (n≥27, **p<0.01). *lin-42(0); daf-7(e1372*) animals were raised at 15°C due to the strong Daf-c phenotype of these animals at 20°C. Shown are analyses of the non-dauer forming population. (n≥18, p=1). **D.** *daf-16/FOXO* is required for suppression of *lin-42(0)* arrest by *daf-2/InR. lin-42(0)* and *lin-42(0);daf-2(e1370);daf-16(mu86)* mutants were raised at 20°C and monitored for developmental progression. (n≥58, p=0.27 Fisher Exact Test).

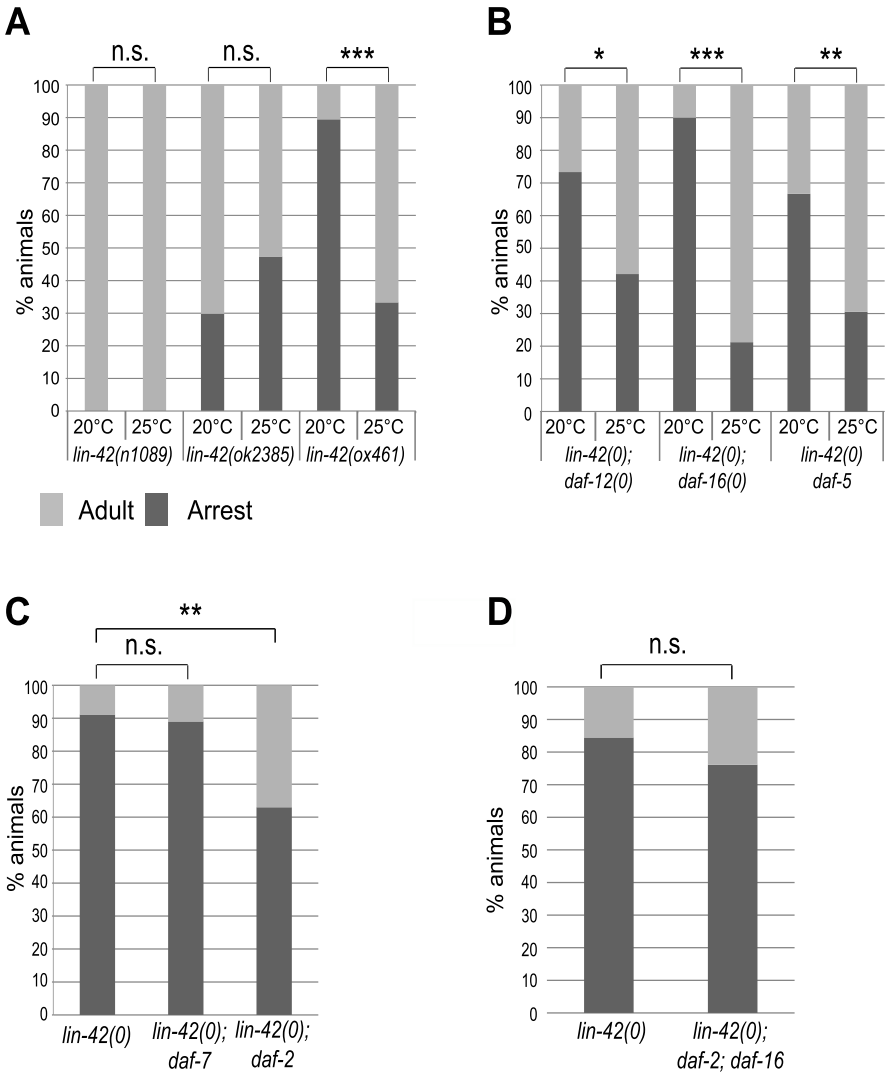

A
n.s.
n.s.
***
% animals
20°C
25°C
lin-42(n1089)
lin-42(ok2385)
lin-42(ox461)
Adult
Arrest
B
*
***
**
lin-42(0); daf-12(0)
lin-42(0); daf-16(0)
lin-42(0) daf-5
C
**
n.s.
lin-42(0)
lin-42(0); daf-7
lin-42(0); daf-2
D
n.s.
lin-42(0)
lin-42(0); daf-2; daf-16

Figure 7

Figure 8: Model for regulation of developmental timing by environmental signals

Temperature and insulin/TGFβ signaling affect *lin-42(lf)* heterochronic phenotypes by parallel pathways. Temperature suppression requires both *daf-12* and *lin-28* function. LIN-28 protein levels are up-regulated at higher temperatures in a *daf-12*-dependent manner. As *lin-42* and *lin-28* act in parallel to regulate timing, there is less requirement for *lin-42* activity under these conditions. Some residual *lin-42* function is also required in this pathway, as *lin-42(0)* animals are not suppressed by high temperature. Decreased insulin and TGFβ signaling also suppress *lin-42(lf),* in parallel to temperature suppression, as these pathways require neither *daf-12* nor any residual *lin-42* function. *lin-42(0)* is also suppressed by *daf-2(lf)* and *daf-7(lf).* In contrast to the dauer formation pathway, *daf-12* functions in parallel with, rather than downstream of, the insulin and TGFβ pathways in modulating developmental timing (See Figure 1B).

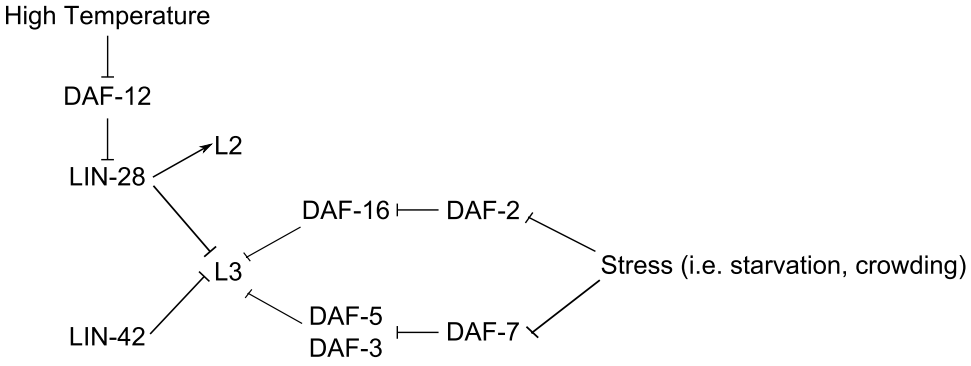

High Temperature
DAF-12
LIN-28
L2
DAF-16
DAF-2
L3
Stress (i.e. starvation, crowding)
LIN-42
DAF-5
DAF-3
DAF-7

Figure 8

Table S1: Stress suppression of *lin-42(lf) heterochronic phenotype*

	Animals with L3 molt alae 20°C (%)				Animals with L3 molt alae 25°C (%)			
	Complete	Partial	No alae	n	Complete	Partial	No alae	n
lin-42(mg152)	45	55	0	60	8	61	31	80
lin-42(n1089)	73	28	0	80	28	63	9	141
lin-42(ok2385)	43	45	12	42	5	29	66	41
lin-42(ve11)	68	32	0	60	25	63	13	80
lin-42(ox461)	67	33	2	85	72	24	3	92
lin-42(RNAi)	99	1	0	100	99	1	0	100
hbl-1(ve18)	51	39	10	41	33	39	27	33
lin-41(ma104)	0	58	42	31	0	47	53	30
lin-42(n1089); daf-16(mu86)	63	37	0	60	32	64	4	120
lin-42(ve11); daf-16(mu86)	82	18	0	60	57	4	3	60
lin-42(ox461); daf-16 (mu86)	57	60	2	47	69	28	3	37
lin-42(n1089); daf-3(mgDf90)	17	8	3	70	4	70	26	120
lin-42(ve11); daf-3(mgDf90)	50	50	0	60	16	76	8	80
lin-42(ve11) daf-5(e1386)	54	46	0	50	15	63	22	60
lin-42(ve11) daf-5(e1386); daf-16(mu86)	33	59	7	27	10	39	52	31
lin-42(n1089); daf-12(rh61rh411)	0	70	30	60	0	68	32	60
lin-42(ve11); daf-12(rh61rh411)	0	55	45	60	0	52	48	60
lin-42(ox461); daf-12(rh61rh411)	0	31	69	35	0	45	55	38
daf-2(e1370)	0	0	100	18				
lin-42(n1089); daf-2(e1370)	0	17	83	47				
lin-42(ve11); daf-2(e1370)	0	40	60	57				
lin-42(ox461); daf-2(e1370)	0	37	63	60				
daf-2(e1370); lin-42(RNAi)	2	55	25	60				
lin-42(n1089); daf-16(mu86); daf-2 (e1370)	35	58	8	66				
lin-42(ve11); daf-16(mu86); daf-2(e1370)	73	27	0	60				
daf-7(e1372) 15°C	0	0	100	17				
lin-42(n1089); daf-7(e1372)	0	28	72	53				
lin-42(ve11); daf-7(e1372)	0	26	74	53				
lin-42(ox461); daf-7(e1372) 15°C	0	11	89	45				
daf-7(e1372); lin-42(RNAi)	10	48	42	60				
lin-42(ve11) daf-5(e1386); daf-7(e1372)	60	38	2	60				
daf-12(rh61rh411); lin-42(RNAi)	10	57	33	60				
daf-7(e1372); daf-12(rh61rh411); lin-42 (RNAi)	0	20	80	60				
daf-2(e1370); daf-12(rh61rh411); lin-42(RNAi)	0	8	92	60				
daf-3(mgDf90); lin-42(RNAi)	66	29	5	80				
daf-5(e1386); lin-42(RNAi)	100	0	0	20				
daf-16(mu86); lin-42(RNAi)	98	2	0	40				

Chapter IV: *lin-42/per* regulates the timing of heterochronic miRNA expression

SUMMARY

miRNAs are small RNAs that regulate gene expression at the post-transcriptional level via interactions with the 3'UTR of target mRNA transcripts. miRNAs were first identified as members of the heterochronic pathway in *C. elegans*, and have since been found in many organisms with roles in differentiation and pluripotency. lin-4 miRNA and miRNAs of the *let-7*-family regulate developmental timing in *C. elegans*, and precise control of the temporal expression of these miRNAs is critical to ensure developmental events occur in the correct sequence. Although much is known about how miRNAs are generated, relatively little is understood about how the timing of their expression is regulated. Here, I demonstrate that the *C. elegans period* homolog, *lin-42*, is important for preventing let-7 miRNA and miR-48 from being expressed too early. Although both *lin-42* and primary transcript levels of *let-7* and *mir-48* oscillate over developmental time, *lin-42* function is not required for these oscillations. Instead, the total levels of these primary transcripts are elevated in *lin-42* mutants. Genetic analyses revealed that *lin-42* likely acts primarily through let-7 family miRNAs in the heterochronic pathway. These data show that a key

function of *lin-42* in developmental timing is to dampen the levels of some miRNA transcripts, preventing their premature expression as mature miRNAs.

INTRODUCTION

miRNAs are highly conserved small RNA molecules (~22nt) that negatively regulate gene expression post-transcriptionally. Work in many systems has discovered a great deal about miRNA biogenesis (For review, see (Bartel, 2004)). Briefly, miRNAs are first transcribed by RNA polymerase III into a long primary transcript (pri-miRNA) (Lee et al., 2004). The pri-miRNA is then cleaved in the nucleus by a multi-protein complex called the Microprocessor producing a pre-miRNA hairpin (Denli et al., 2004;Lee et al., 2002;Lee et al., 2003). The pre-miRNA is exported from the nucleus and further cleaved by the endoribonuclease Dicer into a ~22 nucleotide heteroduplex (Grishok et al., 2001;Hutvagner et al., 2001;Ketting et al., 2001). This molecule is incorporated into a large protein complex called RISC, where the duplex is passively unwound, and the passenger strand discarded (Hammond et al., 2000;Nykanen et al., 2001). The miRNA then directs the miRISC to sites of complementary in the 3'UTR of mRNA transcripts, targeting them for repression (Fabian and Sonenberg, 2012;Stadler et al., 2012;Zhang et al., 2007).

miRNAs were first identified as members of the heterochronic gene pathway in *C. elegans*, and study of miRNAs in the worm continues to contribute to our understanding of miRNA regulation and function (For review, see (Abbott, 2011;Resnick et al., 2010)). The heterochronic pathway functions to regulate the timing of larval developmental programs, and mutations in heterochronic genes result in timing defects in a variety of tissues. Developmental timing of seam cell

development has been most extensively studied. In wild-type animals, these cells divide asymmetrically at the beginning of each larval stage (Figure 1A). At the L2-stage, the asymmetric division is preceded by a symmetric division, causing an increase in seam cell number. At the L4-to-adult transition, the seam cells terminally differentiate and contribute to the formation of an adult cuticle that contains lateral ridges called alae. In precocious heterochronic mutants, early developmental programs are skipped, causing later ones to occur to early. In contrast, retarded mutants cause early programs to repeated, which can result in delaying later events.

miRNAs are critical components of the heterochronic pathway, and control the expression of transcription factors that promote stage-specific developmental programs (Figure 1B). The onset of the developmental timer begins with the expression of lin-4 miRNA, thought to be triggered at least in part by feeding (Baugh and Sternberg, 2006;Feinbaum and Ambros, 1999;Lee et al., 1993). *lin-4* mutants have a retarded heterochronic phenotype, where the L1-seam program is repeated (Figure 1A). lin-4 miRNA down-regulates the *lin-14* transcription factor, promoting the transition to the L2-stage (Ambros and Horvitz, 1987;Arasu et al., 1991). Next, during the L2-stage, miR-48, miR-241, and miR-84 accumulate and function redundantly to promote the L2-to-L3 transition by down-regulating the expression of a gene called *hbl-1*, which encodes a zinc-finger transcription factor homologous to *Drosophila hunchback* (Abbott et al., 2005;Fay et al., 1999). Decreased levels of *hbl-1* allow the L2-to-L3 transition to occur. (Abrahante et al., 2003;Lin et al., 2003). Finally, the let-7 miRNA is up-

regulated at the L3-stage and indirectly promotes the larval to adult transition (Reinhart et al., 2000). Mutation of *let-7* causes a retarded phenotype, where the late larval program is reiterated (Figure 1A). As its name implies, loss of *let-7* function is lethal, and these animals often burst through the vulva as young adults.

let-7 is the founding member of a family of miRNAs that share identity within the 5', or "seed" sequence of the miRNA. This sequence is thought to be critical for targeting miRNAs to specific transcripts. Interestingly, miR-48, miR-241, and miR-84 are also members of this family (Abbott et al., 2005). Although these four miRNAs have identical seed sequences, it is likely that they do not completely overlap functionally. First, they have distinct temporal expression patterns, as let-7 miRNA is detectable a full stage later than its paralogs. Additionally, *let-7* mutants have a somewhat different phenotype from *mir-48 mir-241; mir-84* mutant animals. While mutations of the *let-7* paralogs result in repetition of L2 programs, mutation of *let-7* results in repeating later programs (Figure 1A).

Another highly conserved player in the heterochronic pathway is *lin-42* (Jeon et al., 1999). *lin-42* is the *C. elegans* homolog of *period*, which is a key component of the circadian timekeeper in many organisms, including humans (Yu and Hardin, 2006). *lin-42(lf)* causes an opposite phenotype of miRNA mutants, precocious development (Figure 1A). In *lin-42* mutants, adult alae are observed after the third larval molt instead of the fourth. In addition to its sequence similarity to *period*, *lin-42* also has a dynamic expression pattern. Both

lin-42 mRNA and protein levels oscillate over developmental time, peaking during the intermolt (Jeon et al., 1999;Tennessen et al., 2006). This is uncommon in the heterochronic pathway, as most genes are express early or late depending on their function, and suggests that *lin-42* may have a reiterative role in the pathway. How *lin-42* regulates developmental timing is still unknown.

The timing of expression of miRNAs is vital for promoting larval developmental transitions at the appropriate developmental time. However, little is understood about how the temporal expression of these molecules is regulated. Of the many heterochronic genes identified, only three, *lin-28*; *daf-12;* and *hbl-1,* have been shown to regulate the timing of miRNA expression (Bethke et al., 2009;Hammell et al., 2009a;Roush and Slack, 2009;Vadla et al., 2012;Van Wynsberghe et al., 2011). In this study, I demonstrate that let-7 miRNA and miR-48 are up-regulated precociously in *lin-42(lf)* animals. Analysis of heterochronic pri-miRNA expression revealed that the levels all of these transcripts oscillate over larval development. Surprisingly, oscillations were also observed in *lin-42(lf)*. However, the amplitude of these oscillations for *pri-let-7* and *pri-mir-48* were significantly increased in *lin-42(lf)* compared to wild-type. Genetic analysis indicates that these miRNAs are downstream of *lin-42* in the heterochronic pathway. Taken together, these results show that a key role for *lin-42* in developmental timing is to dampen *pri-let-7* and *pri-mir-48* oscillations, preventing premature accumulation of mature miRNA.

MATERIALS & METHODS

Nematode Maintenance and Strains

C. elegans strains were grown and maintained using standard methods (Sulston and Hodgkin, 1988). Experiments were performed at 20˚C unless otherwise noted. Strains used in this study were: N2, RG280 *lin-42(ve11)*, RG1590 *lin-42(ox461)*, SP231 *let-7(mn112) unc-3(e151)*, VT1066 *mir-48 mir-241(nDf51)*; *mir-84(n4037)*, MT13669 *mir-48 mir-241 (nDf51)*, RG1742 *lin-42(ox461) nDf51*, *RG1743 lin-42(ox461); nDf51; mir-84(n4037),* RG1738 *lin-42(ox461); nDf51; let-7(mn112) unc-3(e151).* During strain construction, *ox461* and miRNA gene deletions were followed by PCR.

RG1738 animals were maintained on "low *hbl-1(RNAi)"* plates, which were standard NGM plates seeded with E. coli HT115 expressing double stranded *hbl-1* RNA (Vadla et al., 2012). This treatment weakly suppresses the lethality caused by *let-7(0)*, allowing maintenance of the strain. To score RG1738 phenotypes, eggs were isolated by hypochlorite treatment from adults grown on low *hbl-1(RNAi)* and plated on NGM plates seeded with *E. coli* OP50. Suppression was not observed in this generation.

qRT-PCR

To synchronize populations, eggs were isolated from gravid adults using hypochlorite treatment and hatched overnight in M9 buffer. Animals were placed on 10cm OP50 seeded NGM plates at a density of ~5,000 to ~10,000 animals

per plate. For long timecourses, animals were plated twice, 12 hours apart, to stagger collection times. Worms were flash frozen in liquid nitrogen for RNA preparation. Biological replicates were performed with animals from independent bleach treatments and grown on different days.

RNA was extracted using Trizol (Life Technologies). For mRNA reverse transcription (RT) reactions, 5 µg total RNA was treated with DNAse I to remove genomic DNA using Ambion Turbo DNAse kit as directed. Roche Transcriptor was used for RT reactions according to manufacturer's instructions with random primers (Promega) and 1 µg of DNAse-treated RNA per reaction. miRNA RT reactions were performed with a Taqman miRNA reverse transcription kit (Life Technologies). Taqman miRNA and gene expression assays were performed according to manufacturer's instructions (Life Technologies).

All qPCR reactions were run in triplicate on an Eppendorf Realplex Thermocycler using a 96-well plate format and data was collected using Realplex 2.0 software. The sample used for normalization (i.e. wild-type 6 hr) was run on every plate. U18 RNA was used as an internal control for miRNA experiments. *ama-1* was used for normalization of pri-miRNAs and *mlt-10* qRT-PCR. Data were analyzed using the ΔΔCt method in Microsoft Excel (Livak and Schmittgen, 2001). Statistical analyses were performed using R. Specificity of miRNA and pri-miRNA assays was determined by analyses of deletion mutants (Figure S1).

RESULTS

let-7 miRNA accumulates precociously in *lin-42(lf)*

Several genetic results suggest that *lin-42* function in the heterochronic gene pathway could involve modulation of miRNA activity. For example, *lin-42* acts antagonistically to *daf-12* to time cell fate decisions in the hypodermis, and one role of *daf-12* is to up-regulate expression of *let-7* family miRNAs (Bethke et al., 2009;Hammell et al., 2009a;Tennessen et al., 2006). In addition, analysis of double mutants between *lin-42* and *lin-28*, a miRNA processing regulator, reveals an enhanced precocious phenotype in the hypodermis, relative to either mutant alone suggesting that these genes may act in parallel to regulate timing (Lehrbach et al., 2009;Vadla et al., 2012;Van Wynsberghe et al., 2011) (Chapter III). Finally, mutual suppression has been observed between mutations in *lin-42* and *let-7* or *lin-4*, suggesting these genes act in opposition to each other (Abrahante et al., 1998;Reinhart et al., 2000). Given these relationships, we tested whether expression of heterochronic miRNAs is altered in *lin-42* mutants relative to wild-type.

Gene expression profiles of *lin-42* mutants are not directly comparable to wild-type animals based on absolute time from hatching because larval stages are lengthened in *lin-42* loss-of-function *(lf).* This delay is due, at least in part, to a molting defect that results in delayed and lengthened larval molts compared to wild type (Monsalve et al., 2011) (Chapter II). To account for this delayed development, expression of endogenous *mlt-10* was measured by qRT-PCR at 2 hour intervals in each strain and the timing of its expression peaks were used to

align stages. *mlt-10* encodes a nematode specific protein that is required for molting, and GFP reporters for this gene exhibit a cyclical expression pattern with a peak once per larval molt (Frand et al., 2005;Meli et al., 2010). *Pmlt-10::gfp* reporter expression has previously been used to monitor larval development of individual *lin-42* mutant animals (Monsalve et al., 2011).

In wild-type animals, *mlt-10* mRNA levels peak once per stage, approximately 4 hours prior to the onset of lethargus, a characteristic molting behavior in which the worm ceasing pharyngeal pumping prior to ecdysis (Figure 2A). In *lin-42(lf)* populations, the oscillation of *mlt-10* expression was delayed, and the delay became progressively worse at each stage. While all four *mlt-10* peaks are observed between 12-58 hours after wild-type larvae are placed on food, only three peaks are detected during the same interval in *lin-42(lf)* mutants. Indeed, for the sample shown in Figure 2A, the third *mlt-10* peak in *lin-42(lf)* was not observed until 44 hrs after animals were placed on food, 10 hours later than the comparable wild-type peak.

let-7 miRNA levels were measured from the same biological samples using Taqman miRNA qRT-PCR (Figure 2B) (see Materials and Methods). In wild-type animals, let-7 miRNA was not detectable above background until the animals were approaching the L1 molt, about 18 hours after being placed on food. During the L2-stage, let-7 miRNA levels remained at this low and fairly constant level. After the L2-molt, however, let-7 miRNA levels rapidly and dramatically increased in agreement with previous northern analysis, and the miRNA levels remained high throughout subsequent larval development (Abbott

et al., 2005;Esquela-Kerscher et al., 2005;Li et al., 2005;Reinhart et al., 2000). In contrast to wild-type, let-7 miRNA levels start to rise during the early L2 in *lin-42(lf)* populations and continue to rise until the early to mid-L3, where, similar to wild-type, levels are high and somewhat variable (Figure 2B). To more directly compare the developmental expression pattern of let-7 miRNA in wild-type and *lin-42(lf)* samples, the data were plotted to focus on the dynamics of the L2-stage. Using the *mlt-10* expression profile to adjust for stage timing and length, 18-28 hr timepoints were plotted for wild-type versus 22-32 hrs for *lin-42(lf)* (Figure 2A, 2C). let-7 miRNA levels rise nearly 150-fold by the mid-L2 in *lin-42(lf),* while they remain just above background in wild-type animals. This analysis reveals that let-7 miRNA levels increase abnormally early, during the L2-stage, in *lin-42(lf)* mutants. This L2 stage increase in let-7 miRNA levels is reproducible and statistically significant across multiple biological replicas (Figure 2D). During the L3-stage, let-7 miRNA levels quickly increase in wild-type, and the levels become more equivalent between the two strains. Although the trend is for let-7 levels to be somewhat higher in *lin-42* mutants, statistical significance was not observed (Figure 2D).

miR-48 is up-regulated in *lin-42(lf)* in the L1 stage

Given the prominent role of miRNAs in the heterochronic gene pathway, we tested whether any of the early-acting miRNAs (lin-4, miR-48, miR-241, and miR-84) are aberrantly expressed in *lin-42* mutants (Abbott et al., 2005;Lee et al.,

1993;Li et al., 2005;Reinhart et al., 2000). In these experiments, samples were collected from 6-38 hrs of development to focus on the first three larval stages of wild-type and Taqman miRNA qRT-PCR was used to measure miRNA levels. In addition to *lin-42(lf),* we also collected samples from a *lin-42* null allele, [*lin-42(0)*] generated by mosDEL technology that removes the entire coding region of *lin-42* (Frokjaer-Jensen et al., 2010) (Chapter II). *lin-42(0)* mutant animals have a stronger molting defect than do *lin-42(lf),* resulting in late L2-arrest in the majority of animals; therefore, these samples were only collected through the L1 stage, ending at 24hrs. *mlt-10* expression was again measured to monitor synchrony and determine approximate stage lengths for these populations. The *mlt-10* profile corroborates the extended L1 stage in *lin-42(0)* animals (Figure 3A, Chapter II).

The wild-type expression patterns of these miRNAs as determined by qRT-PCR closely match their previously established profiles as determined by northern blot analyses (Abbott et al., 2005;Esquela-Kerscher et al., 2005;Feinbaum and Ambros, 1999;Li et al., 2005) (Figure 3B). lin-4 miRNA levels rise in the L1-stage, at approximately 12-hrs while miR-48, miR-84, and miR-241 levels increase somewhat later, with a dramatic rise in the early L2-stage. As with let-7 miRNA, the general expression pattern of these miRNAs is similar between biological replicates (Figure S2). Although the Fold Difference relative to 6hrs can be variable at a specific timepoint, particularly after time of the dramatic increase in levels, these variations are rarely greater than two-fold.

lin-4 miRNA levels have been reported to oscillate during larval development (Kim et al., 2013). A similar oscillation pattern was evident in two out of three of our biological replicates (Figure S2). In addition to lin-4 miRNA, we also observed some oscillation of let-7 miRNA, following up-regulation in the L3-stage in some biological replicates, however, the timing of these oscillations were not reproducible (Figure S2).

Among the early-acting miRNAs, only miR-48 was found to be consistently up-regulated in *lin-42* mutants, with significantly higher levels observed in the mid-to-late L1 stage (Figure 3B). This increase in miR-48 accumulation is particularly evident when the data are plotted to adjust for stage length using the *mlt-10* peaks to align timepoints and is reproducible and statistically significant (Figure 3C, 3D). When averaged over multiple biological replicas, a statistically significant increase in miR-48 levels is observed. During the L2-stage, as miR-48 is being up-regulated in wild-type, levels are similar between strains (Figures 3B, S3). miR-241 and miR-84 were more similar to wild-type, and were up-regulated in the early to mid L2-stage (Figure 3B). Other than the developmental delay, these analyses did not reveal changes significant differences between wild-type and *lin-42* mutants for miR-241 or miR-84 (Figure 3D).

As in wild-type animals, lin-4 miRNA was up-regulated in the late-L1 stage in *lin-42* mutants. When multiple biological replicas are averaged, lin-4 miRNA expression levels are consistently higher in *lin-42* mutants compared to wild-type from the early L1 to the L2 molt; however, these differences were not statistically significant (Figure 3D,S3). Additionally, other miRNAs in *C. elegans* that do not

regulate timing, such as miR-1, do not appear affected by *lin-42(lf)* in the L1 stage (Figure 3D).

pri-miRNA transcript levels oscillate over larval development

Analysis of miRNA expression revealed that let-7 miRNA and miR-48 were up-regulated in *lin-42(lf),* but, a critical question is at which step in miRNA biogenesis *lin-42* acts. Immunostaining experiments have shown that LIN-42 is localized to the nucleus (Tennessen et al., 2006). In addition, in circadian clocks, PERIOD regulates gene expression by binding to transcriptional activators and inhibiting their activity (Lee et al., 1999). Therefore, one possibility is that *lin-42* may regulate pri-miRNA transcription. To address this question, qRT-PCR analysis was used to measure pri-miRNA levels over larval development. Heterochronic pri-miRNA levels were first measured in wild-type to ascertain the standard expression patterns of these transcripts.

Interestingly, we found that all heterochronic pri-miRNA levels oscillated over larval development in wild-type by qRT-PCR (Figure 4). This is consistent with previous analysis using northern blotting that showed that *pri-let-7* levels oscillate over larval development (Van Wynsberghe et al., 2011). Also interesting is that the particular expression patterns of these pri-miRNAs are somewhat different. *pri-let-7* and *pri-lin-4* expression patterns were very similar to each other over several biological replicates (data not shown). The levels of these transcripts peaked once per stage at exactly the same time (Figure 4).

This result raises the possibility of coordinate regulation of *lin-4* and *let-7* transcription, which is intriguing since the expression of mature *let-7* and *lin-4* are dissimilar. lin-4 miRNA is first detected in the L1-stage, while let-7 miRNA levels dramatically increase at the beginning of the L3. In the case of *let-7*, factors such as *lin-28* are required to inhibit *pri-let-7* processing during early larval stages (Vadla et al., 2012;Van Wynsberghe et al., 2011).

pri-mir-48 and *pri-mir-241* had similar expression patterns to each other (Figure 4). The levels of these transcripts do appear to oscillate; however, these oscillations are less pronounced than those observed for *pri-let-7* and *pri-lin-4*. The expression pattern of *pri-mir-84* differed from *pri-mir-48* and *pri-mir-241*. Although the mature forms of these miRNAs are all up-regulated coordinately, *pri-mir-84* appears to oscillate out of phase from *pri-mir-48* and *pri-mir-241*. While *pri-mir-48* and *pri-mir-241* levels peak at mid-larval stages, *pri-mir-84* levels increase later in each stage, often during the molt. This is surprising and suggests that *pri-mir-84* expression is under a different regulatory regime than its paralogs.

Oscillation of primary transcript levels is observed in *lin-42(lf)*

The observed oscillations in levels of heterochronic pri-miRNAs are particularly intriguing in light of *lin-42* expression patterns. *lin-42* mRNA and protein levels also cycle during post-embryonic development in a similar pattern as heterochronic pri-miRNA levels (Jeon et al., 1999;Tennessen et al., 2006).

Furthermore, in the circadian clock, *period* is critical for maintaining the rhythmic expression patterns of circadian-regulated genes, including *period* itself (Hardin et al., 1990). This led us to ask whether *lin-42* function is required for pri-miRNA cycling.

To our surprise, pri-miRNA transcripts levels were still observed to oscillate in *lin-42(lf)* mutants (Figure 5A, S4). Similar to wild-type, pri-miRNA levels in *lin-42(lf)* populations peak once per larval stage. Moreover, taking into account the molting defect of *lin-42(lf)* animals, peaks were observed at similar developmental times as in wild-type, in the mid to late-larval stages. (Figure 5A, S4). Importantly, even in a *lin-42(0)* background, a *pri-let-7* and *pri-mir-48* peak was observed during the L1 (Figure S4). From these data, we concluded that *lin-42* is not required for pri-miRNA cycling.

pri-let-7 and *pri-mir-48* levels increase *lin-42* mutants

Although pri-miRNA cycling is still observed in *lin-42(lf),* the amplitude of some pri-miRNA peaks appeared to increase in *lin-42(lf)* populations (Figure S4). To estimate total expression of these genes, the area under the L1-peak in each strain was measured in pixels using ImageJ. Using this technique, we found a reproducible and statistically significant increase in the area of the *pri-let-7* L1-peak in *lin-42(lf)* compared to wild-type (Figure 5B). The average area of *pri-mir-48* L1-peaks was also mildly, but significantly, increased in *lin-42(lf)* populations. *pri-let-7* levels in particular appeared strongly elevated in the *lin-42(0*), however,

these experiments are extremely challenging due to the highly penetrant molting and egg-laying defects of these animals, and therefore this strain was only examined once (Figure S4) (Chapter II). In contrast to *pri-let-7* and *pri-mir-48*, the *pri-lin-4* peaks were not significantly greater in *lin-42(lf)* than wild-type, similar to what was observed for lin-4 miRNA levels (Figure 5B). *pri-mir-241* peaks were elevated in some biological samples analyzed (Figure S4). However, this effect was not reproducible over several biological replicates. The fact the miR-241 levels are also not reproducibly elevated in mutants argues against *pri-mir-241* being regulated by *lin-42*. These data support a role for *lin-42* in regulating *pri-let-7* and *pri-mir-48* transcription.

Genetic interaction between *lin-42* and *let-7*-family miRNAs

Investigation of miRNA expression patterns indicated that let-7 miRNA and miR-48 were significantly up-regulated in *lin-42(lf)* populations. We next investigated the genetic relationship between *lin-42* and *let-7*-family miRNAs to determine whether they acted downstream of *lin-42* in the heterochronic pathway. Previous analysis showed that *lin-42(RNAi); let-7(0)* animals were mutually suppressed for their heterochronic phenotypes, suggesting that they function in opposition to regulate timing, but not in a simple linear pathway (Tennessen et al., 2006). We also observed mutual suppression in *lin-42(0); let-7(0)* double mutant animals (Table 1, Figure 6). At the L3-molt, most double

mutants did not have alae. Importantly, in those animals that did form precocious alae, the alae were weak and indistinct compared to *lin-42(0)* mutants alone. In double mutants at the L4-molt, the *let-7(0)* retarded heterochronic phenotype was also strongly suppressed, and most of these mutants formed complete alae similar to wild-type animals (Table 1, Figure 6). Additionally, the lethality associated with the *let-7* mutation is also strongly suppressed by *lin-42(0),* and these animals can be maintained as doubly mutant homozygotes (data not shown).

Our analyses of miRNA expression patterns suggested that increased expression of *mir-48* may contribute to *let-7(0)* suppression by *lin-42(0).* Both miR-48 and let-7 miRNA promote developmental transitions in *C. elegans,* and, because they have identical "seed" sequences, they may also share some targets, such as *hbl-1* (Abbott et al., 2005;Abrahante et al., 2003;Li et al., 2005;Lin et al., 2003;Reinhart et al., 2000). Indeed, over-expression of *mir-48* from multi-copy arrays or by *mir-48* regulatory mutations has been shown to suppress *let-7(0)* phenotypes (Li et al., 2005). Therefore, it is possible that over-expression of *mir-48* caused by *lin-42* mutations may compensate for loss of *let-7* activity. A *lin-42(0); nDf51, let-7(0)* triple mutant was analyzed to test if *let-7* paralogs are required for suppression of *let-7(0)* phenotypes by *lin-42(0). nDf51* is a deficiency that removes both the *mir-48* and *mir-241* loci (Abbott et al., 2005). The *lin-42(0); mir-48 mir-241; let-7(0)* strain was not suppressed for the *let-7* lethality defect, and needed to be maintained on low *hbl-1 RNAi* to survive (Vadla et al., 2012). At the L4-molt, the triple mutant exhibited a strong retarded

defect in contrast to the *lin-42(0); let-7(0)* double mutant. Not only was the extent of the alae very incomplete, but the quality of the alae was weak and difficult to discern, similar to *let-7* mutants alone (Table 1, Figure 6). This genetic result indicates that let-7-family miRNAs are downstream of *lin-42* in the heterochronic pathway, and supports the model that a key function of *lin-42* is to negatively regulate the expression of these miRNAs.

DISCUSSION

miRNAs play critical roles in regulating developmental timing in *C. elegans* by promoting developmental transitions. Although much is known about miRNA biogenesis, little is understood regarding how the temporal expression patterns of these small RNAs are maintained. In this study, we show the *C. elegans lin-42/period* is important for regulating temporal expression of some let-7-family miRNAs.

The expression patterns of heterochronic miRNAs in wild-type as measured by qRT-PCR was similar to that observed using northern blotting analyses (Abbott et al., 2005;Esquela-Kerscher et al., 2005;Lee et al., 1993;Li et al., 2005;Reinhart et al., 2000). Interestingly, analysis of pri-miRNA levels by qRT-PCR every two hours over development showed that the transcript levels of all of these miRNAs cycle over larval development, peaking once per stage. Save for *pri-let-7*, these oscillations have not been previously reported (Van Wynsberghe et al., 2011). This is likely because most developmental northern

experiments have typically analyzed just a few timepoints per stage, and were not done at the temporal resolution performed here.

Only let-7 miRNA and miR-48 were reproducibly up-regulated in *lin-42(lf)* mutants compared to wild-type, and *lin-42* likely regulates let-7 miRNA and miR-48 accumulation at the level of transcription. Although the severe developmental defects of the *lin-42(0)* strain precluded analysis of multiple biological replicates, *pri-let-7* levels in particular appeared strongly up-regulated in this strain, even compared to the *lin-42(lf)* animals. This likely contributes to the increased penetrance of precocious defects in *lin-42(0)* animals compared to *lin-42(lf)* mutants (Chapter II).

Taken together, these data suggest that *lin-42* is required to dampen pri-miRNA oscillations, acting as a buffer to ensure that they are not over-expressed sufficiently to overcome downstream regulation of pri-miRNA processing. Therefore, when *lin-42* is absent, mature miRNAs accumulate aberrantly. However, our analyses measured global levels of pri-miRNAs, therefore, it is also a possibility that *lin-42* blocks pri-miRNA expression in a specific tissue (i.e. seam), which is detected as an overall change in levels by qRT-PCR.

lin-4 miRNA at some timepoints appeared to increase in *lin-42(lf),* however, this was not a strong or reproducible effect, and is likely due to the variability of lin-4 miRNA expression. Whereas the expression patterns of let-7-family miRNAs were generally consistent between biological replicates, the timing of lin-4 miRNA up-regulation was not. Why lin-4 miRNA expression might be more erratic than others in the heterochronic pathway is unclear. One

possibility is that the variability of lin-4 miRNA levels is a result of *pri-lin-4* processing. As lin-4 miRNA is up-regulated at the same time as the first *pri-lin-4* peak is observed, it is possible that *pri-lin-4* processing is not as tightly regulated as that of other pri-miRNAs.

Analysis of pri-miRNA expression with the sensitivity of qRT-PCR and high temporal resolution revealed surprising results. Especially striking was the fact that the cyclical expression patterns of pri-miRNAs in the heterochronic pathways were often un-coupled from their miRNA accumulation patterns. This is particularly true of *let-7*: its primary transcript is strongly expressed during the mid-L1 stage, two stages prior to the up-regulation of the miRNA. Interestingly, *pri-lin-4* has a similar expression pattern to *pri-let-7*, suggesting that there may be co-regulation of these genes at the transcriptional level. Therefore, *pri-let-7* transcripts are clearly under tight post-transcriptional regulation to ensure that the miRNA does not accumulate too early. *lin-28* has been shown previously to be critical for control of *pri-let-7* processing, and let-7 miRNA is expressed precociously in *lin-28* mutants (Lehrbach et al., 2009;Vadla et al., 2012;Van Wynsberghe et al., 2011). LIN-28 binds to pri- and pre- let-7 miRNAs, indicating it blocks processing at one or both steps (Vadla et al., 2012;Van Wynsberghe et al., 2011). Our data show that *lin-42* is another gene that is critical in regulating let-7 miRNA expression at the transcriptional level.

pri-mir-48 and *pri-mir-241* also cycle over larval development, and peak expression during each stage occurs at the same time as that of *pri-lin-4* and *pri-let-7*. Interestingly, the shapes of the curves for *pri-mir-48* and *pri-mir-241* from

the same biological replicate were similar to each other. Given that the *mir-48* and *mir-241* sequences are within 1.7kb of each other, this suggests that they may be co-transcribed. However, in *lin-42(lf),* only *pri-mir-48* levels were significantly and consistently up-regulated, not *pri-mir-241* levels. This implies that there are differences in transcription and/or processing of *pri-mir-48* and *pri-mir-241*. Another interesting observation was that *pri-mir-84* levels oscillated out of phase with other pri-miRNA transcripts, indicating that *mir-84* transcription is regulated differently from its paralogs. This may be due, at least in part, to specific roles for *mir-84* in regulating vulval fate specification (Hayes et al., 2011;Johnson et al., 2005).

How is the cycling of primary transcripts achieved? Although LIN-42 levels oscillate over larval development, it is not required for pri-miRNA cycling (Tennessen et al., 2006). This was surprising given that PERIOD is important in maintaining oscillations of circadian regulated genes in other systems. In *C. elegans*, however, the oscillation of pri-miRNA transcript levels is likely promoted by one or more transcription factors whose activities are negatively regulated by LIN-42. A recent report indicates that a large proportion of the *C. elegans* transcriptome oscillates over larval development, and many genes whose mRNA levels cycle are involved in molting (Kim et al., 2013). Given that *lin-42* also regulates molting, it is likely that factors the regulate transcription of genes involved in molting may also regulate the oscillation of pri-miRNA levels (Chapter II). A small RNAi screen for genes that may affect the expression of a

Plet-7::gfp reporter has identified several factors that may regulate *pri-let-7* (Kai et al., 2013).

A very interesting candidate for regulating pri-miRNA expression is *nhr-25*. This gene encodes a nuclear hormone receptor that is required for molting and several other developmental events (Gissendanner and Sluder, 2000). Like *lin-42*, *nhr-25* mRNA levels oscillate over larval development, therefore, it is possible that *nhr-25* may periodically promote pri-miRNA transcription in concert with the larval molt cycle (Gissendanner and Sluder, 2000). *nhr-25(RNAi)* results in multiple defects including molting and developmental timing (Chen et al., 2004;Hada et al., 2010). *nhr-25(RNAi)* also causes embryonic and larval lethality, therefore a strong loss-of-function null allele would likely be lethal. However, RNAi experiments have shown that knockdown of *nhr-25* results in a retarded heterochronic phenotype, opposite of *lin-42.* Additionally*, nhr-25(RNAi)* in a *lin-42(lf)* background partially suppresses *lin-42* heterochronic phenotypes, suggesting they act in opposition to each other in timing (Hada et al., 2010). *nhr-25(RNAi)* also abrogated the expression of a *Plet-7::gfp* reporter, therefore, it may be necessary to up-regulate *pri-let-7* (Kai et al., 2013). Whether *nhr-25* affects the expression of other miRNA promoter fusions has not been tested. In addition to *nhr-25*, several other genes involved in molting also affected expression from the reporter (Kai et al., 2013). It will be intriguing to investigate which of these genes are involved in the cycling of pri-miRNA transcripts.

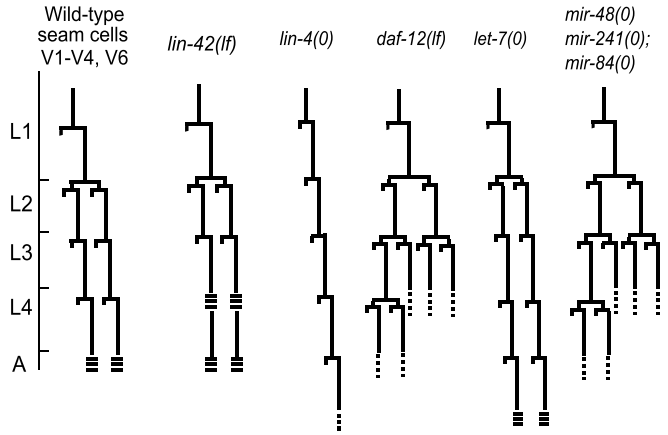
Gene expression studies have found that some miRNAs are regulated by circadian clocks in *Drosophila* and mammals, and this is dependent on the

period circadian feedback loop (Reviewed in, (Hansen et al., 2011;Kojima et al., 2011)). Therefore, regulation of miRNAs by PERIOD-like proteins appears to be conserved. Further investigation of *lin42/per* activity in regulating miRNA expression will provide further insight into how PERIOD-like proteins regulate gene expression and development.

Figure 1: miRNAs regulate developmental timing in C. elegans

A. Lineage diagrams of a seam cell in wild-type animals, *lin-42* mutants, and heterochronic miRNA mutants. Vertical lines denote time, while horizontal bars represent a cell division. **B.** Simplified representation of the heterochronic gene pathway. See text for details. miRNAs promote developmental transitions in C. elegans post-embryonic development by inhibiting expression of transcription factors that promote specific larval programs.

A


Wild-type
seam cells
V1-V4, V6
lin-42(lf)
lin-4(0)
daf-12(lf)
let-7(0)
mir-48(0)
mir-241(0);
mir-84(0)
L1
L2
L3
L4
A

B

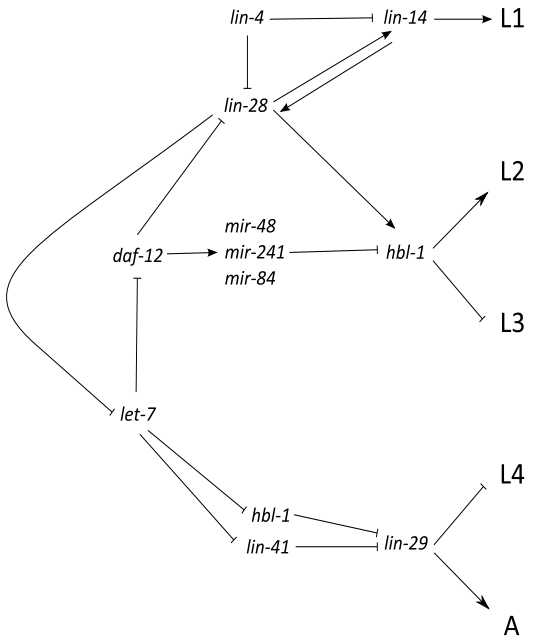

lin-4
lin-14
L1
lin-28
L2
mir-48
mir-241
mir-84
daf-12
hbl-1
L3
let-7
L4
hbl-1
lin-41
lin-29
A

Figure 1

Figure 2: let-7 miRNA accumulates precociously in *lin-42(lf)* mutants

A. Graph of fold change in *mlt-10* expression levels assayed by Taqman qRT-PCR in wild-type and *lin-42(lf)* mutants. *mlt-10* levels were measured at two-hour intervals starting 12hrs after starved L1s were placed on food and extending to 56 hrs. All timepoints are normalized to wild-type at 12hrs. Approximate stages based on *mlt-10* expression are indicated with blue bars for wild-type and red bars for *lin-42(lf*). A representative biological replicate is shown**. B**. Graph of fold change in let-7 miRNA levels in wild-type and *lin-42(lf)* mutants across the 18-56 hr samples analyzed in A by Taqman qRT-PCR. All time-points are normalized to wild-type at 18hr, when let-7 miRNA is detectable above background, but expressed at low and relatively constant levels. Data are representative of three biological replicates (See Figure S2). **C**. the L2 data from (B) are replotted to emphasize the early rise in let-7 accumulation observed in *lin-42(lf)*, using the *mlt-10* expression profile to normalize stages. The onset of the L2 was delayed by approximately 4hrs in *lin-42(lf),* starting at ~22hrs rather than ~18hrs, but was the same duration, ~10hrs. Therefore, to compare developmentally similar time-points the wild-type axis extends from 18-28 hrs, while *lin-42(lf)* axis spans 22-32 hours. The data sets were plotted using the scatterplot function in Microsoft Excel. **D.** The level of let-7 miRNA accumulation at the time of *mlt-10* peak during the L2 and L3-stage of *lin-42(lf)* and wild-type was averaged over several biological replicates. For example, in the biological replicate shown in (A), the let-7 miRNA levels at 24hr in wild-type and 28hr in *lin-42 (lf)* are compared. At these time points, the populations are developmentally similar. let-7 miRNA levels were reproducibly and significantly elevated in the L2 in *lin-42(lf)* compared to wild-type. At the L3 *mlt-10* peak timepoint, there was no significant difference between the two strains. (**p=0.002 Welch's t-test. Error bars represent S.D. n=3 biological replicas with three technical replicates for each timepoint.)

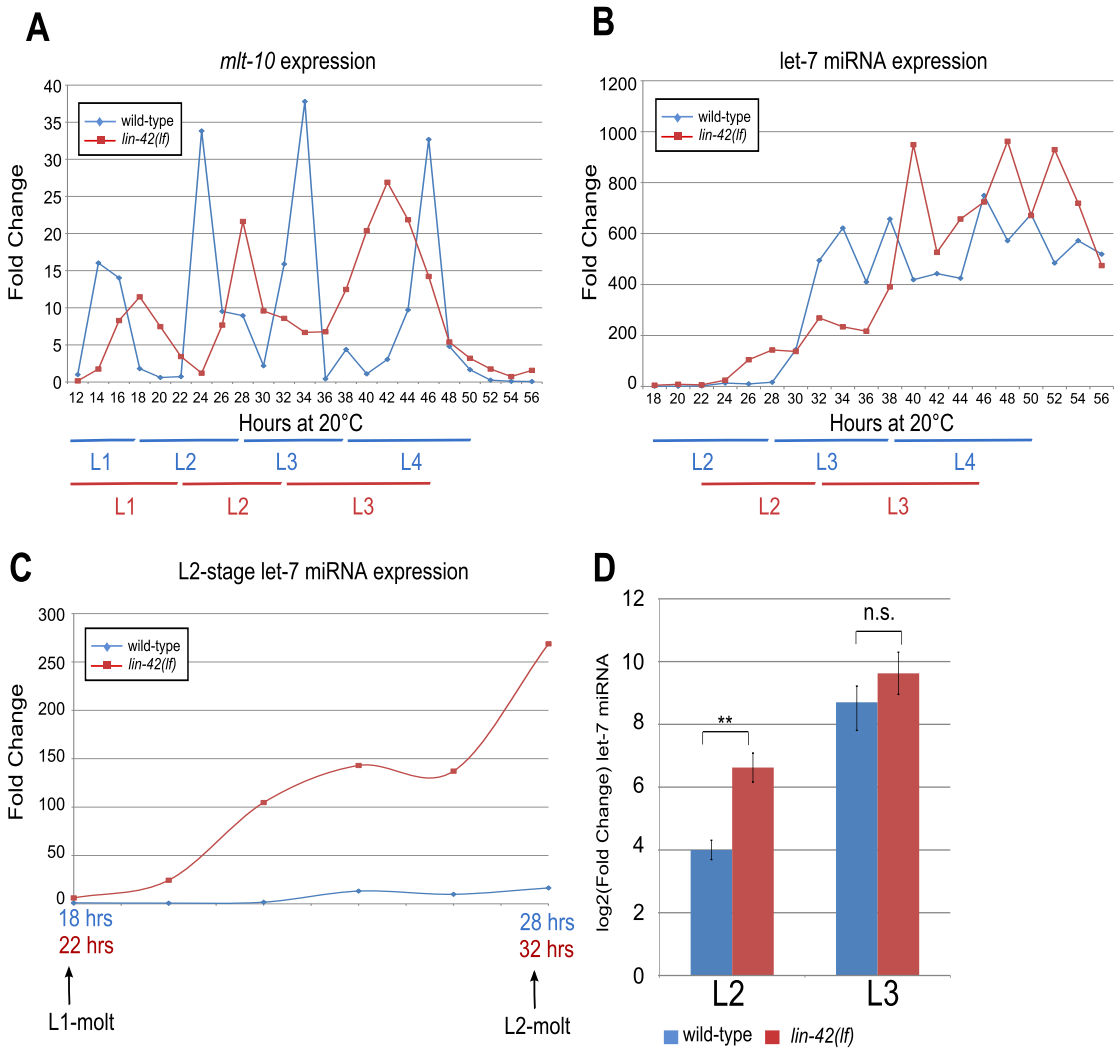

A
mlt-10 expression
wild-type
lin-42(lf)
Fold Change
Hours at 20°C
L1
L2
L3
L4
B
let-7 miRNA expression
wild-type
lin-42(lf)
Fold Change
Hours at 20°C
L2
L3
L4
C
L2-stage let-7 miRNA expression
wild-type
lin-42(lf)
Fold Change
18 hrs
22 hrs
28 hrs
32 hrs
L1-molt
L2-molt
D
log2(Fold Change) let-7 miRNA
**
n.s.
L2
L3
wild-type
lin-42(lf)

Figure 2

Figure 3: miR-48 levels are increased in the mid-to-late late L1 in *lin-42* mutants, but other tested miRNAs are not.

A. *mlt-10* mRNA levels were tracked to approximate the length of each stage in wild-type, *lin-42(lf)* and *lin-42(0)* mutant animals. Samples were collected from 6-38 hrs to span the L1-L3 stage in wild-type, with the exception of *lin-42(0)* animals which were collected up to 24 hrs, after which these mutants begin to arrest development due to a molting defect. All timepoints are normalized to wild-type at 6hrs. Approximate stage lengths are indicated by blue bars for wild-type, red bars for *lin-42(lf),* and green bars for *lin-42(0).* Representative of ≥3 biological replicates, except for lin-42(0), where n=2. **B.** Graphs of fold change in levels of lin-4 miRNA, miR-241, miR-84, and miR-48 in synchronized populations of wild-type, *lin-42(lf),* and *lin-42(0)* animals used in (A). Approximate larval stage durations for each strain based on *mlt-10* expression are indicated by bars along the x-axis. For all panels, wild-type is blue, *lin-42(lf)* is red*,* and *lin-42(0)* is green. **C.** miR-48 data for the L1 stage from (B) was replotted to emphasize the increased levels in *lin-42* mutants relative to wild-type.The L1 stage was 1.1X longer than wild-type in *lin-42(lf)* and 1.2X longer in *lin-42(0)* animals. The timepoints for these mutants were compressed by these factors to fit the mutant time-scales onto wild-type, allowing developmentally similar stages to be compared. **D.** Graph of average miRNA levels during the L1 stage plotted as $\log_2$ (Fold Change). As in Figure 2D, the timepoint of the *mlt-10* expression peak was compared. (n=3 biological repetitions, with 3 technical replicas per timepoint. * $p<0.05$. Welch's t-test. n.s., not significant.)

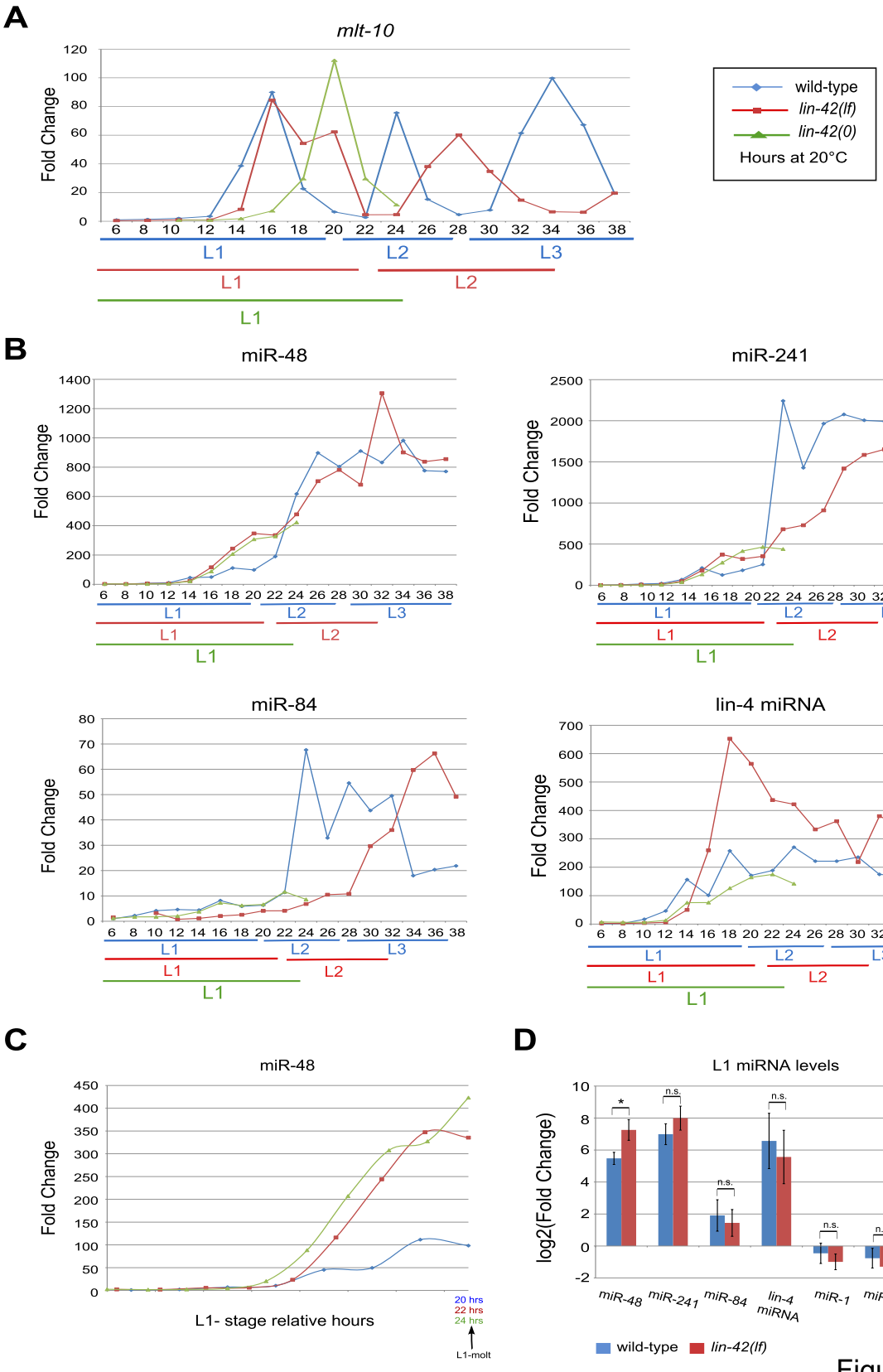

A
mlt-10
Fold Change
wild-type
lin-42(lf)
lin-42(0)
Hours at 20°C
L1
L2
L3
B
miR-48
miR-241
miR-84
lin-4 miRNA
C
miR-48
L1- stage relative hours
20 hrs
22 hrs
24 hrs
L1-molt
D
L1 miRNA levels
log2(Fold Change)
miR-48
miR-241
miR-84
lin-4 miRNA
miR-1
miR-51
miR-58
wild-type
lin-42(lf)

Figure 3

Figure 4: Heterochronic pri-miRNA transcript levels oscillate during larval development

Graphs of fold change in primary transcript accumulation for *let-7*, *lin-4*, *mir-48*, *mir-241*, *mir-84*, and *mlt-10* in wild-type animals from the mid-L1 to L3 stages. Samples analyzed are the same as in Figure 3. Data are representative of three biological replicates. *mlt-10* expression is shown with *pri-let-7* to indicate staging.

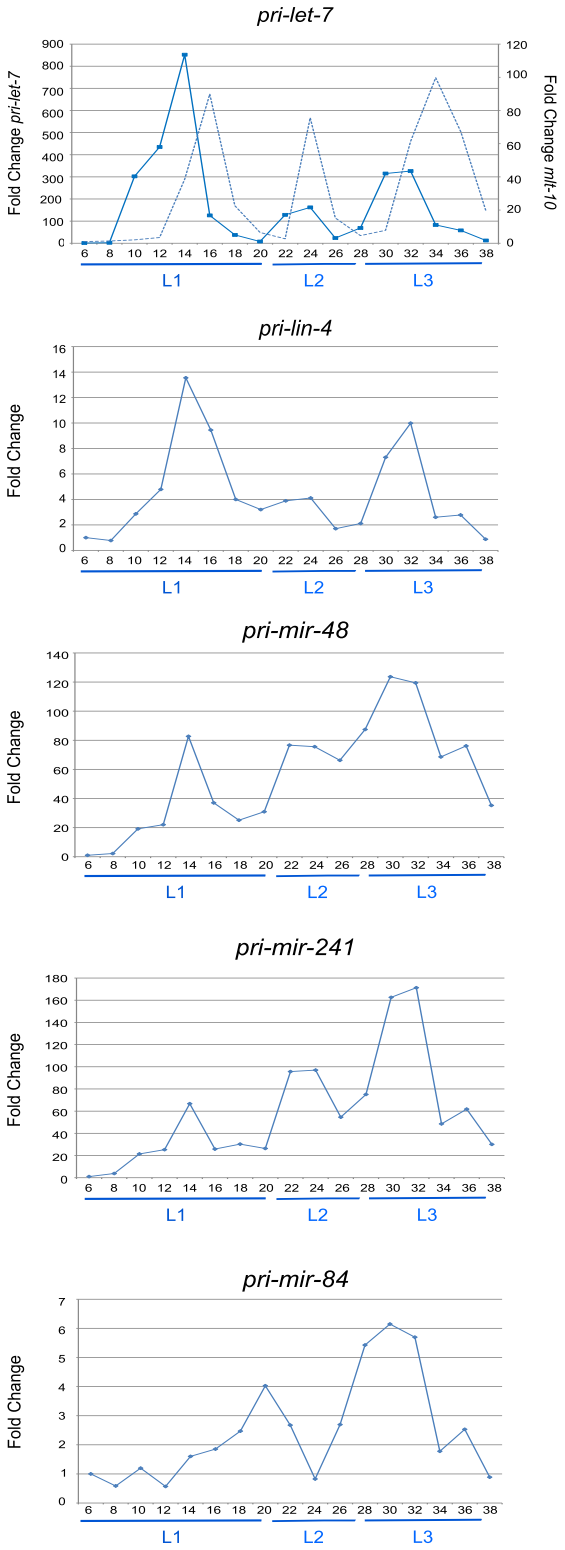

pri-let-7
Fold Change pri-let-7
Fold Change mlt-10
pri-lin-4
Fold Change
pri-mir-48
Fold Change
pri-mir-241
Fold Change
pri-mir-84
Fold Change
L1
L2
L3

Figure 4

Figure 5: Amplitude of *pri-let-7* and *pri-mir-48* expression during the L1-stage is significantly increased in a *lin-42(lf)* mutant.

pri-miRNA levels were assayed by Taqman qRT-PCR in total RNA isolated from synchronized populations of wild-type or *lin-42(lf)* mutants and graphed as fold change. All timepoints are normalized to wild-type at 6hrs. Stage lengths as determined by *mlt-10* expression are indicated by blue bars for wild-type and red bars for *lin-42(lf).* **A**. pri-let-7 levels in wild-type and *lin-42(lf)* mutants harvested at 2 hour intervals from 0-38 hours. In both wild-type and *lin-42(lf), pri-let-7* levels peak once per stage. **B.** *pri-lin-4*, *pri-mir-48*, and *pri-let-7* levels during the L1 and early L2 stage are graphed to the left in each panel. The amplitudes and lengths of the pri-let-7 and pri-mir-48 peaks are increased in *lin-42(lf)* animals. To obtain a relative measure of expression during the stage, the area under each L1 stage peak was measured as pixels^2 using ImageJ software (Rasband, 1997-2012). Bar graphs show the average of ≥3 biological replicates where each data point was analyzed in triplicate (**p<0.01, *p<0.05, Welch's t-test).

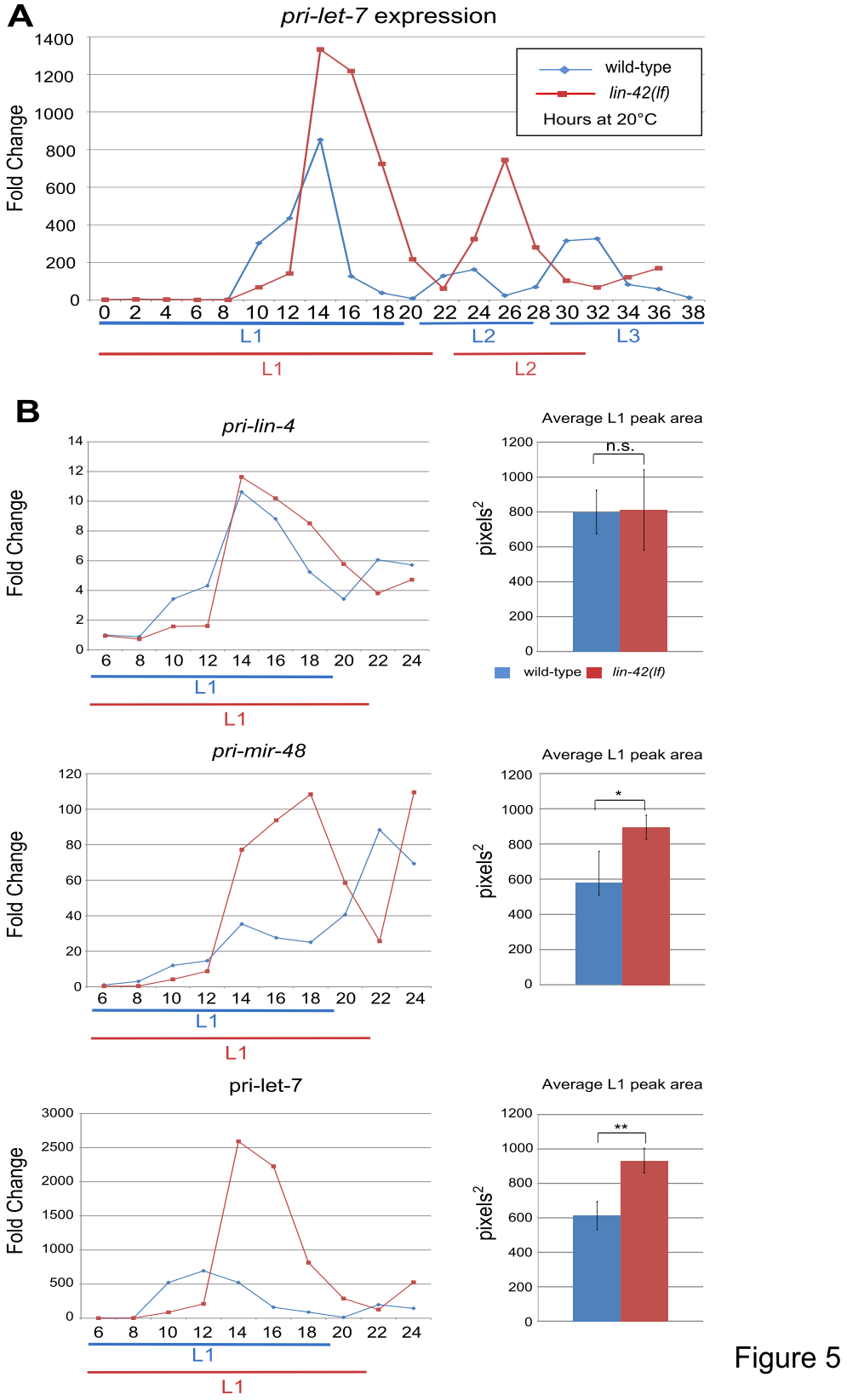

A
pri-let-7 expression
Fold Change
wild-type
lin-42(lf)
Hours at 20°C
L1
L2
L3
B
pri-lin-4
Average L1 peak area
n.s.
pixels2
wild-type
lin-42(lf)
pri-mir-48
*
pri-let-7
**

Figure 5

Figure 6: *lin-42(ox461)* suppresses the cuticle phenotype of *let-7 unc-3* but not *mir-48 mir-241; let-7 unc-3* mutants. Micrographs of the left lateral surfaces of representative worms of the indicated genotypes. Adult cuticle is secreted during the L4 molt of wild-type animals, during the L3 molt of *lin-42* mutants, and fails to be made during the L4 molt of *let-7* mutants. White bars indicate regions where hints of alae could just be discerned by eye. This weak alae is indicated as + in Table 1.

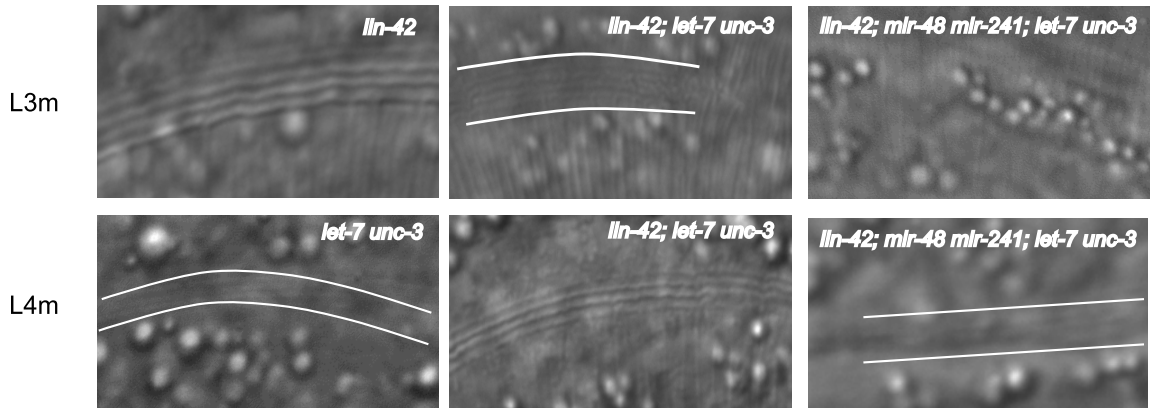

L3m
L4m
lin-42
lin-42; let-7 unc-3
lin-42; mir-48 mir-241; let-7 unc-3
let-7 unc-3
lin-42; let-7 unc-3
lin-42; mir-48 mir-241; let-7 unc-3

Figure 6

Table 1: Genetic interaction of *lin-42(0)* with let-7 miRNAs

	Animals with L3 molt alae (%)					Animals with L4 molt alae (%)				
	Complete	Partial	No alae	Alae Quality[a]	n	Complete	Partial	No alae	Alae Quality[a]	n
lin-42(ox461)	66	32	2	+++	87	nd[b]	nd	nd	nd	
let-7 unc-3	nd	nd	nd	nd		11	26	63	+	38
lin-42(ox461); let-7 unc-3	0	15	85	++/+	62	83	14	3	+++	59
nDf51; mir-84	nd	nd	nd	nd		0	57	43	++/+	35
lin-42(ox461); nDf51; mir-84	0	2	98	+	41	83	17	0	+++	35
nDf51	nd	nd	nd	nd		2	88	19	++	42
ox461; nDf51	0	0	100		25	97	3	0	+++	29
lin-42(ox461); nDf51; let-7 unc-3	0	13	87	+	30	19	22	59	+	37

[a] Alae varied in quality between strains but consistant within strains, with +++ being strongest to + being very weak and indistinct

[b] nd=not determined

Figure S1: Specificity of miRNA and pri-miRNA Taqman Assays

miRNA and pri-miRNA qRT-PCR Taqman analyses of RNA collected from mixed populations of N2 wild-type, VT1066 *mir-48 mir-241(nDf51); mir-84(n4037*), DR721 *lin-4(e912),* MT13650 *mir-48(n4097*), MT13651 *mir-84(n4037);* MT13897 *mir-241(n4316).* All measurements are normalized to wild-type.

A.-D. miRNA analysis in wild-type and indicated deletion strains.

E-F. pri-miRNA anlaysis in wild-type and indicated deletion strains.

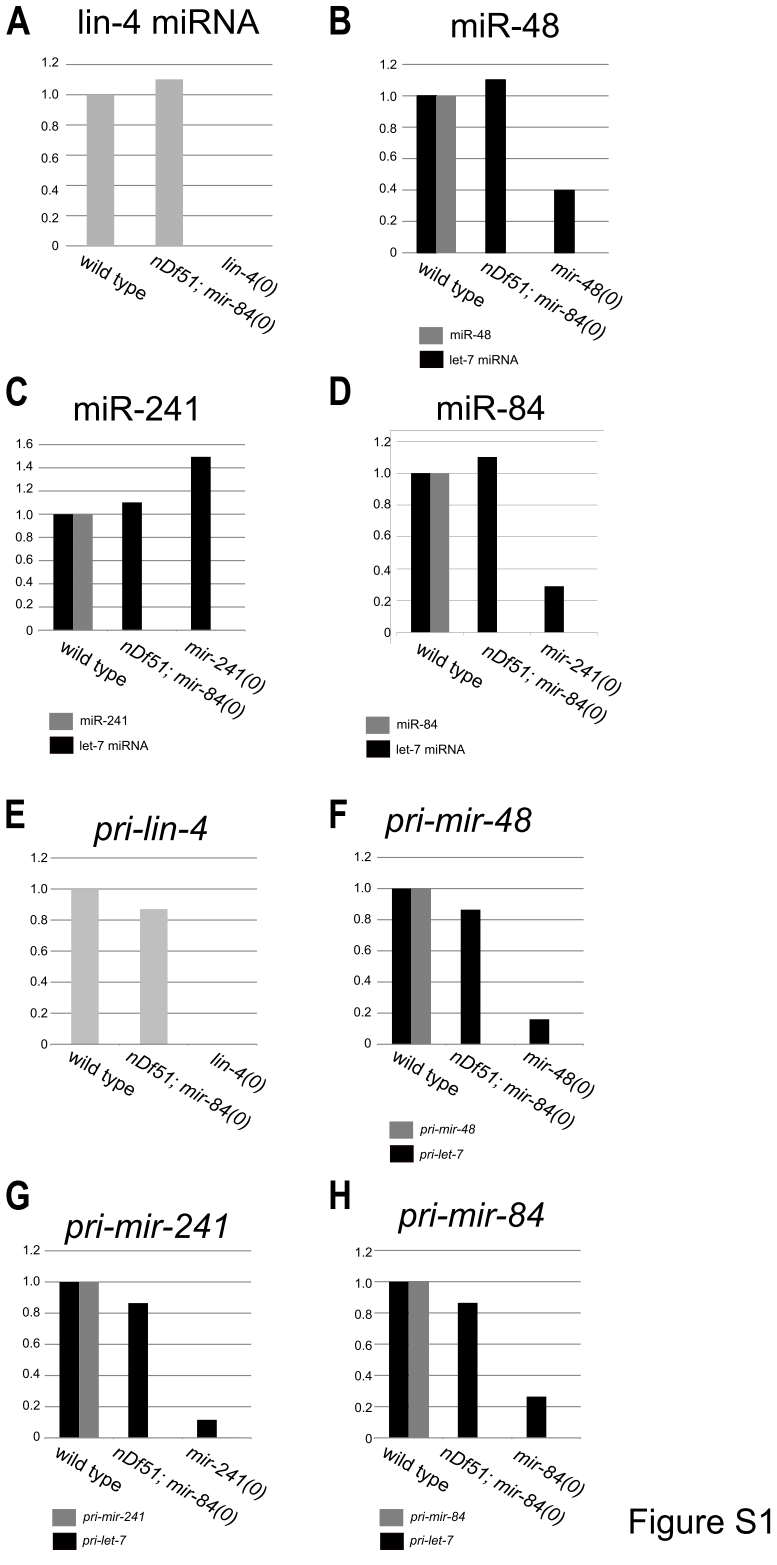

A
lin-4 miRNA
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
lin-4(0)
B
miR-48
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-48(0)
miR-48
let-7 miRNA
C
miR-241
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-241(0)
miR-241
let-7 miRNA
D
miR-84
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-241(0)
miR-84
let-7 miRNA
E
pri-lin-4
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
lin-4(0)
F
pri-mir-48
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-48(0)
pri-mir-48
pri-let-7
G
pri-mir-241
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-241(0)
pri-mir-241
pri-let-7
H
pri-mir-84
1.2
1.0
0.8
0.6
0.4
0.2
0
wild type
nDf51; mir-84(0)
mir-84(0)
pri-mir-84
pri-let-7

Figure S1

Figure S2: Biological replicates of miRNA expression patterns. The expression of lin-4 miRNA, miR-48, and let-7 miRNA in wild-type animals was measured in three biological replicates and normalized to wild-type at 6hrs. Although the general expression pattern of each miRNA is consistent between biological replicates (i.e. miR-48 is up-regulated at 20-24 hrs), the levels at a specific timepoint can vary.

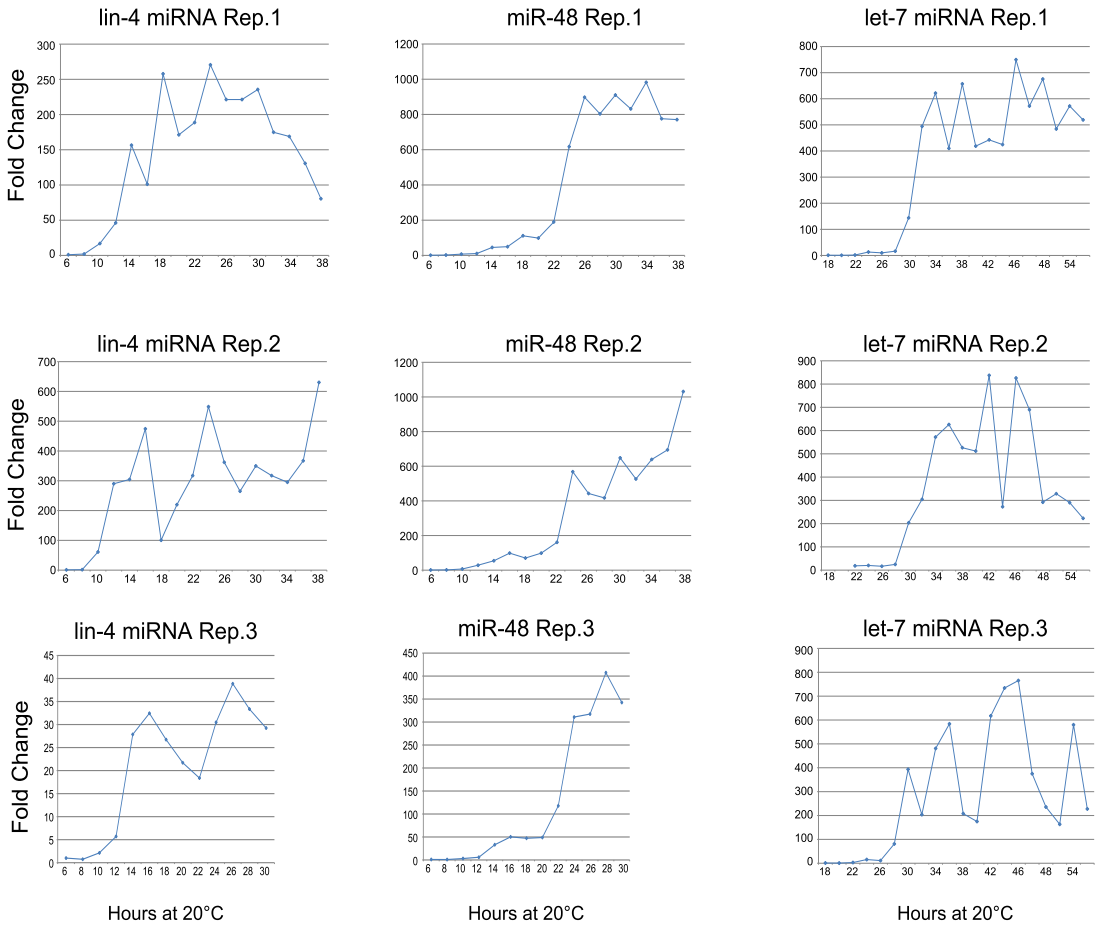

lin-4 miRNA Rep.1
miR-48 Rep.1
let-7 miRNA Rep.1
lin-4 miRNA Rep.2
miR-48 Rep.2
let-7 miRNA Rep.2
lin-4 miRNA Rep.3
miR-48 Rep.3
let-7 miRNA Rep.3
Fold Change
Hours at 20°C

Figure S2

Figure S3: let-7 miRNA and miR-48 are significantly up-regulated early in *lin-42(lf)*

Expression of lin-4 miRNA, miR-48, and let-7 miRNA was averaged over three biological replicates. *mlt-10* expression levels were used to identify mid larval stages and molts in each strain. The *mlt-10* peak was used as the mid-larval stage the molt sample was taken four hours later, and early-L1 is 2 hrs prior to the *mlt-10* peak. In these assays, average miRNA levels were consistently higher in *lin-42* mutants relative to wild-type, but a significant difference was only measured for miR-48 (early L1) and let-7 (L2 and L2 molt). ** < 0.01, * < 0.05.

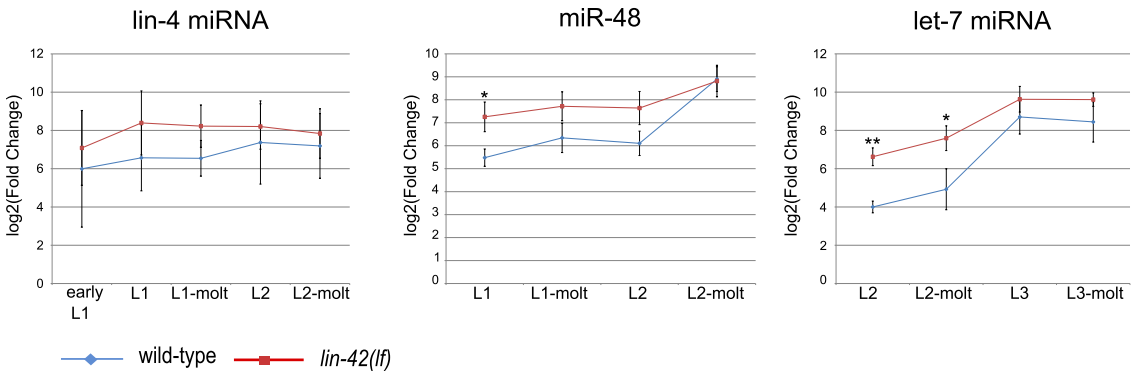

lin-4 miRNA
miR-48
let-7 miRNA
log2(Fold Change)
early L1
L1
L1-molt
L2
L2-molt
L3
L3-molt
*
**
wild-type
lin-42(lf)

Figure S3

Figure S4: Heterochronic pri-miRNA transcripts oscillate in *lin-42* mutants

Graphs of primary transcript levels for *mir-48*, *mir-241*, *mir-84* and *lin-4* in wild-type and *lin-42* mutants. All analyses for each strain were performed from the same biological replicate and data were normalized to the 6hr wild-type timepoint. The length of each stage is indicated by a blue bar for wild-type, a red bar for *lin-42(lf),* and a green bar for *lin-42(0)* based on *mlt-10* expression patterns.

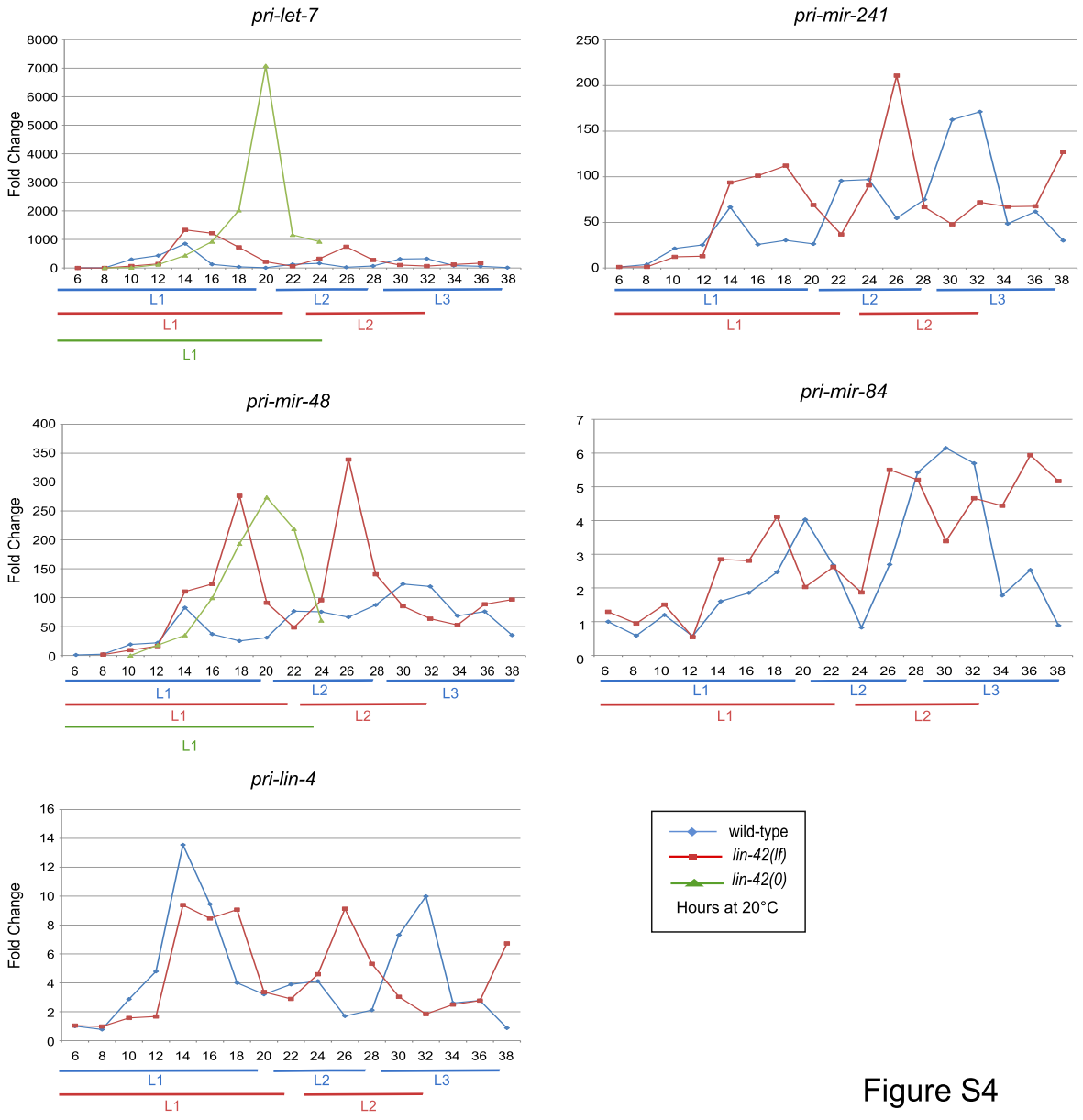

pri-let-7
pri-mir-241
pri-mir-48
pri-mir-84
pri-lin-4
Fold Change
L1
L2
L3
wild-type
lin-42(lf)
lin-42(0)
Hours at 20°C

Figure S4

CHAPTER V: PROSPECTUS

SUMMARY

The research described in this thesis reveals roles for *lin-42/per* in *C. elegans* developmental timing and molting pathways. I show that a critical function of *lin-42* in developmental timing is to regulate the expression of *let-7*-family miRNAs, likely at the transcriptional level. This places *lin-42* upstream of let-7-family miRNAs in the heterochronic pathway. I also show that *lin-42* has a more global role in maintaining robustness of developmental timing and molting pathways against environmental fluctuations. Here, I discuss the broader significance of these findings and suggest future directions.

PROSPECTUS AND FUTURE DIRECTIONS

Structure function analysis of *lin-42*

To aide in investigation of *lin-42* function in developmental timing and molting, we generated a null allele using mosDEL technology (Frokjaer-Jensen et al., 2010). This allele is the first described *lin-42* mutation that deletes the entire *lin-42* coding region. This lesion results in more severe heterochronic and molting defects than previously characterized hypomorphic alleles.

The *lin-42(0)* allele provides the opportunity to fully investigate the contribution of particular *lin-42* isoforms and sequences to *C. elegans*

development. All previous experiments were performed in a background with one of the isoforms still intact, complicating these analyses (Jeon et al., 1999;Monsalve et al., 2011;Tennessen et al., 2006). Here, we were able to test rescuing ability of different isoforms in isolation. These results were similar to earlier studies, in which only multi-copy arrays expressing Lin-42a rescued *lin-42(0)* mutants. However, this rescue may be due to over-expression. *lin-42(n1089)* mutants, in which Lin-42a is presumably intact, have precocious heterochronic defects, indicating this region is important in regulating developmental timing. Using the *lin-42(0)* background, we will be able to more fully test Lin-42a function by using a single-copy transgene to express this isoform at endogenous levels.

Recent advances in genome engineering allow for the relatively simple and stable introduction of single copy *lin-42* transgenes at specific genomic positions. CRISPR-Cas genome editing technology has become the method of choice to introduce specific lesions or transgenes into the *C. elegans* genome (Chen et al., 2013;Dickinson et al., 2013;Friedland et al., 2013;Katic and Grosshans, 2013;Lo et al., 2013;Tzur et al., 2013;Waaijers et al., 2013). This technique utilizes the Cas9 nuclease, a component of the prokaryotic adaptive immune system, to generate sequence specific double strand breaks (DSB) that are repaired by homologous recombination from a supplied template. Using this system, specific *lin-42* sequences could be introduced into the null background at single-copy to test for rescue. Therefore, we can ask whether specific isoforms rescue the *lin-42(0)* mutants when expressed at endogenous levels.

It will also be of interest to determine which residues in the LIN-42 conserved domains are required for *lin-42* activity. Surprisingly, none of the extant *lin-42* alleles isolated in our lab or elsewhere have isolated missense alleles, and the requirement for specific domains of LIN-42 in regulating development in unknown. The PAS domains are the most highly conserved between LIN-42 and PERIOD (Jeon et al., 1999). These domains are protein interaction domains, and, indeed, LIN-42B and LIN-42C isoforms containing these domains can bind each other in yeast-2-hybrid experiments, indicating they may dimerize *in vivo* (Gardner, 2005). In addition, a conserved tryptophan residue within the PAS domain is required for homodimerization of LIN-42B *in vitro* (C. Partch, personal communication), indicating that this residue may be critical for function of LIN-42, similar to PERIOD, in which this residue is necessary for homodimerzation (Hennig et al., 2009;Kucera et al., 2012). CRISPR-Cas technology can modify endogenous sequences, and it would be of interest to alter this residue in *lin-42* and test if mutation of the conserved tryptophan produces a phenotype.

The C-terminal LT and SYQ domains are short domains that are also conserved between LIN-42 and PERIOD. The requirement of all of these domains for LIN-42 function can be tested by introducing alanine mutations into these sequences and determining whether extra-chromosomal arrays expressing mutated *lin-42* can rescue *lin-42* phenotypes. Modification of the endogenous locus with CRISPR-Cas could be used to validate any potentially interesting mutations.

In addition to testing isoform-specific rescue, tissue-specific rescue experiments in the *lin-42(0*) background would be of interest, where the only *lin-42* expressed is from transgenic DNA. It will be intriguing to investigate more thoroughly from which tissues *lin-42* regulates heterochronic and molting defects. For example, over-expression of Lin-42a from its native promoter rescues both the heterochronic and molting phenotypes of *lin-42(0)* animals. Because *lin-42(0)* mutations cause defects in molting behaviors as well as ecdysis, it is possible that *lin-42* may regulate molting via a neuro-endocrine system. Therefore, a key question is if Lin-42a is over-expressed in the hypodermis only, will rescue of both molting and heterochronic phenotypes still be observed? Such studies will provide insight into the mechanism by which *lin-42* regulates these different developmental pathways.

lin-42 maintains robustness of *C. elegans* molting and developmental timers against environmental fluctuations

Work in this thesis shows that *lin-42* functions to confer robustness on developmental timing and molting pathways against environmental fluctuations. Although here I describe a mechanism by which the heterochronic phenotypes are modulated by temperature, still unclear is how insulin and TGFβ signaling may modulate developmental timing. ChIP and other gene expression analyses have been performed to identify regulatory targets of *daf-16*, the downstream transcriptional output of the insulin signaling pathway (McElwee et al.,

2003;Murphy et al., 2003). These studies were primarily carried out in adults to identify factors involved in longevity caused by decreased insulin signaling, but this timeframe is not relevant for identifying genes involved in larval developmental timing. Performing gene expression analyses, such as RNA-seq, in *daf-2(lf)* mutants compared to wild-type larvae may reveal heterochronic genes with altered expression patterns in conditions of reduced insulin signaling. Similar experiments could be performed to understand which heterochronic genes may be regulated by changes in TGFβ signaling.

In addition to developmental timing, *lin-42(lf)* reveals that molting can also be affected by temperature, as the molting defects of *lin-42(0)* animals is strongly suppressed when animals are raised at 25°C rather than 20°C. How the molting cycle is modulated by temperature stress is unknown, as little is understood about how molting is regulated at the systemic level in *C. elegans*. Genome-wide RNAi screens have revealed some players; however, most of the genes identified are necessary for construction and shedding of the cuticle (Frand et al., 2005). Although some genes, such as transcription factors, that may globally regulate molting were identified in this screen, they have not been organized into a regulatory pathway. The sensitized *lin-42* background provides an opportunity to identify additional genes involved in the molting pathway. One possible method to identify such factors would be to perform a genetic screen for genes required for the temperature suppression of *lin-42(0)* molting defects. Genes isolated in such a screen may function in parallel with *lin-42* to regulate molting, which might be lethal at lower temperatures in combination with *lin-42.* This may

reveal new players in the molting pathway. In addition, a suppressor screen preformed at 20°C would be useful in identifying genes that function downstream of *lin-42* to regulate molting.

Regulation of miRNA accumulation by *lin-42/per*

I have established that *lin-42* primarily regulates developmental timing by inhibiting the expression of *pri-let-7* and *pri-mir-48*. The levels of these transcripts, and the mature miRNAs derived from them, are elevated in *lin-42(lf)* compared to wild-type. A key future question, then, is how LIN-42 acts to inhibit pri-miRNA expression. One possibility is that LIN-42 negatively regulates transcription by binding to transcription factors and inhibiting their activity. In *Drosophila*, PERIOD proteins regulate transcription by binding to CLK::CYC heterodimers, interfering with up-regulation of circadian-regulated genes (Lee et al., 1999). The fact the LIN-42 is localized to discrete puncta in nuclei suggests that LIN-42 may function similarly (Tennessen et al., 2006). ChIP experiments should reveal if LIN-42 localizes to *let-7* and *mir-48* promoter regions. Such studies could be facilitated by use of CRISPR-Cas technology to introduce an epitope tag for biochemical purifications at the endogenous *lin-42* locus (Lo et al., 2013).

In addition to determining whether LIN-42 is associated with miRNA promoter sequences, another key question is whether LIN-42 interacts with other proteins to regulate transcription. Several proteins that control heterochronic miRNA expression have been identified that *lin-42* may interact with to regulate

pri-miRNA transcription. For example, ligand-bound DAF-12 directly promotes the transcription of *let-7*-family miRNAs during early larval stages (Bethke et al., 2009). Therefore, it is possible that LIN-42 may interact with DAF-12 to affect its regulation of pri-miRNA transcription. Indeed, DAF-12 and LIN-42B have been shown to bind each other in a yeast-2-hybrid system, consequently, they may also interact *in vivo* (Tennessen et al., 2010).

Additionally, LIN-28 has been shown to regulate *pri-let-7* processing co-transcriptionally and, by ChIP analysis, binds to endogenous *let-7* sequence (Vadla et al., 2012;Van Wynsberghe et al., 2011). An attractive possibility is that LIN-28 may interact with LIN-42 at the *let-7* locus. ChIP or coIP experiments could be used to test if LIN-42 and LIN-28 interact to regulate let-7 miRNA accumulation.

lin-42 and pri-miRNA oscillations

A striking finding in this work is that all heterochronic pri-miRNA transcripts oscillate over time. This is surprising as the expression patterns of primary transcripts do not correspond to the expression patterns of the miRNAs derived from these transcripts. For example, *pri-let-7* transcript levels peaks once per stage, even during the L1, two stages prior to up-regulation of the let-7 miRNA itself. It is likely that the transcription of these miRNAs is regulated by similar factors, however, post-transcriptional mechanisms, such as LIN-28 regulation of *pri-let-7* processing, must be in place to ensure that specific mature miRNAs are

produced at the appropriate time of development (Vadla et al., 2012;Van Wynsberghe et al., 2011).

The timing of the most of the heterochronic pri-miRNA oscillations, save for *pri-mir-84*, overlaps with the *lin-42* mRNA and protein expression pattern. For example, *pri-let-7* and *pri-mir-48* levels as well as LIN-42 protein levels are high at approximately 12 hrs of development in the L1-stage (Tennessen et al., 2006). Therefore, LIN-42 is expressed at the appropriate time to regulate these oscillations. It was a surprise to find that *lin-42* function is not required for pri-miRNA oscillations, indicating other factors are necessary for these patterns. How are these oscillations achieved? Is *lin-42* transcription also regulated by the same factors? These are critical questions to be resolved.

Several lines of evidence suggest *nhr-25* and *nhr-23* may be involved in regulating the expression of *lin-42* and pri-miRNAs of the heterochronic pathway. *nhr-25* and *nhr-23* encode orphan nuclear hormone receptors with roles in molting and heterochrony. RNAi knockdown of *nhr-25* results in severe molting defects and mild retarded heterochronic phenotypes (Gissendanner and Sluder, 2000;Hada et al., 2010;Hayes et al., 2006). Additionally, *nhr-23(RNAi)* results in molting defects and larval arrest (Kostrouchova et al., 1998). RT-PCR and transcriptional reporters indicate that *nhr-25/23* levels oscillate over development in a similar pattern as *lin-42* and heterochronic pri-miRNAs (Gissendanner and Sluder, 2000;Kostrouchova et al., 1998). As NHRs primarily function as transcription factors, these genes are primary candidates for regulating oscillations of *lin-42* and pri-miRNA transcripts. Therefore, *lin-42*, *nhr-25*, and

nhr-23 may be key factors that integrate the molting and developmental timing pathways of *C. elegans* larval development.

qRT-PCR experiments similar to those performed Chapter IV could be used to determine if *nhr-25* or *nhr-23* is necessary for oscillation of *lin-42* and/or pri-miRNA transcript levels. *nhr-25(ku217)* is a loss-of-function allele that results in molting defects without resulting in a highly penetrant of embryonic or larval arrest as observed when *nhr-25* is knocked down by RNAi (Chen et al., 2004). Synchronized larval population of these *nhr-25* mutants could be collected to measure expression of *lin-42* isoforms, as well as pri-miRNAs levels in these mutants compared to wild type, and determine if these expression patterns are altered when activity of this NHR is reduced.

nhr-23 mutants are inviable, however, progeny of adult wild-type animals fed *nhr-23(RNAi)* can progress through the first molt prior to onset of larval arrest, similar to *lin-42(0*) animals (Kostrouchova et al., 1998). Collecting sufficient samples for qRT-PCR experiments would be very challenging in this case. However, monitoring the expression of transcriptional reporters for *lin-42* and miRNAs in animals treated with *nhr-23(RNAi)* may also be useful to determine if *nhr-23* regulates the expression of these genes.

Also of interest is to understand further the expression patterns of specific *lin-42* isoforms. Initial analysis of *lin-42* expression using RT-PCR only measured expression of PAS-encoding isoforms, and found that the levels of these transcripts oscillate over larval development (Jeon et al., 1999). Additionally, the antibody showing the LIN-42 oscillated with the molts was raised

against the C-terminal sequence of the protein, which could detect both LIN-42A and LIN-42B isoforms (Tennessen et al., 2006). However, RT-PCR experiments to specifically detect Lin-42a indicate that this isoform may be expressed at or near the onset of each lethargus (Monsalve et al., 2011). Comprehensive analyses of the developmental expression patterns of each of these isoforms would clarify when specific isoforms are expressed during development and indicate if these isoforms have specific functions in molting or developmental timing. One straightforward approach to addressing this question would be to measure the levels of specific isoforms in wild-type animals over time by qRT-PCR, similar to analyses of pri-miRNA oscillations shown in Chapter IV. Additionally, using the CRISPR-Cas system, multiple fluorescent tags could be inserted into the endogenous locus to label each isoform. Although less quantitative than qRT-PCR, mRNA patterns may not necessarily reflect protein expression patterns. Additionally, monitoring accumulation of the protein would reveal if specific isoforms are expressed in different tissues during development.

lin-42/per integrates developmental timing, molting, and stress response pathways in *C. elegans*

My work describes roles for *lin-42/per* in developmental timing and molting. In addition, *lin-42* is necessary to confer robustness on developmental pathways, and *lin-42* phenotypes are susceptible to environmental stress. Finally, *lin-42* regulates developmental timing by dampening the oscillation of *let-*

7 and *mir-48* primary transcript levels. Although this work identifies through which genes *lin-42* acts to regulate developmental timing, key future questions include elucidation of the molecular mechanism of *lin-42* function, as well as identifying other factors are involved in regulating miRNA expression and molting in concert with *lin-42.*

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Appendix I. Identification of potential LIN-42 binding partners by co-immunoprecipitation and mass spectrometry

lin-42 is a vital component of the *C. elegans* heterochronic gene pathway, and mutation of *lin-42* results in severe precocious heterochronic phenotypes (Abrahante et al., 1998;Jeon et al., 1999;Tennessen et al., 2006). In addition to its roles in developmental timing, *lin-42* also is required for execution of larval molts (Monsalve et al., 2011). Research described in this thesis provides a clearer picture of how *lin-42* functions and where it fits in the heterochronic pathway. An important role for *lin-42* is to inhibit expression of pri-miRNA transcripts. However, still unclear is the molecular mechanism by which *lin-42* regulates the expression of pri-miRNAs.

lin-42 is the *C. elegans* homolog of *period* genes of flies and mammals. *period* is a key player in the circadian timekeeper, and functions primarily by binding to transcriptional activators in the nucleus and inhibiting their activity. Evidence to date suggests that LIN-42 is likely to have a similar function in worms. First, LIN-42 has been shown to localize to the nucleus by immunostaining (Tennessen et al., 2006). Additionally, although LIN-42 does not contain any clear DNA-binding domains, it does encode two domains with homology to PERIOD that are thought to be important for protein interactions in the circadian clock (Jeon et al., 1999;Tennessen et al., 2006). Finally, as shown in Chapter IV, *lin-42(lf)* results in increased expression of *pri-let-7* and *pri-mir-48* transcripts, suggesting it does indeed regulate transcription. Taken together,

these data indicate that LIN-42 regulates gene transcription through protein interactions. To identify *in vivo* partners of LIN-42, we generated an epitope-tagged LIN-42 transgenic line to immunoprecipitate (IP) LIN-42 from whole worm lysates. Purified LIN-42 complexes can then be subjected to mass spectrometry analyses to identify potential binding partners.

METHODS

Construction of lin-42::LAP transgenic strains

To generate LAP-tagged *lin-42* , the LAP-tag DNA sequence, which encodes S –TEV-GFP, was PCR amplified from pCS410 with primers inserting 5' EcoR1 site and 3' Not1 site (Oldenbroek et al., 2013). Primers used were KM40/KM29: AAACGAATTCTTAAGGAGACAGCTGCA/CAGCGGCCGCTCATTTGTAT. The PCR product was digested with EcoR1 and Not1 and replacing the GFP tag in pJT97, generating pKM30, which encodes LIN-42B with a C-terminal LAP-tag (Tennessen et al., 2010).

To construct transgenic strains, 2.5 ng/ul of pKM30 and 100 ng/µl pBX (*pha-1*+ rescue) were injected into RG1607 *lin-42(ve11);pha-1 (e2123)* animals and transformation was identified by *pha-1* rescue. Extra-chromosomal arrays were integrated by UV irradiation to generate stable lines. ~100 L4 transgenic animals were transferred into 1ml M9 buffer, washed twice in M9, and dropped onto a 10cm unseeded NGM plate. After drying, the plate was irradiated with

300 j/m2 using a Stratalinker. After irradiation, OP50 was pipetted onto plates and worms were placed at 20°C to recover. ~250 F1s were singly picked and progeny were screened for high transmittance of the array. From those plates showing high transmittance, 7-8 F2s were singly picked and these plates were screened for 100% transmission of the array to F3. This strain was RG1615 *lin-42(ve11); pha-1(e2123); veIs56* was used for analysis.

Liquid culture and Protein Preparation

For more detailed protocols, see Appendix 2.

Generation and collection of synchronized populations for protein preparations was performed essentially as described (Seggerson et al., 2002)). Approximately 2-3L of worms were raised in liquid S complete media supplemented with HB101 bacteria until gravid adults were present (approximately 3 days). Eggs were isolated by hypochlorite treatment, and were allowed to hatch overnight in M9 buffer. Hatched L1s were cultured in HB101 liquid cultures for 24hrs. Worms were then sucrose floated and drop-frozen in liquid nitrogen in an equal volume of lysis buffer (50mM HEPES, 1mM EGTA, 1mM $MgCl_2$, 100mM KCl, 10% glycerol, 0.05% NP-40, with 2X protease inhibitors). Frozen worm pellets were stored at -80°C.

To isolate proteins, the frozen worm pellet was ground in a mortar and pestle into a fine powder. This was transferred to a microfuge tube and an equal volume of lysis buffer + protease inhibitors was added. This mixture was sonicated for 15s total, 5s with 30s breaks three times at 15% amplitude.

Lysates were spun at >12,000g in the cold for 5 minutes to clear debris. The supernant was used in IPs.

Immunoprecipitation

Several antibodies were used in various experiments to isolate GFP-tagged LIN-42 protein (See Appendix 2). In the experiment described here, the GFP-TRAP system was used (Chromotek). This system utilizes a recombinant GFP antibody, which was originally raised in Llama. These animals have very small, single chain IgG molecules, allowing for its cloning and expression in bacteria.

50µl of GFP-TRAP bead slurry was combined with 1ml of worm lysate in a 1.5ml microfuge tube. Five tubes in total were used, for a total of 5ml lysate and 250µl beads. The lysate was incubated with beads for 1hr at 4°C with rotation. Beads were washed 3X in wash buffer and then incubated with 50µg TEV protease for 2hrs at RT. The supernatant was removed to a new tube and protein concentrated by TCA precipitation. 50µl of the TEV elution was saved for western blot analysis. A 1/10 volume of 100% TCA was added to the TEV elution and the tube incubated at -20°C for two hours. The tube was spun and the pellet was washed three times with acetone. Washed protein pellet was sent for MudPIT analysis in the laboratory of James Wohlschegel at UCLA as described (Duchaine et al., 2006;Law et al., 2010).

Western Blotting

Samples were boiled in SDS sample buffer for 5 minutes (Bowen et al., 1980). SDS-PAGE and transfer was performed with a BioRAD Mini-Protean system (Bio-Rad Laboratories). Samples were run on a precast 4-15% gradient gel (Bio-Rad laboratories). Protein was transferred to a PVDF membrane for immunoblotting (Millipore). Blots were probed with either rabbit anti-GFP (Santa Cruz Biotechnology sc-8334) or rabbit anti-S tag (abcam 18588). A 1:2500 dilution of anti-rabbit 680 (Life Technologies) was used as secondary antibody and blots were imaged with a LiCor Fluorescent Imaging System.

RESULTS & DISCUSSION

LIN-42B::LAP is expressed in the hypodermis

Co-immunoprecipition coupled with mass spectrometry analysis was used to identify *in vivo* LIN-42 binding partners. For isolation of LIN-42 from whole worm lysates, transgenic lines carrying an integrated multi-copy array expressing LIN-42B::LAP, was generated. The LAP tag is a multi-component epitope tag that encodes GFP-TEV-S (Cheeseman and Desai, 2005). The TEV sequence is a cleavage site that can be digested by TEV protease during purification (Figure A1). The *lin-42b::*LAP transgene is expressed in hypodermal nuclei and can rescue *lin-42(lf)* phenotypes (Figure A2).

Isolation of LIN-42B from whole worm lysates

Large-scale immunoprecipitation using GFP-TRAP beads was performed in parallel from wild-type and LIN-42::LAP strains (see Material and Methods for

details). Synchronized worms were grown to the mid-L2 stage for collection. We focused on the L2 stage for several reasons. First, LIN-42 protein is periodically expressed for just a few hours each stage (Tennessen et al., 2006). As synchronized cultures progress through development, they become progressively asynchronous, therefore, in older populations, fewer transgenic worms would be expected to express LIN-42B::LAP at the same time. Secondly, genetic experiments described in this thesis and elsewhere have shown that *lin-42* interacts with several genes whose key function is during the L2, such as *lin-28* and *daf-12* (Antebi et al., 1998;Moss et al., 1997;Tennessen et al., 2006;Tennessen et al., 2010). Therefore, we were interested in identifying LIN-42 interactors at this stage. LIN-42B::LAP protein was very weakly detectable by anti-GFP western blot in whole worm lysates (Figure A3). After purification and TEV treatment, LIN-42-S was detected in the supernatant while only GFP is detected in the pellet, indicating successful cleavage of LIN-42-S off the beads. Supernatants were TCA precipitated and analyzed by MudPIT to identify proteins that co-purified with LIN-42B::LAP. The wild-type samples were also analyzed as a negative control.

This experiment was difficult to replicate for several reasons. Due to the extremely low abundance and dynamic expression of LIN-42 during larval development, obtaining sufficient sample for downstream analyses proved to be extremely challenging. In addition, we found that a great deal of the LIN-42B::S sample bound non-specifically to the GFP-TRAP beads after cleavage. However, other anti-GFP antibodies tested did not bind LIN-42B::LAP at all. As a result of

these difficulties, only one result is shown here, where LIN-42 was detectable at all stages by western blot.

Primary candidates

Mass Spectrometry and bio-informatic analyses of wild-type and LIN-42B::S samples were performed as described, utilizing SEAQUEST and DTASelect contrast algorithms (Law et al., 2010;Tabb et al., 2002) (Table A1). Identified proteins were ranked based on normalized spectral abundance factor (NSAF) metrics (Florens et al., 2006). NSAF accounts for the fact that larger proteins are likely to contribute more peptides/spectra than smaller proteins. 18 unique LIN-42 peptides were identified with 37% sequence coverage. Any proteins that were present in both wild-type and LIN-42::S samples were discarded. The LIN-42::S specific analysis identified 92 proteins (Table A1). *mec-7,* was ranked higher on this list than *lin-42.* Although this protein was not detected in the wild-type sample, it has been detected by mass spectrometry in other wild-type samples after immunoprecipitation with anti-GFP antibodies (C. Spike, personal communication). Therefore, this protein was treated as non-specific. After these analyses, there remain several proteins in the LIN-42 specific list that are intriguing.

Of all proteins identified in this analysis, *kin-20* is the most interesting. *kin-20* is the *C. elegans* homolog of casein kinase 1. This kinase, called *doubletime* in *Drosophila,* phosphorylates PERIOD to promote its degradation in the

circadian clock (Price et al., 1998). The dynamic expression pattern of LIN-42 also suggests it may be regulated similarly to PERIOD, and is possible that LIN-42 accumulation and/or localization may be regulated by *kin-20.* Interestingly, *kin-20(RNAi)* has been reported to cause precocious seam cell fusion as observed by *ajm-1::gfp* reporter (Banerjee et al., 2005). Additionally, *kin-20(RNAi)* moderately enhances the precocious alae phenotype of *lin-42(lf).* This suggests that *kin-20* may function in the developmental timer, possibly in parallel with *lin-42*. However, these proteins do not interact in yeast two hybrid, therefore they may not directly interact, but be parts of a complex (Gardner, 2005). Further exploration of the interactions between *kin-20* and *lin-42* will be of interest.

dpy-30 is another potentially interesting candidate. *dpy-30* encodes essential component of the dosage compensation complex and mutations in this gene causes XX-lethality (Hsu et al., 1995). Dosage compensation mechanisms ensure the correct expression levels of X-linked genes by specifically silencing regions of X-chromosomes. Interestingly, there have some recent connections between the heterochronic gene pathway and dosage compensation. Mutations in *sea-2* have been reported to cause a retarded heterochronic defect (Huang et al., 2011). *sea-2,* which encodes a zinc-finger transcription factor, was first identified as an autosomal signal element that is important for regulating the expression of dosage compensation complex components (Meyer, 2005). Interestingly, *lin-28* function was found to be required for *sea-2* XX-lethality, suggesting it is necessary for *sea-2* function in dosage compensation. These data indicated that lin-28 may function is dosage-compensation (Huang et al.,

2011). As *lin-42* functions in parallel with *lin-28* in the heterochronic pathway, investigation of *dpy-30* may be of future interest.

Figure A1: Experimental design of co-immunoprecipitation with LAP-tagged protein

Shown is the basic experimental design for LIN-42::LAP co-immunoprecipitation to identify interacting proteins. The LIN-42::LAP protein is purified using the GFP-tag. Bound LIN-42::LAP is cleaved by TEV protease, releasing LIN-42B::S and any protein(s) bound to it. This complex can be further purified on S-protein beads; however, this step was omitted due to loss of material. This eluted protein was analyzed by mass spectrometry.

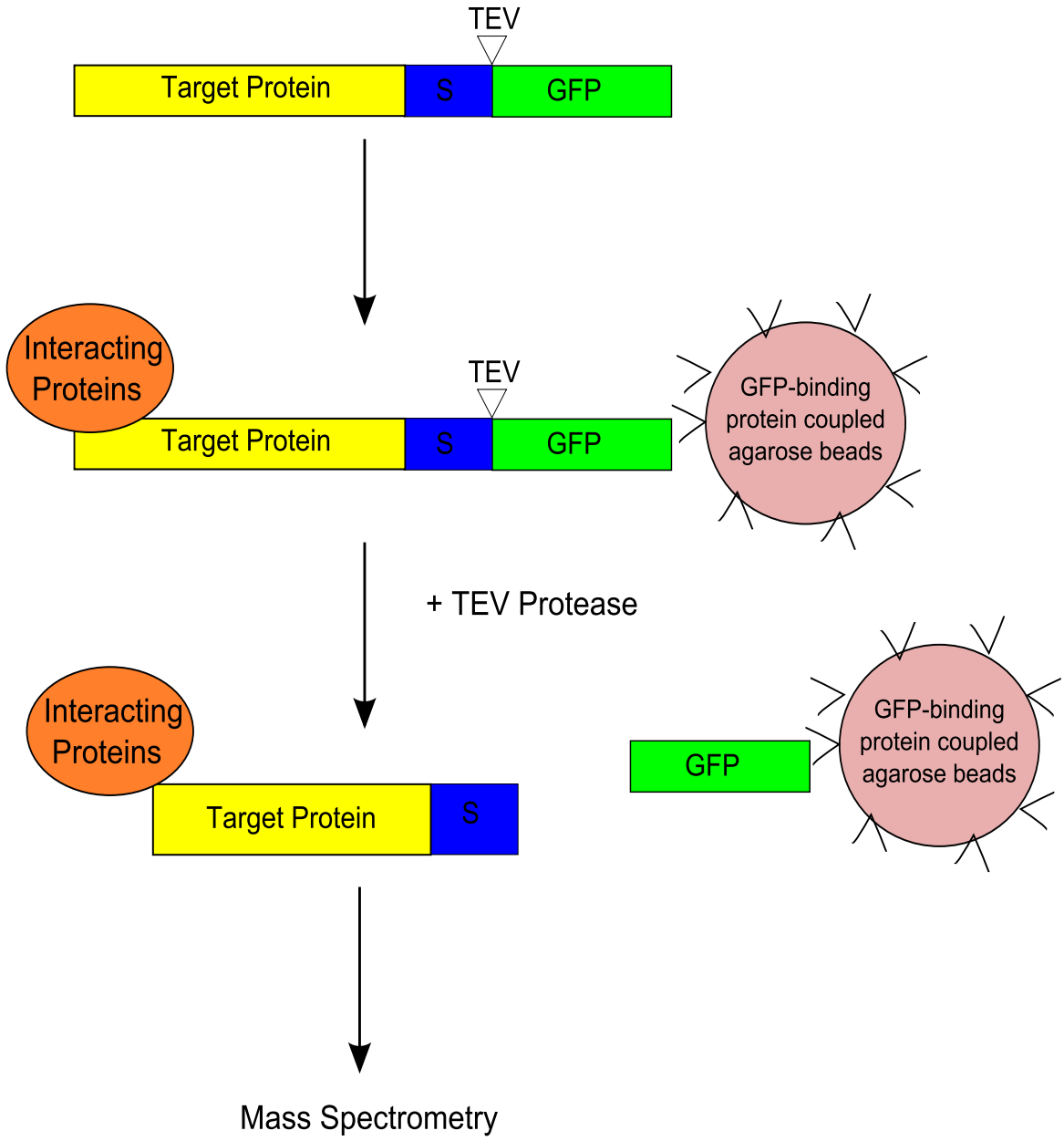

TEV
Target Protein
S
GFP
Interacting Proteins
TEV
Target Protein
S
GFP
GFP-binding protein coupled agarose beads
+ TEV Protease
Interacting Proteins
Target Protein
S
GFP
GFP-binding protein coupled agarose beads
Mass Spectrometry

Figure A1

Figure A2: LIN-42B::LAP is expressed in the hypodermis

Shown is the expression of LIN-42B::LAP in an L2-stage animal by fluorescence microscopy. LIN-42B::LAP is localized to hypodermal nuclei in a punctuate pattern, similar to endogenous LIN-42 as determined by antibody staining (Tennessen et al., 2006).

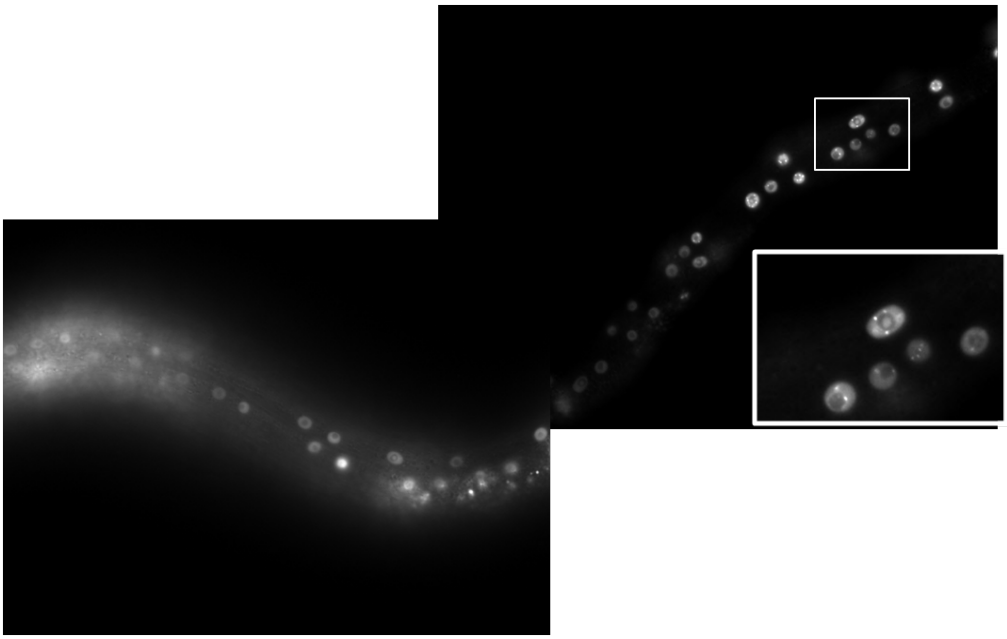


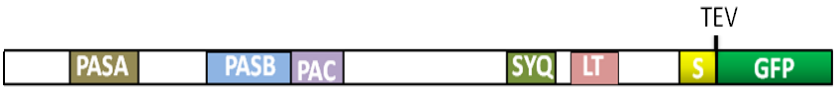
Figure A2

Figure A3: Purification of LIN-42B::LAP from whole worm lysates

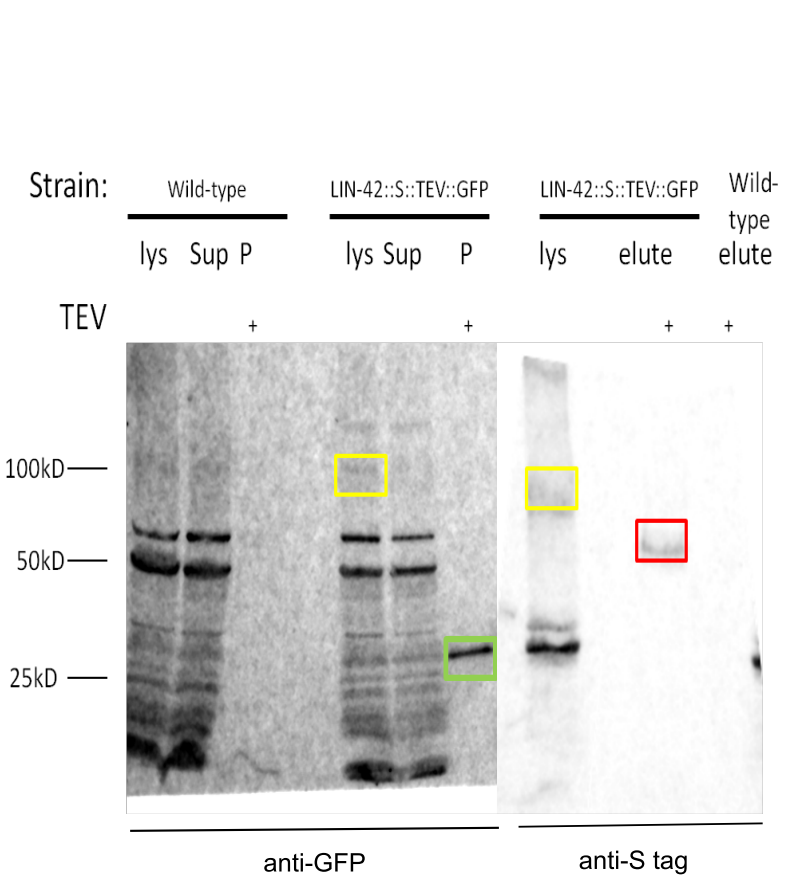
A. Cartoon diagram of LIN-42B::LAP protein, indicating the S-TEV-GFP epitope.

B. Analysis of LIN-42B::LAP purification by western blot. Two separate blots are shown, one probed with anti-GFP the second probed with anti-S tag. The purification steps are shown on the right, with each protein form color coded and outlined in the same colored box on the blot. lys=lysate, sup=supernatant, p=pellet, elute= TEV elution.

A


TEV
PASA
PASB
PAC
SYQ
LT
S
GFP

B


Strain:
Wild-type
LIN-42::S::TEV::GFP
LIN-42::S::TEV::GFP
Wild-type
lys Sup P
lys Sup P
lys elute
elute
TEV
+
+
+
+
100kD
50kD
25kD
anti-GFP
anti-S tag

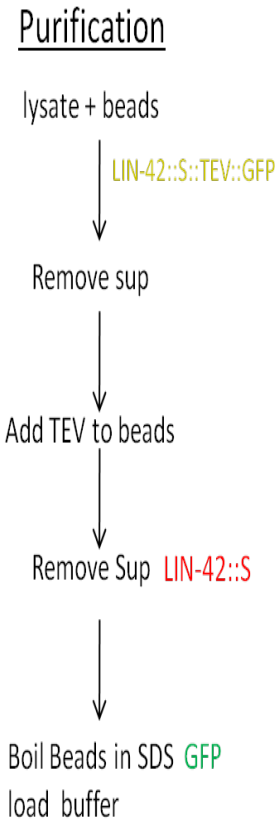

Purification
lysate + beads
LIN-42::S::TEV::GFP
Remove sup
Add TEV to beads
Remove Sup LIN-42::S
Boil Beads in SDS GFP
load buffer

Figure A3

Table A1: MudPIT analysis of LIN-42B::LAP co-IPs

LocusID	Gene	L[a]	MW[b]	pI[c]	Rank	LIN-42 NSAFe5[d]	N2 NSAFe5[d]
ZK154.3	*mec-7*	441	49260	5	91	529.375	0
F47F6.1b	*lin-42*	597	65255	8.1	144	376.563	0
F35G12.11		113	12972	5.9	253	191.293	0
Y119D3B.15	*dss-1*	82	9502	4.4	292	158.167	0
Y66D12A.22	*tin-10*	86	9608	5.5	310	150.81	0
C48E7.1		174	18677	8.1	314	149.077	0
T26A5.9	*dlc-1*	89	10344	7.4	320	145.727	0
T28D9.10	*snr-3*	126	13625	11.7	335	137.245	0
W08E3.1	*snr-2*	160	16742	10	338	135.101	0
F52E1.7	*hsp-17*	148.5	17496		366	116.451	0
Y43F8B.1		284.5	32655.5		383	106.371	0
ZK863.6	*dpy-30*	123	12837	4.5	385	105.445	0
F13D12.7	*gpb-1*	340	37406	6	394	101.723	0
F43C1.6	*mrpl-1*	171	19070	6.9	396	101.128	0
F02E8.1	*asb-1*	305	34937	8.7	399	99.222	0
F54D5.3		186	20533	5.4	417	92.973	0
F53E10.6		191	21520	5.1	420	90.539	0
F10B5.2		357	41683	6.4	433	84.769	0
F43D9.4	*sip-1*	159	17839	8.1	442	81.57	0
F27C1.7	*atp-3*	217.5	23668.5		450	79.508	0
B0564.1b	*tin-9.2*	111	12456	5.2	457	77.896	0
F56H11.4	*elo-1*	288	33562	9.7	472	75.056	0
Y54E10A.10		297	33460	9.8	478	72.782	0
ZC97.1a	*mtx-2*	244	28161	6.5	481	70.873	0
W02A11.2	*vps-25*	183	21473	6.2	482	70.873	0
T04G9.5	*trap-2*	188	21563	9.1	489	68.988	0
Y39C12A.1		276	30516	7.4	506	62.656	0
F46F2.2	*kin-20*	454.8	52268.8		526	57.035	0
Y48A6B.3		163	18091	6.8	538	53.046	0
R119.3		253	26539	6.3	543	51.264	0
C10H11.8		382	43209	8	572	45.269	0
F55B12.1	*ceh-24*	299	33077	6.8	577	43.377	0
Y39G10AR.9		401	44239	5.9	578	43.124	0
ZK1236.2	*cec-1*	304	33784	4.4	579	42.663	0
C08F11.14		927	104047	6.6	583	41.973	0
F52H3.7a	*lec-2*	1245	131936	4.4	586	41.67	0
Y77E11A.13a	*npp-20*	313	34692	7	587	41.437	0
C33H5.7	*swd-2.2*	326	36311	5.5	597	39.784	0
M01D7.7	*egl-30*	329	38837		598	39.422	0
F08G12.2		331	36822	7.4	600	39.183	0
Y18D10A.9		337	38190	5.1	601	38.486	0
Y54G9A.6	*bub-3*	343	38127	7.9	606	37.813	0
C08B6.9	*aos-1*	343	38738	5.4	607	37.813	0
C47E12.4	*pyp-1*	360.8	39459.8		618	35.947	0
R12E2.3	*rpn-8*	362	40687	6.5	619	35.828	0
Y66H1A.4		244	24626	10.9	623	35.436	0
C06G3.5		370	41379		629	35.053	0
C39E9.8		247	27958.5		630	35.006	0
R53.1	*flad-1*	514.5	58391		635	33.611	0
Y47D3A.15	*aakb-2*	274	30740	5.2	645	31.556	0
F40E10.3	*csq-1*	417	48327	4.3	646	31.102	0
F55G1.9		299	32057	6.1	661	28.918	0
F35G12.10	*asb-1*	301	34368	8.9	662	28.726	0
ZK863.7	*rnp-1*	305	33049	8.3	665	28.349	0
C24G6.8		316	33689	4.6	671	27.362	0
R07H5.2a	*cpt-2*	646	72547	7.9	672	26.769	0
Y63D3A.5	*tfg-1*	486	49739	5.6	673	26.687	0
Y75B8A.30	*pph-4.1*	333	37359	5.3	676	25.965	0
K08E3.5		504.7	56291.3		678	25.698	0
C33F10.4		338	37079.5		679	25.581	0
C32F10.5	*hmg-3*	693	78193.5		683	24.954	0
B0412.2	*daf-7*	350	39533	6.7	685	24.704	0
B0310.5	*ugt-46*	531	60204	7.3	686	24.425	0
F13C5.2		374	40881	4.9	689	23.119	0
C25B8.3	*cpr-6*	378.5	42340		691	22.844	0
C29F5.1		383	43264	6.2	694	22.576	0
C05D11.12	*let-721*	597	65335	6.7	698	21.725	0
C02B10.1	*ivd-1*	419	45642	8	704	20.636	0
C56G2.1	*akap-1*	638	71248.5		708	20.329	0
F56H1.4	*rpt-5*	430	48126	5.3	710	20.108	0
Y63D3A.6	*dnj-29*	665.5	76706.5		714	19.489	0
T09A5.11	*ostb-1*	445	48760	8.8	716	19.43	0
F08A8.2		679	77311	8.2	717	19.101	0
Y87G2A.3	*atg-4.1*	493	56621	5.1	726	17.538	0
R05G6.8	*plc-4*	751	85869	6.4	727	17.27	0
R13A5.12	*lpd-7*	531	61626	9.4	732	16.283	0
K07H8.10		798	86819	4.8	733	16.253	0
Y71H2AM.13		550	61645	6.7	734	15.721	0
C43E11.1	*acin-1*	580	65495	5.1	741	14.908	0
F09B12.3		582	66638	7.3	743	14.856	0
W03F8.10	*lam-1*	587	64444	5	745	14.73	0
W05E10.4	*tre-3*	588	67783	5.7	746	14.705	0
Y50D7A.7	*ads-1*	597	66559	6.8	748	14.483	0
K02F2.3	*teg-4*	1220	135100	5.6	750	14.175	0
C34E10.5	*prmt-5*	734	83292	6.3	763	11.78	0
T12A2.2	*stt-3*	757	85122	8.8	765	11.422	0
ZK550.3		772	90344	6.6	766	11.2	0
C25A1.10	*dao-5*	833.5	87247		773	10.374	0
Y106G6H.5		855	94942	6.6	774	10.113	0
Y17G7B.5a	*mcm-2*	881	99323	5.1	776	9.814	0
F49E2.5		1008	111178.3		784	8.578	0
C49C3.4		1566	169745	4.7	786	8.282	0
F29G6.3	*hpo-34*	1112.5	125253.5		792	7.772	0
T25C12.3		2014	221261	4.9	805	4.293	0
ZC247.1		4404	498362	5.2	806	3.927	0

a=protein length (AA)
b=protein molecular weight
c=isoelectric point
d=normalized spectral abundance factor:
Peptide counts are normalized to protein size as larger proteins have higher probability of detection.

Appendix 2: *C. elegans* Biochemistry Protocols

Described in this section are various protocols for biochemical methods in C. elegans including large-scale synchronized cultures and immunoprecipitation protocols

PART 1: Egg isolation and liquid culture of worms

Growth of synchronized worm cultures has only been taken out to mid-to-late L2 stage. I have not gone beyond that stage, therefore it is unknown how long synchrony is maintained

This protocol is adapted from (Seggerson et al., 2002).

DAY 1-3

1. From starved medium plates, chunk to 4X peptone plates seeded with HB101 bacteria (this is more nutritious than OP50, and allows for growth of denser populations and is less prone to contamination and degradation of the agar). Plate x+3-5 large plates for total flasks of worms you need. For large scale preps, x=5-10.
2. The day before the plates starve, prep 500ml o/n HB101 cultures/flask of worms in 2L Erlenmeyer flasks.

DAY 4-6

1. The next day, spin down each 500ml culture in a sterile 250ml centrifuge bottle. I spin at 6000rpm for 10minutes in the Beckmann J2 centrifuge.
2. Re-suspend bacterial pellet is S-complete media:

S-basal(1L): 5.9g NaCl
50ml 1M Potassium Phosphate pH6.0
1ml of cholesterol (5mg/ml in EtOH)

To make S-complete: to 1L add
10ml 1M Potassium citrate pH6.0
10ml trace metals solution
3ml 1M CaCl2
3ml 1M MgSO4
Store at 4 degrees

Trace metals solutions:
Disodium EDTA 1.86g (5mM)
FeSO4 7H20 0.69g(2.5mM)
MnCl24H20 0.20g(1mM)
Zn SO4 7H20 0.29g(1mM)
CuSO4 5H20 .025g(0.1mM)
Dissolve in 1L water; aliquot into 50ml conical and store in dark

1M Potassium Citrate, pH6.0:
210.1g citric acid monohydrate, water to 900ml
Adjust pH to 6.0 using 10N KOH and bring to 1L
Autoclave and store at RT

3. Wash worms off plates in water. Again, I use X+3-5 larges plates, where X is the total number of flasks. For a sick strain, I would try starting form even more plates. Transfer to 15ml conical.
4. Wash worms with water 2X
5. Add worms to re-suspended bacteria in sterile 2L Erlenmeyer flasks.
6. Put on shaker in 20 degree room, spin at about 150 rpm.
7. Check worms after 2-3 days. When most are gravid adults, you are ready to bleach.
8. Pour cultures into 50ml conical. Let settle on ice or spin in J(?) at 2000rpm for 2minutes.
9. Transfer to 15ml conical. Optimally, you want less than 1ml of worms per tube.
10. Bleach using Jason's protocol. With practice, you can do 4-6 tubes at a time.
11. Let the worm starve o/n in M9.
12. Prepare HB101 cultures as above. For about 24 hours of growth, you'll need 150ml culture per 1L flask.
13. Add starved L1s to sterile 1L flask with 150ml re-suspended HB101. I add all L1s from one 1 large flask of adults to 1 small flask. For L2-stage, I usually start between 10-12 in the morning.

PART 2: Freezing down synchronized worms

1. Pour cultures into 50ml conical tubes
2. Spin 2minutes in J-6 centrifuge at 2000rpm in the cold.
3. Once all of the worms are in conical, transfer to 15ml conical tubes (1-2ml per tube)
4. Wash worms 2X in water
5. Resuspend worms in 6ml of water
6. Add 6ml of 70% sucrose (to make 35%)
7. Mix well
8. Spin worms at low speed for 5 minutes
9. Transfer worms using Pasteur pipette to another 15ml tube filled halfway with water
10. Once all worms have been transferred, spin down and wash with water until sucrose is gone.
11. Wash worms on final time in lysis buffer (w/o NP40) + 2X protease inhibitors
12. Drop-free worms in liquid nitrogen in 50ml conical tube (make sure tube is COLD before starting or worms will stick tightly to tube). Store at -80.

PART 2: Preparation of Worm Lysates (METHOD 1-SONICATION)

There are many methods for preparing worm lysates and several should be tried to figure out what work best for your samples. Sonication will break up worms and organelles, but it doesn't break up the worms very well. Dounce homogenizer will break up the worms really well, but often leaves organelles such as the nucleus intact.

******ALL LYSIS AND WASH BUFFERS SHOULD BE SUPPLEMENTED WITH 2X PROTEASE INHIBITORS!!!!!!!

1. Grind frozen worms in liquid nitrogen with a mortar and pestle into a fine powder
2. Transfer to 2ml eppendorf tube and thaw quickly in water bath.
3. Add equal volume of lysis buffer (w/NP40) + 2X protease inhibitors to worm slurry
4. Sonicate at 15% amplitude, 5s 3X with 30s rest
5. Spin 5 minutes in cold at top speed (optional second spin in Connor Lab tabletop ultracentrifuge. Use special microfuge tubes for this step. Talk to Titus Lab or Connor Lab before using. I've done 100,000g for 10min)
6. Add KCL to final concentration of 300mM to extract (i.e. 65ul 3M/ml lysate)
7. Proceed to IP

PREPARATION OF WORM LYSATES (METHOD 2-HOMOGENIZATION)

1. Thaw worm pellet at 37C water bath
2. Add equal volume 100mM lysis buffer
3. Break up in dounce homogenizer
    i. break up 1ml at a time
    ii. 60-70 strokes per ml
4. Spin cold for 20 minutes at 10,000rpm
5. Add KCL to final concentration of 300mM to extract
6. Proceed to IP

PART 3: IMMUNOPRECIPITATION

Described below is a very standard GFP immunoprecipitation. However, salt concentrations, incubation times, amount of beads added may need to be altered depending on specific GFP-tagged protein. Also, most preps were done with magnetic beads.

1. Add 1ml lysate/50-75ul beads in a 1ml microfuge tube (either GFP-TRAP or GFP-sepharose, see supplementary protocol "conjugating antibodies to sepharose)
    i. For large scale preps (i.e. mass spec) use at least 5mls of lysate, 1ml/tube
2. Incubate with rotation 1 hr at 4°C
3. Place on magnet and remove supernatant. SAVE
4. Wash beads 3X in 1ml wash buffer (incubate beads in buffer for a few minutes)
5. Combine beads into 1 tube
6. Boil beads in 100-150ul SDS sample buffer

    TEV CLEAVAGE:

    1. If your protein has a TEV cleavage site, transfer to 2ml tube. Add 50ug TEV protease to tube.
    2. Incubate at RT for 2hr with rotation
    3. Place on magnet and remove supernatant.

4. Wash beads with 250ul wash buffer and add to supernatant
5. Boil beads in equal volume of SDS sample buffer

Lysis/Wash Buffer

50mM HEPES pH 7.4

1mM EGTA

1mM MgCl2

100mM KCl (wash buffer, 300mM KCl)

10% glycerol

0.05% NP-40

**Before use, add protease inhibitors to 2X

**Store at 4C

Supplementary Protocol 1: TCA Precipitaiton

1. To supernatant (i.e. from TEV elution, Urea elution), add 1/10 volume 100% TCA
2. Incubate 1-2hr at -20°C
3. Spin?
4. Wash in 1ml cold acetone 2x
5. Leave third acetone wash overnight at -20C
6. Resuspend in ½ starting volume lysis buffer or SDS sample buffer (mass spec samples can be shipped dry)

Supplementary Protocol 2: Conjugating antibodies to sepharose beads

To prep 500ul of beads (slurry)

1. Binding antibody to beads
    i. Add 100ul beads to tubes (5 tubes, 500ul total)
    ii. Place on magnet or spin and pipette off supernatant
    iii. Add 10ug of antibody in 400ul PBS-T
    iv. Incubate at RT for 10min. with rotation
    v. Place on magnet or spin and remove supernatant
    vi. Wash 2X in conjugation buffer
2. Cross-linking
    i. Add 475 ul conjugation buffer to each tube of beads

ii. Add 25ul 100mM DSS solution (216ul DMSO + 8mg DSS). Allow DSS to come to RT before adding to DMSO
iii. Incubate 30 minutes with rotation at RT
iv. Quench reaction with 25ul quenching buffer
v. Incubate at RT 15 minutes with rotation
vi. Wash 3X in PBST
vii. Combine beads, final bead slurry volume 500ul

Conjugation Buffer
To make 50ml:
0.15g Na_2HPO_4
0.45g NaCl
pH 7-9

Quench Buffer
1M Tris-HCl pH7.5