Characterization of *lin-42/period* transcriptional regulation by the *Ikaros/hunchback*-family transcription factor ZTF-16 in *Caenorhabditis elegans*

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Abstract

The gene *lin-42* is an ortholog of the mammalian *period* gene, a component of the circadian pathway that converts environmental stimuli into behavioral and physiological outputs over 24 hours. Mammalian *period* also regulates adult stem cell differentiation, although this function is poorly understood. The structure, function and expression of *lin-42* are all similar to *period*. Therefore, we are studying *lin-42* regulation and function during *C. elegans* larval development as a model for understanding *period* control of mammalian stem/progenitor cell development.

Previous work has shown that ZTF-16 is a regulator of *lin-42* transcription. The *lin-42* locus encodes three isoforms, and we have characterized *lin-42* isoform specific regulation by ZTF-16 through phenotypic assays and analysis of transcriptional reporter strains. Our data show that ZTF-16 regulates the cyclic expression of *lin-42A* and *lin-42B* during larval development. However, *ztf-16* is not expressed during the adult stage and does not regulate *lin-42C*, which is expressed only in adults and may be responsible for the circadian functions of *lin-42*. We also show that *ztf-16* reduction-of-function mutations phenocopy loss-of- function phenotypes of the *lin-42A*/*B* isoforms. Finally, we have found that deletion of a putative ZTF-16 transcription factor binding site within the *lin-42BC* promoter abolishes tissue-specific expression patterns. Together, these data indicate that ZTF-16 is required to regulate the expression of *lin-42A/B* during *C. elegans* development, and may do this by direct binding to the *lin-42BC* promoter. Our

findings pave the way for testing the possible regulation of *period* expression by HIL-family transcription factors in mammalian tissues.

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Chapter 1: Literature Review

The field of stem-cell research has expanded considerably in recent years, but there is still relatively little known about the process of stem cell differentiation. Dividing stem cells have two options: to maintain their potency and the ability to self-renew, or to differentiate. The molecular mechanisms that control the decision between self-renewal versus differentiation are poorly understood; until more is learned about the factors that influence this decision, the process cannot be manipulated or controlled and stem cell therapies will be of limited use. We are studying *C. elegans lin-42*, which regulates the developmental timing of stem cell differentiation in larval *C. elegans*, as a model to investigate fundamental and conserved mechanisms of stem cell differentiation.

C. elegans lin-42 is the nematode ortholog of *period*, a key component of the circadian timing pathway that controls periodic patterns of physiology and behavior in vertebrates and invertebrates in coordination with daily cycles of environmental inputs such as light and temperature[1, 2]. The circadian physiological and behavioral functions of *period* have been extensively described in a wide array of animals and the molecular mechanisms of the circadian timing pathway are well characterized. Novel developmental functions for *period*, specifically in regulation of stem and progenitor cell differentiation, have recently been reported[3-5]. However, the mechanisms of *period* regulation and function in stem cell differentiation pathways have yet to be thoroughly investigated.

Like its ortholog *period, C. elegans lin-42* regulates both circadian patterns of behavior [6, 7] and cellular development, but unlike *period*, the developmental functions of *lin-42* are more extensively described in *C. elegans*. In addition to many others, *lin-42* is a heterochronic gene that is responsible for coordinating the timing of developmental events during post-

embryonic larval development. Specifically, *lin-42* regulates the timing of stem/progenitor cell division and terminal differentiation, as well as other developmental events such as the periodic replacement of the outer cuticle, called molting, the timing of periodic entry into a sleep-like state called lethargus, and the decision to commit to the alternative, developmentally quiescent stage called dauer in response to developmental stress [2, 8, 9]. Study of *lin-42* regulation and function during *C. elegans* post-embryonic development may lead to insights into the developmental regulation and function of *period* in mammals, and ultimately, insights into the molecular mechanisms that regulate stem cell development.

Circadian physiological roles of *period*

The circadian timing pathway or circadian clock in animals is a conserved molecular mechanism that coordinates physiological and behavioral responses with environmental cues such as daily-light and temperature changes. The circadian clock is responsible for regulating a variety of periodic metabolic and physiological states and behaviors, including sleep/wake cycles, appetite, activity levels, blood glucose levels, and hormonal release [10]. In mammals, the primary circadian clock is located in the Suprachiasmatic Nuclei (SCN) of the hypothalamus, which receives visual inputs from the retina [11, 12]. Period protein is critical for the operation of the circadian molecular clock. Loss of function of *period* genes in *Drosophila melanogaster* or mouse models typically results in an alteration to the length of the circadian period or in behavioral arrhythmia. In mammals, which have three period genes (*Per1*, *Per2*, and *Per3*), *Per1* loss of function results in a shorter circadian period compared to their wildtype counterparts [13]. *Per2* loss of function also results in a shorter circadian period and arrhythmic behavior in constant darkness [14]. Interestingly, *Per3* appears to lack significant function in the circadian

clock, which implies that it has an alternate non-circadian function [15]. This alternate role of *Per3* may be developmental.

Period protein (PER) expression cycles rhythmically over 24 hours with a peak in expression at night [16]. The cyclic nature of this expression pattern is controlled by a feedback loop that regulates rates of transcription and translation of key clock proteins [reviewed in [17]] (**Figure 1**). In the cell nucleus, the transcription factor proteins CLOCK (CLK) and BMAL (CYCLE in Drosophila) form a complex that binds to E-box motifs in the promoters of *period (per)* and *cryptochrome (cry),* or *timeless (tim)* in Drosophila, to activate transcription. As these target proteins begin to accumulate in the cytoplasm, PER forms a heterodimer with Casein Kinase I ε/δ (CKIε/δ) and, depending on its phosphorylation status, can also associate with either cryptochrome (CRY) in mammals or Timeless (TIM) in Drosophila. The PER/CRY or PER/TIM complexes then translocate to the nucleus, where PER inhibits its own transcription by inactivating the CLK/BMAL transcription complex. Another layer of clock regulation includes other proteins activated by the BMAL/CLK complex, such as REV-ERBα and RORα, which act antagonistically to regulate *Bmal* transcription through promoter elements upstream of the *Bmal* gene [18]. REV-ERBα is a repressor of *Bmal* transcription, whereas RORα is an activator of *Bmal* transcription. When the PER/CRY complex translocates into the nucleus, PER also represses *Rev-erbα* transcription, thus allowing RORα to bind to the *Bmal* promoter and activate *Bmal* transcription [19]. These *period*-based transcriptional/translational loops result in cyclic expression of not only *period* transcript and protein but also of BMAL transcription factor, which is expressed in anti-phase to *period*.

An important aspect of maintaining the accuracy of the circadian clocks involves tightly regulating the stability of clock proteins, which is done primarily through protein modifications

including phosphorylation, dephosphorylation, methylation, and acetylation [10]. PER phosphorylation by CKI ε/δ and Glycogen Synthase Kinase 3β (GSK3β), as well as dephosphorylation by Protein Phosphatase 2A(PP2A) and Protein Phosphatase 1(PP1) is vital for modulating the stability of the protein [Reviewed in [10, 20]]. In fact, mutations in the kinases or phosphatases that control PER stability can influence the duration of the circadian period. For example, humans with Familial advanced sleep-phase syndrome (FASPS) have a shortened, offset, circadian period. Mutations linked to this syndrome include mutations to CKIδ or mutations to PER2 that affect the ability of CKI ε/δ or PP1 to phosphorylate/dephosphorylate the protein [21, 22]. These mutations affect the half-life of the protein, thereby altering the amount of time that it takes to down-regulate its own transcription.

The transcription/translation feedback loops maintained by circadian proteins define the basic backbone of the circadian molecular clock in mammals and many other eukaryotes. Nevertheless, our understanding of the circadian clock is still evolving and there are many interactions that have only been partially defined. We are aware of many proteins that modulate the activity of PER and other circadian proteins, but have only a limited idea of how these additional regulators fit into the larger framework of the clock and its function. Parsing out the functions of these circadian regulators is complicated by the fact that the circadian clock is tightly integrated with many other signaling pathways and physiological systems. Other recently identified regulators of *period* include Ubiquitin specific peptidase 2 (USP2), Glucocorticoids, and the aryl hydrocarbon receptor (AhR) [23-26]. USP2 has been shown to de-ubiquitinate PER1 in mice [23]. The post-translational modification does not affect the stability of PER1, so it is thought that it may instead regulate PER1 function or localization. This hypothesis has not been confirmed; however, USP2 does increase the locomotory period of mutant animals during

conditions of free-running darkness, demonstrating that the mutation does affect circadian clock function [23]. Glucocorticoids are steroid hormones that both regulate and are regulated by the circadian clock. Glucocorticoid levels are regulated by the circadian clock to maintain glucose homeostasis in animals [24]. A feedback loop is established because glucocorticoids are able to bind to response elements in the *per1* and *per2* promoters to regulate their transcription in peripheral tissues [24]. Inhibition of glucocorticoids results in attenuation of *period* transcription and overall dampening of the circadian rhythms in those tissues [24]. Finally, AhR down-regulates *per1* expression by disrupting the binding of CLK/BMAL complex to the *per1* promoter [26]. The mechanism controlling this regulation is largely unknown, although it is thought that AhR binds to BMAL, thereby preventing the CLK/BMAL heterodimer from forming [26]. Many of these regulators operate only in peripheral tissues and their exact function within the larger context of the molecular clock is still under investigation.

Developmental roles of *period*

In recent years *period* has been found to function as a regulator of adult stem/progenitor cell differentiation and is involved in controlling cancer cell proliferation. In mice, PER2 and PER3 regulate endothelial progenitor cell (EPC), neural progenitor cell (NPC), and mesenchymal stem cell (MSC) differentiation [3-5]. PER3 inhibits MSC adoption of the adipocyte cell fate in mice; this appears to be its primary function, as its function in the circadian clock is limited [4]. PER3 interacts with PPARγ, presumably via its PAS domain, to inhibit the adipocyte cell fate in MSCs [4]. PPARγ is a nuclear receptor that influences the transcription of the *ap2* gene. AP2 is necessary to allow for MSC differentiation into adipocytes, and inhibition

of PPARγ is sufficient to prevent MSC differentiation. Glucocorticoids down-regulate PER3 expression to relieve PPARγ inhibition and permit adipocyte cell fate adoption [4].

PER2 functions as a regulator of *Bmal* transcription in the circadian clock. Mutation of PER2 results in circadian phenotypes including a shorter circadian period and the loss of circadian rhythmicity in constant darkness [18]. Interestingly, while PER2 is cyclically expressed in the Suprachiasmatic Nuclei (SCN), it is constitutively expressed in the dentate gyrus of the hippocampus, where it regulates NPC proliferation and differentiation [5]. Mutation of *Per2* in mice results in an increase in proliferation and differentiation of NPCs and, surprisingly, a decrease in EPC growth and proliferation [3, 5]. This suggests that *Per2* regulation of progenitor cells is cell-type dependent.

In addition to its roles in stem cell development, *period* has been found to function as a tumor-suppressor. Expression of *period* is reduced in some tumor types, while overexpression of *period* increases apoptosis resulting from DNA damage and results in an overall decrease in tumor growth[27]. Genetic mutations or other perturbations to the normal function of the circadian clock also result in an increased incidence of tumorigenesis in humans, and mice with *Per2* loss of function are more likely to develop tumors following gamma radiation exposure [28, 29]. Tumorigenesis is thought to increase in *Per2* mutant animals because p53-mediated apoptosis of cells with extensive DNA damage is reduced [29]. These results indicate that *period* function is required to inhibit proliferation of cancer cells.

Worth noting is the fact that some of the regulators of *period* in the circadian pathway are also regulators of stem cell development, which implies that there may be cross-regulation between circadian and developmental pathways. GSK3β, for instance, is known to modulate stem cell differentiation in both hematopoietic and mesenchymal cell lines in mammals [30]. In

the circadian pathway, GSK3β modulates *period* stability and inhibition of GSK3β results in a longer circadian period [20]. Similarly, ATF4, a transcriptional regulator of *per2* that is itself regulated by CLK/BMAL, is also required for osteoblast differentiation in mammals [31, 32].

C. elegans lin-42 as a model for mammalian *period*

The relative advantage of using *C. elegans lin-42* as a model for *period* stems from the difficulties associated with the study of *period* function in mammalian or even other invertebrate models. Stem cell development strongly relies on signaling from surrounding tissues, meaning that the study of mammalian stem cells *in vitro* would likely not accurately reflect *in vivo* interactions. Mammalian *in vivo* studies are complicated by the challenge of finding and observing rare populations of stem cells in complex multicellular organisms, as well as the difficulty of inducing genetic mutations to observe the effects on stem cell development. *C. elegans* has a number of anatomical and developmental features that overcome the difficulties associated with stem cell studies in mammals. The adult *C. elegans* consists of 959 terminally differentiated somatic cells whose developmental lineages are fully delineated. The *C. elegans* cell fate map, the only complete cell fate map available for any eukaryote, allows for the identification of mutant cell division and differentiation patterns over the entire time-course of embryonic and post-embryonic development. In addition, *C. elegans* has an experimentally accessible adult stem/progenitor cell lineage, the seam cell lineage located in the single cell layer non-pigmented epidermis of worms (**Figure 2**). This seam cell lineage has distinct spatio-temporal patterns of division and differentiation that take place during larval development, thus allowing for the evaluation of the interaction between physiological and stem cell fate pathways during post-embryonic development (**Figure 2**). Finally, there are a number of molecular tools

available to increase the ease of genetic manipulations in *C. elegans*, including a large array of mutant and fluorescently labeled strains and an extensive RNA interference (RNAi) library. Additionally, transgenic lines can be made more easily in *C. elegans* than in most other model organisms by introducing the foreign DNA into the gonad using microinjection or microbombardment. The simple anatomy, stereotypical development and ease of genetic manipulation in *C. elegans*, and the accessibility of *C. elegans* cells, including seam cells, to *in vivo* real-time imaging makes the investigation of *lin-42/period* roles in stem cell development more tractable in *C. elegans* than in most other model organisms.

A number of structural and functional parallels between *C. elegans lin-42* and *period* in Drosophila or mammals make *lin-42* an appropriate model for studying the developmental roles of *period*. At the molecular level, LIN-42 contains the PAS protein-protein interaction domain (PERIOD/ARNT/SIM), which is characteristic of circadian proteins [1]. Furthermore, *lin-42* and *period* are both expressed cyclically, although on different time scales; *period* expression oscillates over 24-hours, whereas *lin-42* levels oscillate over the time course of *C. elegans* larval development, peaking once every larval stage [1, 2, 10]. More recently, the functional similarities between *lin-42* and *period* have become apparent as circadian and developmental functions have been reported for both. The role of *period* as a regulator of stem cell differentiation mirrors the role of *lin-42* in *C. elegans* larval development. In adipose and other tissue types, *period* down-regulation is required for differentiation of the stem/progenitor cells [4]. Similarly, *lin-42* loss of function results in precocious terminal differentiation of epidermal progenitor cells, whereas *lin-42* gain of function causes a delay in differentiation and allows the inappropriate continuation of larval stage traits such as continued self-renewal into the adult stage [2]. Thus, *lin-42* mutant phenotypes indicate that the gene is required to promote stem cell

self-renewal/proliferation during post-embryonic development and its down-regulation is required to promote terminal differentiation. Several other features of *lin-42* make it easier to study than its vertebrate counterpart. First, three independent *period* loci have been identified in mammals, while the *C. elegans* genome only has one *lin-42* locus. Null mutants can therefore be produced with more ease in *C. elegans* than in other mammalian model organisms because only one gene must be inactivated. Second, some of the developmental functions of *lin-42* have already been described and investigated at the genetic and molecular levels, whereas very little is known about the developmental functions of *period* in mammals. The parallels in function between *lin-42* and *period* and the ease with which *C. elegans* can be manipulated makes *lin-42* uniquely suited to define the developmental roles of period.

C. elegans lin-42 isoform structure, expression and functions

Although there is only one *lin-42* locus in the *C. elegans* genome, there are three alternatively spliced isoforms of *lin-42*, designated *lin-42A*, *lin-42B* and *lin-42C*, which differ in structure, function, and expression (**Chapter 2, Figure 1 and Table 1**). Two of the isoforms, *lin-42A* and *lin-42B*, are expressed coordinately throughout larval development, with peaks of expression during each of the four larval stages in *C. elegans* (**Chapter 2, Figure 2**), while *lin-42C* is expressed only during the adult stage (**Chapter 2, Figure 2 and Figure 10**). Expression of *lin-42A* and *lin-42B* is repressed on transition to the adult stage, during which all somatic lineages terminally differentiate, and during entry to dauer, a developmentally quiescent alternate life-stage that is adopted in response to environmental stressors [9], (Banerjee, unpublished). *lin-42* is broadly expressed and *lin-42* expression has been reported in the hypodermis and seam cells, vulva muscle cells, distal tip cells, intestine, muscle, and some head and tail cells [1, 2].

In addition to the differences in spatio-temporal expression patterns, each *lin-42* isoform differs in its protein structure. The longest isoform, LIN-42B, consists of a portion of the exonic regions of both *lin-42A* and *lin-42C*, and the protein contains SYQ and LT motifs, as well as the PAS domain characteristic of circadian proteins[1, 2](**Chapter 2, Figure 1**). The LIN-42A isoform, which is comprised of exons 7-12 of the genomic region, includes only the SYQ and LT motifs. Only the PAS domain is present in LIN-42C, which is made up of exons 1-6 of the genomic region. The two amino acid motifs, SYQ and LT, and the PAS domain are all conserved in the human PER1 protein [1, 2]. The structural differences among the *lin-42* isoforms suggest that their molecular interactions could vary and that they fulfill different molecular functions.

Further evidence that *lin-42* isoforms have different functions can be found in the loss of function phenotypes of various *lin-42* mutants (**Chapter 2, Table 1**). In all of the known mutants, the *lin-42B* isoform is presumably eliminated, as it contains exons from both the *A* and *C* isoforms [2]. For example, *lin-42(mg152)* and *lin-42(n1089)* are mutations that affect the *B* and *C* isoforms, leaving only the *A* isoform intact [2]. These two mutations have milder phenotypes resulting only in precocious terminal differentiation. Conversely, mutations such as *lin-42(ve11)* and *lin-42(ok2385),* which eliminate the *A* and *B* isoforms, are much more severe and result in precocious terminal differentiation and lethality [2, 8]. These differences indicate that *lin-42A* and *lin-42B* are important for coordinating developmental processes, while *lin-42C* is not vital for larval development.

The structure of the *lin-42* locus indicates that expression of the *lin-42* isoforms may be differentially regulated transcriptionally and post-transcriptionally. The *lin-42B* and *lin-42C* isoforms share a common promoter, but have different 3'UTRs. Similarly, *lin-42B* and *lin-42A*

share the same 3'UTR, but have different promoter regions. As a result, it may be predicted that *lin-42B* and *lin-42C* may have the same or similar transcriptional regulation, while *lin-42B* and *lin-42A* may have similar post-transcriptional regulation. Indeed, our research group has shown that *lin-42A* and *lin-42B* are post-transcriptionally regulated by the *let-7* family microRNAs while *lin-42C* is not [33]. The variety in the structure, regulation, expression, and function of *lin-42* isoforms indicate that they are not redundant.

Circadian Function of *C. elegans lin-42*

Circadian rhythms were not described in *C. elegans* until 2002 [7, 34]. Subsequent studies showed that adult *C. elegans* exhibit circadian rhythms dependent on temperature, osmotic stress, and to a lesser degree, light [6, 7, 35, 36]. Little is known about the role of *lin-42* in circadian timing, although *lin-42* is the only reported circadian homolog in *C. elegans* that exhibits an aberrant circadian phenotype when mutated. Loss of function mutation of *lin-42* results in a locomotory period in adult mutants that is longer than that of adult wildtype worms, suggesting that *lin-42* has a circadian timing function that is distinct from its developmental timing functions [6]. However, neither *lin-42* nor any other *C. elegans* circadian homologs exhibit cyclic expression patterns in the adult stage [36]. Few other circadian homologs have been tested for loss of function effect on circadian rhythms. The only reported homolog to be tested is *aha-1*, the *C. elegans* homolog of *Bmal* or *Clock*. No circadian rhythm defects were observed in *aha-1* mutants, suggesting that the *C. elegans* circadian timing pathway, while still involving *lin-42/period*, may be regulated differently than the Drosophila or mammalian circadian clock[6].

Developmental Functions of *C. elegans lin-42*: seam cell terminal differentiation and the heterochronic pathway

The heterochronic pathway consists of a network of genes and microRNAs that regulate the timing of developmental events. Mutations in the heterochronic pathway result in changes to the timing of cell fate programs. Mutations can either prematurely turn on development programs, resulting in the precocious enactment of later stage events, or they can delay the activation of developmental programs, resulting in the reiteration of events from the previous stage and the ultimate retardation of stage appropriate events until a later stage. It is important to note that heterochronic mutations do not change the fates of the cells, for example hypodermal cells do not instead adopt intestinal cell fates, but only affect the timing of when the cell fates are adopted. Heterochronic mutations are often studied in the *C. elegans* epidermal stem/progenitor cells known as seam cells. The seam cells are a line of cells that run the length of the lateral sides of the worm [37] **(Figure 2**). With the exception of the L2 stage, these stem cells divide asymmetrically at the start of each larval stage. The anterior daughter cell differentiates and migrates away from the seam to become part of the hypodermis. Conversely, the posterior daughter maintains its ability for self-renewal. This pattern is maintained at the start of every larval stage except at the beginning of the L2 stage, when the seam cells undergo a proliferative cell division prior to the normal asymmetrical cell division. At the beginning of the adult stage, all of the seam cells terminally differentiate, fuse to form a hypodermal syncytium, and begin secreting adult cuticle, called alae. The consistent division pattern of these stem cells makes them ideal for studying the effects of heterochronic mutations as changes in the seam cell number or the timing of the terminal differentiation event are indicative of heterochronic mis-regulation.

One common regulatory theme in the heterochronic pathway is the down-regulation of gene products via the microRNAs (miRs). miRs are small, non-coding RNAs that bind to complementary portions of the 3'UTR of target transcripts, thereby preventing the translation of the mRNA. This type of post-transcriptional regulation is used frequently in the heterochronic pathway to down-regulate genes and allow progression to the next developmental stage (**Figure 3**). For example, at the L3 to L4 transition, the *let-7* family miRs, *mir-48*, *-84*, and *-241*, function redundantly to down-regulate *hbl-1* and allow progression from the L3 stage. Loss of function of the *let-7* family microRNAs results in reiteration of L3 stage cell fates. Two other instances of miR regulation of the heterochronic pathway are known: 1) *lin-14* and *lin-28* regulation by *lin-4* miR in the L1 and L2 stages, and 2) regulation of a number of factors, including *lin-41* and *lin-42*, by the *let-7* miR during the L4 to adult transition [Reviewed in[38, 39]] (**Figure 3**).

Whereas most heterochronic genes are expressed stage-specifically to act as switches for developmental events, *lin-42* is reiteratively expressed at each larval stage, suggesting that it functions at multiple developmental stages. Loss of function of *lin-42* results in precocious developmental events in multiple cell types. In the seam cells, *lin-42* loss of function mutations do not change seam cell division patterns until the L4 stage, at which point the L4 seam cells undergo precocious terminal differentiation [2]. However, *lin-42* does exhibit stage specific phenotypes earlier in development if the *lin-42* loss of function mutation is placed in combination with other heterochronic mutations. For example, *lin-42(n1089); lin-14(n179ts)* double mutants skip the L2 proliferative seam cell division, a phenotype that is not observed in either single mutant [38, 40]. Knock-down of *lin-42* expression by RNA interference reveals early larval stage precocious phenotypes in non-hypodermal tissues, most notably in the gonad and vulva [2]. In *lin-42(RNAi)* animals, precocious division of the vulval precursor cells is often

observed by the end of the L2 stage [2]. Gonad migration is also affected in *lin-42(RNAi)* animals. In wildtype worms the gonad migrates distally until the late L3 stage. During the late L3 stage, the distal tip cells (DTCs) of the gonad turn and migrate dorsally, and finally flex back towards the mid-body. During the L4 stage, the DTCs migrate back towards the midbody/vulva, forming a U-shape [41]. In many *lin-42(RNAi)* worms, the DTCs have already started migrating towards the midbody during the L3 stage, rather than at the start of the L4 stage [2]. The L2 and L3 heterochronic phenotypes seen in *lin-42(lf)* mutants and in *lin-42(RNAi)* animals indicate that *lin-42* function is necessary for both early and late larval development.

Developmental Functions of *C. elegans lin-42*: the Dauer Decision

Dauer is an alternative larval stage that is induced in animals experiencing adverse environmental conditions such as high temperatures, increased population density, or low food availability (**Figure 4**). To enter the dauer stage requires precise coordination of developmental and environmental response programs. Not only must the larvae sense the poor environmental conditions, but normal developmental programs must be suspended and dauer development, which includes extensive physiological remodeling, must begin. Environmental conditions are relayed to developmental and physiological programs via inputs from the TGFβ, steroid and insulin signaling pathways [Reviewed in [42]].

Each of the signaling pathways that regulate dauer converges on the nuclear receptor DAF-12, which acts as the coordinator between continuous development programs, dauer development programs, and environmental signals (**Figure 3**). In favorable environmental conditions, ingested cholesterol is converted into dafachronic acid (DA), a ligand for the DAF-12 nuclear receptor protein. The DAF-12::DA complex promotes continuous larval development.

Conversely, if environmental conditions are unfavorable, less Dafachronic acid is produced and DAF-12 interacts with DIN-1. An excess of the DAF-12::DIN-1 complex inhibits continuous larval development and instead promotes dauer entry. Without mediation from another molecule, even a small excess of the DAF-12::DIN-1 complex can push an animal towards dauer entry. *lin-42* acts as that mediator and has been shown to be sufficient to prevent dauer entry in conditions of mild environmental stress [9].

Down-regulation of *lin-42* expression is required prior to dauer entry and exogenous *lin-42* expression in animals at the L2d stage, an extended L2 stage in which larvae assess their environmental conditions and prepare for dauer formation, can prevent dauers from forming [9] (**Figure 4**). Other studies have shown that the *lin-42A* promoter contains DAF-12 binding sites and is directly regulated by DAF-12 [43]. The other developmentally important isoform, *lin-42B*, does not contain any DAF-12 binding sites in its promoter; however, *lin-42A* and *lin-42B* expression cycles in tandem during larval development, suggesting that their expression is co-regulated in some manner.

Developmental Functions of *C. elegans lin-42*: lethargus and molting

Larval development in *C. elegans* is comprised of four distinct stages (designated L1 to L4) during which cell division and differentiation take place (**Figure 4**). The larval stages are separated by periods of behavioral quiescence, called lethargus, after which the larvae replace their outer cuticles in a process called molting. The four periods of lethargus that are part of each larval stage are characterized by reduction in locomotion and cessation of feeding. *C. elegans* lethargus has all of the behavioral hallmarks of sleep, meaning that lethargus is a reversible behavioral state, results in a reduction in responsiveness to mechanical stimuli, and is

homeostatic in nature [44]. Lethargus and molting are periodically timed events, with lethargus and molts occurring at the end of each of the four larval stages and typically 8-10 hours apart if the animals are raised at 25˚C [45]. Despite the well-coordinated nature of molting and lethargus, only a few molecules involved in the control of the molt timer have been identified.

Wildtype *C. elegans* exhibit a peak of *lin-42* expression during each larval stage, or intermolt, followed by a steady decrease until the molt, after which *lin-42* expression begins to increase again [2]. The only known heterochronic gene to exhibit a cyclic expression pattern that is so well coordinated with the timing of molts is *lin-42*, suggesting that there may be connection between *lin-42* expression and the molting timer. Indeed, recent studies have shown that *lin-42A* expression is required to maintain the rhythmic patterns of lethargus and molting [8]. Specifically, the *lin-42(ok2385)* mutant, in which *lin-42A* exons are deleted, shows irregular molts. The *lin-42(ok2385)* mutant animals often spend considerably more time in lethargus than wildtype animals and may enter lethargus more than once prior to molting. Exogenous expression of *lin-42A* in these mutants restores regular molt cycles; however, *lin-42A* overexpression also results in asynchronous and often atypical molts. These results indicate that lethargus and molting behaviors are dependent on LIN-42A protein function, and just as importantly on the periodic expression of *lin-42A*.

Summary and Rationale for Study

The mammalian gene *period*, which has a well-defined role in the circadian pathway, has recently been shown to function in regulating stem cell differentiation. Little is currently understood about the mechanisms that control *period* regulation of stem cells. We are studying

the *C. elegans period* ortholog, *lin-42*, as a model for understanding the developmental functions of *period*.

C. elegans lin-42 has a circadian phenotype in adult worms and regulates a number of developmental timing events during *C. elegans* post-embryonic development, including seam cell terminal differentiation, dauer entry, lethargus and molting. While *lin-42* has been implicated in the regulation of these developmental processes, the mechanisms that control *lin-42* expression and function have still not been fully defined. Previous work from our lab has shown that ZTF-16, a zinc-finger transcription factor and member of the *Hunchback/Ikaros-like* (HIL) family of proteins, is a candidate transcriptional regulator of *lin-42* expression [33]. The aim of this study is to characterize the regulation of *lin-42* by ZTF-16.

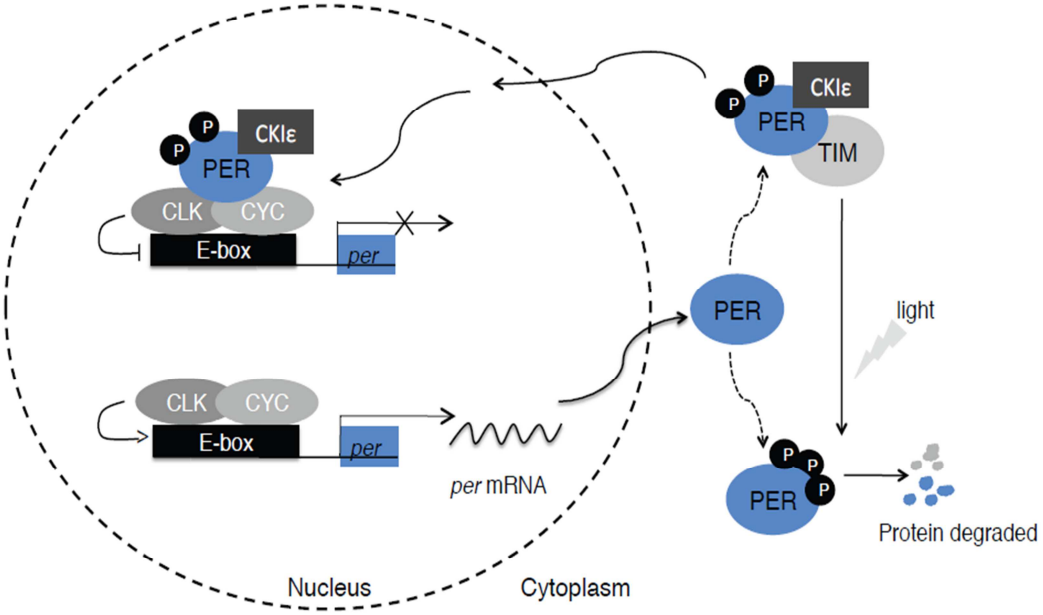

Figure 1. Transcription-translation feedback regulation of *period* in the Drosophila circadian timing pathway.

A complex transcriptional/translational feedback loop involving auto-inhibition of *period* transcription by the PER protein is at the core of the molecular circadian clock. The transcription factors CLK/CYC activate *period* transcription in the nucleus. The PER protein then begins to accumulate in the cytoplasm, where it is either degraded or binds to TIM and CKIε. The PER heterodimer then translocates to the nucleus to inhibit its own transcription by inactivating the CLK/CYC complex. Figure from [33].

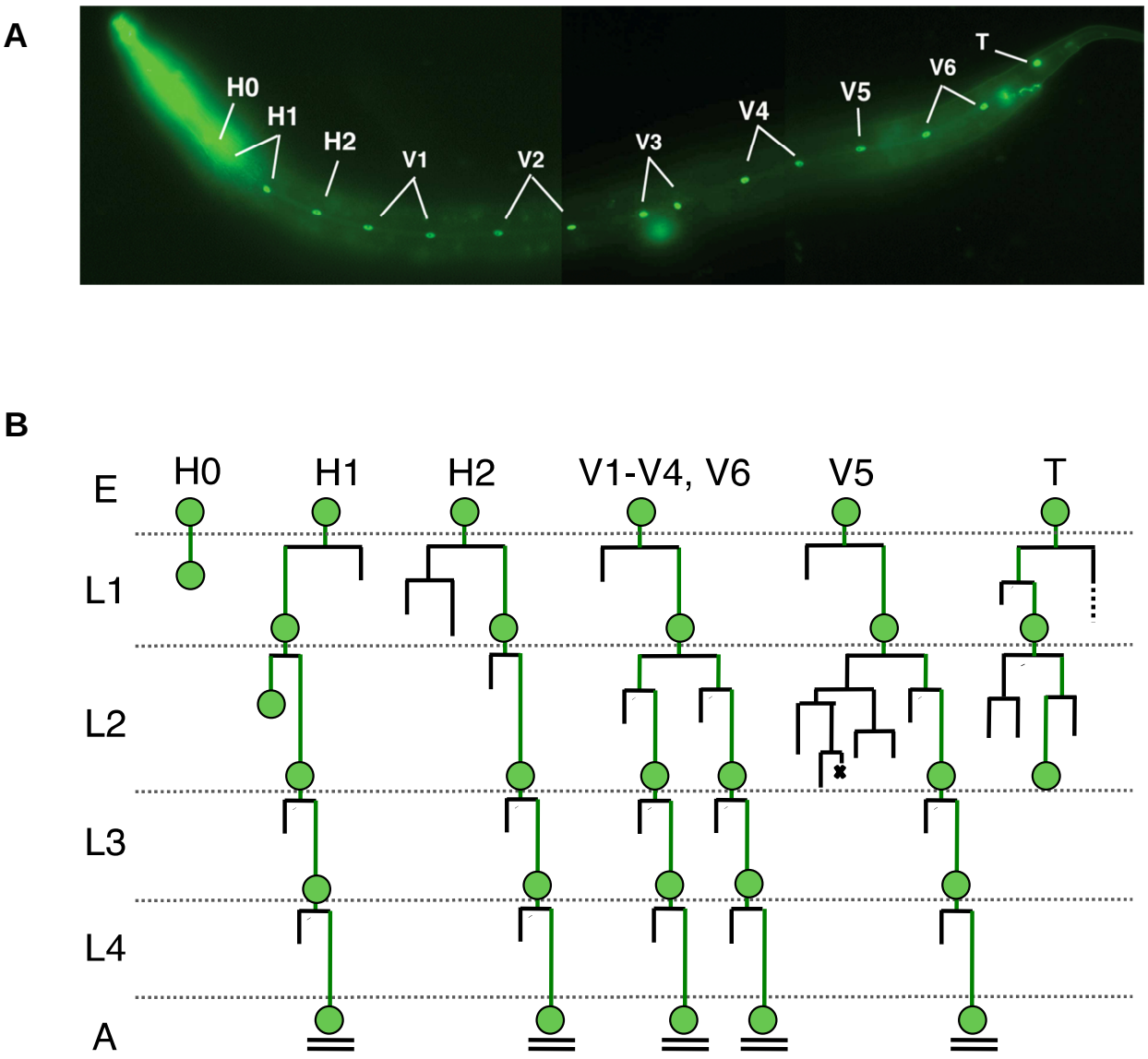

Figure 2. *C. elegans* Seam Cell Lineages (H0-2, V1-6, T).

(A) Adult *C. elegans* of *wIs78* strain with GFP marking seam cell nuclei. (B) Seam cell division and differentiation patterns during larval development. Double horizontal bars represent terminal differentiation in the adult stage. An X represents cell apoptosis. These seam cell division patterns are stereotypical in wildtype *C. elegans*. For that reason, the results of heterochronic mutations can be easily followed using strains with fluorescently labeled seam cell nuclei. Figure from [33].

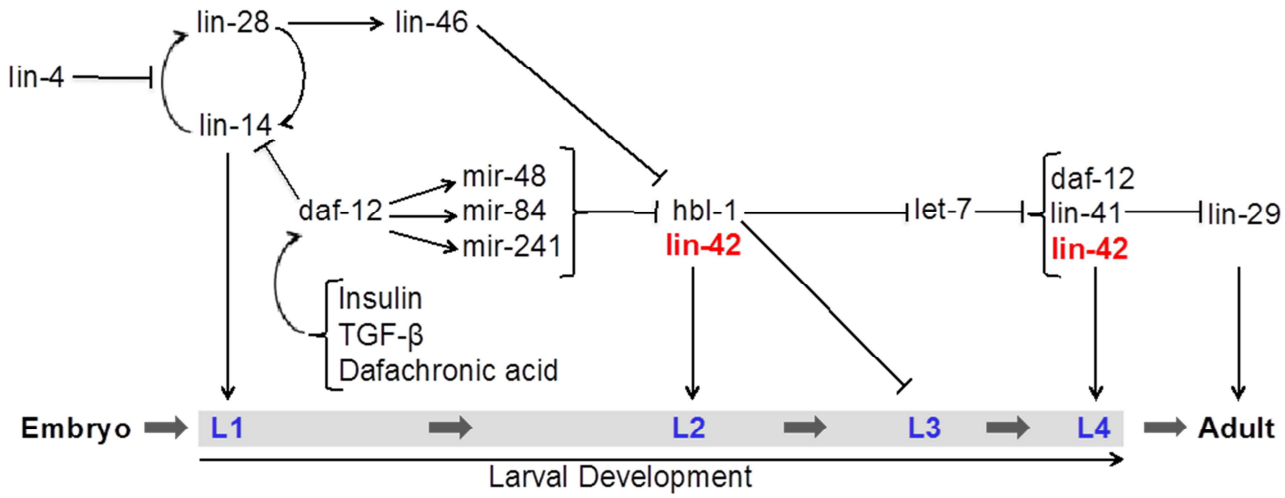

Figure 3. *lin-42* function in the *C. elegans* heterochronic pathway.

This schematic illustrates the known actions of heterochronic genes during *C. elegans* larval development. At both the early and late larval stages, *lin-42* acts to regulate larval development. Arrows indicate activation, whereas lines with bars on the end indicate inhibition. Figure from [33].

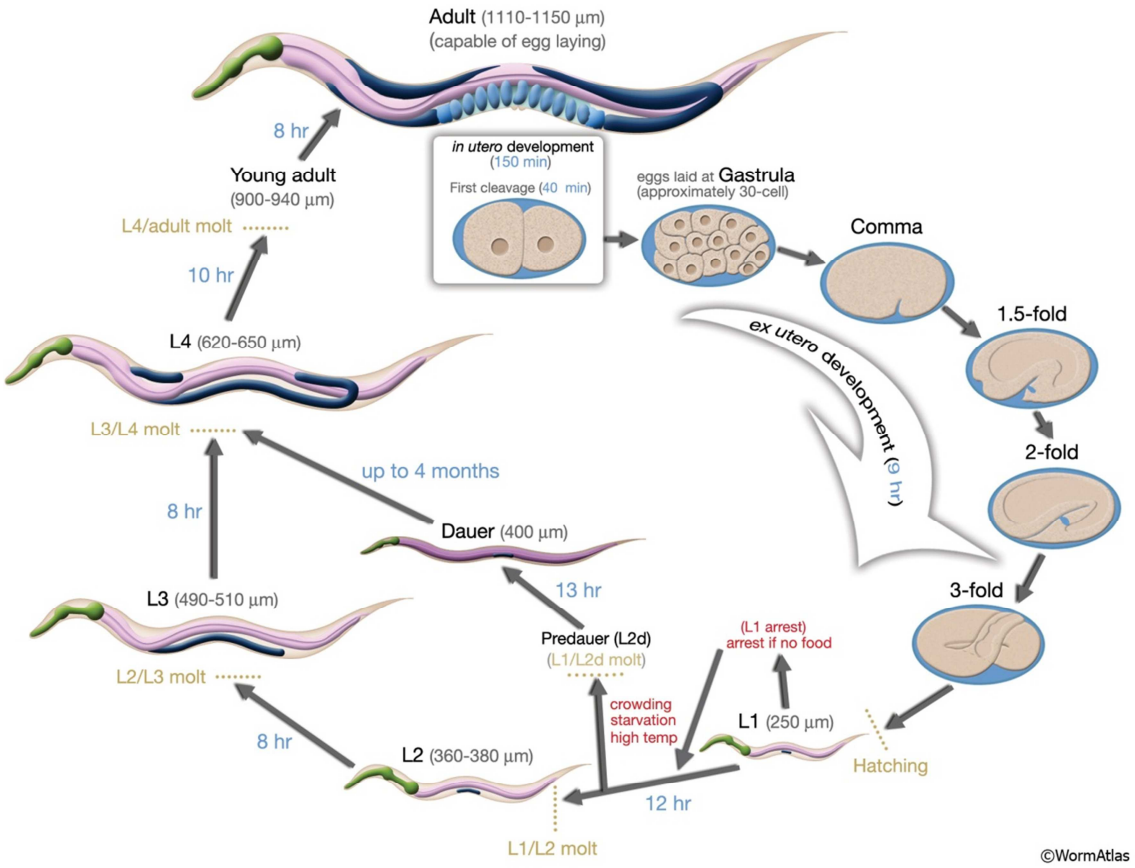

Figure 4. *C. elegans* life cycle showing alternative Dauer developmental stages.

During adverse environmental conditions, *C. elegans* larvae can enter an alternative L3 stage called dauer. Dauer entry is preceded by a lengthened L2 stage, called L2d, during which the worms assess the environmental conditions and prepare for dauer entry if required. Expression of *lin-42A* and *B* must be down-regulated to allow dauer entry to occur. Figure from [46].

Chapter 2: Characterization of ZTF-16 control of *lin-42* isoforms

BACKGROUND AND RATIONALE

The gene *period* is a key member of the molecular circadian clock that is conserved in most animals. The circadian clock is responsible for controlling a number of physiological and behavioral responses to daily environmental signals such as light and dark cycles and changes in temperature. In most animals, the clock is responsible for regulating behaviors such as sleep/wake cycles, activity levels, and even feeding rhythms [Reviewed in [16]]. Recent studies have also implicated *period* in post-embryonic cellular development, specifically the regulation of differentiation of adult and tissue stem cells and progenitor cells. For example, *period* has been shown to regulate neural progenitor cell differentiation, adoption of the adipocyte cell fate by mesenchymal stem cells, and proliferation of endothelial progenitor cells in mammals [3-5, 47]. In the circadian pathway, the regulatory network that controls *period* expression and function is very well defined at the molecular level [10, 16, 17, 48]. However, little is currently known about *period* regulation and function in stem cell developmental pathways. There are reports that *period* is not cyclically expressed in all tissue types but rather exhibits constitutive expression in some tissues, like in the dentate gyrus of the brain [5]. Additionally, there is evidence that *period* expression is controlled in a number of cell types by factors other than CLOCK and BMAL, including *GSK3β*, *ATF4* and glucocorticoids [20, 24, 31, 32]. Many of these *period* regulators have dual functions in the circadian clock and in stem cell development [20, 24, 31, 32]. Our aim is to define the expression patterns, regulation, and functions of *period* in controlling post-embryonic stem cell development. To that end, we have studied the regulation of the *C. elegans period* ortholog *lin-42* during post-embryonic development as a model for *period* regulation of adult stem cell development in mammals.

Like *period, lin-42* has both circadian physiological and developmental roles. Unlike *period*, however, the developmental functions of *lin-42* are better described than the circadian functions. *C. elegans* larval development is comprised of four stages (L1-L4) during which most active cell division and differentiation take place. The end of each larval stage and the beginning of the next stage is marked by a period of behavioral quiescence followed by shedding or molting of the outer cuticle. During the behaviorally quiescent period, called lethargus, the worms synthesize a new cuticle in preparation for ecdysis [44]. Expression of *lin-42* oscillates throughout larval development, with peaks in expression during the larval stages and a reduction in expression during lethargus and molting [1]. A number of stage-specific and reiterative developmental processes are regulated by *lin-42*, including terminal differentiation of epidermal stem cells, called seam cells, the timing of lethargus, and entry into dauer, an alternative larval stage in which the animal is developmentally arrested due to poor environmental conditions.

The proper timing of seam cell terminal differentiation is reliant upon *lin-42*. Seam cells divide asymmetrically at the beginning of each larval stage with the exception of one proliferative symmetric division at the L2 stage. In these asymmetric divisions, one daughter cell differentiates and migrates into the hypodermis, while the other cell remains part of the self-renewing seam. At the beginning of the adult stage, all seam cells terminally differentiate, fuse together into a syncytium and begin to secrete adult cuticle. This terminal differentiation is regulated by *lin-42*, and *lin-42* loss of function results in a timing defect in which the seam cells undergo precocious terminal differentiation and cuticle secretion at the L4 larval stage [1]. In addition to controlling the correct developmental timing of terminal differentiation, *lin-42* also functions as a regulator of lethargus [8]. Lethargus is a periodic behavior that typically occurs after each of the larval stages. One of the three *lin-42* isoforms, *lin-42A*, is required to maintain

the proper timing of this behavior. *lin-42A* loss of function or constitutive overexpression results in desynchronization of lethargus in which the worms may spend an abnormally long time in lethargus or may enter and exit lethargus several times before molting [8]. Finally, *lin-42* is involved in the dauer induction pathway. In order for worms to enter dauer, *lin-42* must be down-regulated at the L2 stage [9]. Overexpression of *lin-42* at this stage is sufficient to inhibit dauer induction. Additionally, *lin-42* loss of function sensitizes worms to dauer formation in conditions in which they would otherwise not form dauers. These developmental phenotypes caused by *lin-42* loss of function or mis-expression clearly indicate that temporal cyclic pattern of *lin-42* expression that mirrors the larval stages is of paramount importance to normal development. Therefore, it is imperative to determine how the dynamic expression pattern of *lin-42* is controlled.

In the circadian timing pathway, *period* expression is regulated by a complex set of feedback loops that are generated through a combination of transcriptional and post-translational control [Reviewed in [10, 16]. Transcription of *period* is activated by a protein heterodimer comprised of the cyclically expressed transcription factors CLOCK and BMAL (CYCLE in Drosophila). In the cytoplasm of mammalian and Drosophila cells, the PERIOD protein (PER) forms a dimer with CRYPTOCHROME (CRY) or TIMELESS (TIM), respectively. The stability and localization of the individual PER protein and the PER/CRY or PER/TIM complex is regulated through phosphorylation by a number of kinases and phosphatases, including Casein Kinase I ε/δ and Protein Phosphatase 1A. Phosphorylation of the PER/CRY complex results in translocation to the nucleus, where PER subsequently inactivates the CLOCK/BMAL complex to inhibit its own transcription.

There are number of *C. elegans* homologs of the mammalian and Drosophila circadian genes, several of which interact genetically with *lin-42*, including *kin-20*, the homolog of Casein Kinase 1, *tim-1*, the homolog of the Drosophila *timeless* gene, and homologs of Protein Phosphatase 1A [49], (Banerjee Lab, unpublished). Nevertheless, evidence indicates that a different mechanism of regulation is employed for *lin-42* expression during *C. elegans* development that does not involve the *C. elegans* homologs of circadian genes. First, although *lin-42* is expressed cyclically during *C. elegans* development, the oscillations are not on a 24-hour timescale. Rather, *lin-42* oscillations are on a 6-10 hour timescale that correlates with the developmental progression of the *C. elegans* larval stages [1]. Furthermore, *C. elegans* lack the *cryptochrome* gene which is responsible for the transduction of light to the machinery of the circadian clock [10, 49]. Therefore, the input pathways that drive *lin-42* expression in *C. elegans* larvae are more likely to be controlled by developmental signals as opposed to circadian environmental signals. Additionally, the translational feedback auto-inhibition of *period* expression in Drosophila and mammalian circadian pathways appears to be absent in *C. elegans*. The *lin-42*(*mg152)* mutant contains a frame-shift mutation in *lin-42* that results in production of full-length mRNA but no functional protein [1]. The levels of *lin-42* mRNA in the *lin-42(mg152)* mutant cycle normally even in the absence of functional LIN-42 protein, suggesting that *lin-42* auto-inhibition is not part of the developmental regulation of *lin-42* expression [1]. Finally, the *C. elegans* homologs of *Clock, Cycle,* and *Bmal* do not control *lin-42* expression, according to RNA interference screens performed in the search for *lin-42* regulators [33]. Therefore, our aim was to identify transcriptional regulators of *lin-42* and characterize the function of these regulators in the generation of dynamic temporal *lin-42* expression patterns during *C. elegans* larval development.

We previously identified the zinc-finger transcription factor ZTF-16 as a regulator of *lin-42* expression during *C. elegans* larval development based on both molecular interaction and genetic interaction screens [33]. EMSAs (Electrophoretic Mobility Shift Assays) were used to identify three 50-100 base pair regions (BR1-3) within the *lin-42BC* promoter that were specifically bound by *C. elegans* nuclear proteins, which may have included transcription factors. Analysis of these three BRs by bioinformatics revealed transcription factor binding site (TFBS) motifs that were conserved between *C. elegans*, *C. briggsae*, and *C. remanei*. A list of *C. elegans* homologs of the Drosophila and mammalian transcription factors that bind the TFBS motifs was then constructed. These candidate *C. elegans* transcription factors were tested through RNA interference (RNAi) screens for genetic interactions with a known regulator of *lin-42* expression. This set of assays identified ZTF-16 as a positive regulator of *lin-42* transcription. A putative TFBS within a 65 bp region (BR2) of the *lin-42BC* promoter was also identified, and ZTF-16 is predicted to bind to this putative TFBS.

In addition to the identification of ZTF-16 via the molecular and genetic interaction screens, *ztf-16* is an attractive candidate for *lin-42* regulation for other reasons. First, *ztf-16* is expressed throughout larval development and in the epidermal progenitor cells, as is *lin-42* [1, 50]. Also, ZTF-16 is a member of the *Hunchback/Ikaros-like* (HIL) family of proteins. Hunchback and Ikaros are both transcription factors with known roles in development in Drosophila and mammals [51, 52]. The aims of this study were to characterize the transcriptional regulation of *lin-42* by the zinc-finger transcription factor ZTF-16. More specifically, we sought to characterize whether ZTF-16 is necessary and sufficient for *lin-42* expression, whether ZTF-16 differentially regulates the three *lin-42* isoforms, and whether *ztf-16* loss of function affects development in ways similar to *lin-42* loss of function in *C. elegans*.

MATERIALS AND METHODS

GFP Transcriptional Reporter Constructs

To generate full length GFP transcriptional reporter constructs, PCR was used to amplify approximately 3 kb of the upstream *lin-42A* promoter and approximately 2.0 kb of the upstream *lin-42BC* promoter. GFP-PEST and the *unc-54* 3'UTR were amplified from the plasmid pAF207 (courtesy of Alison Frand). The PCR promoter fragments were purified using gel purification (Qiagen, Catalog Number 28704) and cloned into TOPO vectors using Invitrogen TOPO XL cloning kits (Invitrogen, Catalog Number K4750-10). The promoter sequences, GFP-PEST, and *unc-54* 3'UTR were then amplified from their vectors and the reporter constructs were created using the Invitrogen MultiSite Gateway cloning kit (Invitrogen, Catalog Number 12537-023). Vector sequences were confirmed by sequencing.

To construct the *lin-42BCΔTFBS* transcriptional reporter, overlap extension PCR was used [53]. The promoter sequence on either side of the desired 65bp deletion was amplified from the TOPO clones containing the full length (2.0 kb) *lin-42BC* promoter using primers that contained overlapping T7 primer sequence. These overlapping T7 sequences were then allowed to anneal and a secondary PCR reaction was used to amplify the complete sequence. The deletion sequence was cloned into the pENTR vector from the Invitrogen MultiSite Gateway kit and the reporter construct was created and confirmed as previously described. Primer sequences used in reporter gene construction can be found in **Appendix A.**

C. elegans Reporter Strain Construction

C. elegans strain preparation: *unc-119(ed3)* mutant worms were used in the reporter strain transformation. In preparation for transformation, *C. elegans* were chunked on to NEP plates

(enriched peptone agar supplemented with 10,000u/mL Nystatin) seeded with NA22 *E. coli*. When the plates were freshly starved and the majority of worms were L1 larvae, they were washed from the plates using M9 buffer and transferred to tubes. The worms were then held on ice for 10 minutes to allow the larger, adult worms to fall to the bottom of the tube. The supernatant containing the L1 larvae was transferred to a fresh tube. This was repeated two additional times to obtain a population of primarily L1 larvae. The larvae were then washed twice with M9, plated immediately on NEP plates and allowed to grow to egg-laying stage, at which time the eggs were harvested and synchronized using the hypochlorite method [54]. The synchronized L1 larvae were subsequently placed onto 10 NEP/NA22 plates at a density of ~20,000 worms per plate and allowed to grow at 25˚C for 52-54 hours or until a single row of eggs could be seen in the worms. At this point the 200,000 worms were transferred to 2 unseeded NEP plates for ballistic transformation.

Ballistic Transformation: Microbombardment was used to create worm strains with genomically integrated transcriptional reporters [55]. Two constructs were simultaneously bombarded into the worms: the desired transcriptional reporter and pMM571, the *C. briggsae unc-119* mutation rescue construct which restores wildtype movement to the worms if successfully transformed. 5µg of each plasmid was used in the bombardment. Both were linearized by restriction enzyme digestion and purified prior to use. The complete bombardment protocol is described in **Appendix B**.

Screening and Strain Confirmation: Post-bombardment, the worms were allowed to recover at 15˚C for 1 hour, after which they were transferred to NEP/NA22 plates. The plates were allowed to dry overnight at room temperature and were placed at 25˚C the following day. To screen for transformed worms, the plates were allowed to completely starve (~7-10 days) after which NA22

bacterial food source was reintroduced to the plates. After 3 to 4 days, the plates were screened for worms with wildtype movement. Any worms exhibiting wildtype movement were picked onto individual plates and allowed to propagate. To confirm the presence of the reporter construct, genomic DNA was extracted using the organic extraction method and PCR using GFP-PEST specific primers was performed.

Reporter Strain Crosses: The full length transcriptional reporter strains (*lin-42AFPp::GFP-PEST::unc-54* and *lin-42BCFLp::GFP-PEST::unc-54*) were crossed into the *ztf-16(ok3028)lf* background by generating reporter strain males and crossing them with *ztf-16(ok3028)* hermaphrodites. The two reporter strains were initially crossed with wildtype males. Male progeny expressing GFP were selected using fluorescence microscopy and crossed with *ok3028* hermaphrodites. F1 hermaphrodite progeny from that cross were picked onto individual plates and allowed to self-fertilize. F2 progeny exhibiting *ztf-16(lf)* phenotypes were picked onto individual plates and screened for GFP expression by fluorescence microscopy. Once the F2 progeny laid eggs, they were screened for *ztf-16(lf)* homozygosity using PCR. GFP expression was not visible in the *lin-42BCFLp::GFP-PEST::unc-54;ztf-16(ok3028)* F2 worms, so GFP screening was also done using PCR once the F2 progeny laid eggs. Progeny expressing GFP and containing the *ztf-16(lf)* mutation were allowed to reproduce and their lines were followed for several generations to ensure GFP homozygosity.

Fluorescence Microscopy

glo-1 and *glo-3* RNAi constructs: Initial attempts at fluorescence microscopy with the transcriptional reporter strains in the wildtype and *ztf-16(lf)* backgrounds were inhibited by gut granule auto-fluorescence (**Appendix E**). To reduce this auto-fluorescence, RNA interference

constructs targeting the genes responsible for the formation of gut granules, *glo-1* and *glo-3*, were made [56, 57]. *glo-1* and *glo-3* were amplified from cDNA synthesized from mixed stage N2 total RNA (See **Appendix C** for primer sequences). The PCR products were then ligated into linearized pL4440 RNAi vector by TA cloning. The ligation reaction was transformed into *E. coli* strain HT115 and transformants were confirmed using PCR.

Fluorescence Microscopy: The transcriptional reporter strains were synchronized using the hypochlorite method. The synchronized L1 larvae were plated onto Nematode Growth Media (NGM) plates supplemented with 1mM IPTG and 100mg/L Ampicillin, and seeded with *glo-1* and *glo-3* RNAi bacteria. The two types of bacteria were mixed at a 1:1 ratio prior to seeding and the mixture was allowed to dry on the plates for 48-72 hours prior to use. The strains were placed at 15˚C and fluorescence microscopy was performed during larval and adult stages.

Quantitative Real-Time PCR (qRT-PCR)

C. elegans larval staging and harvests: The *lin-42A* and *lin-42BC* reporter strains in the wildtype and *ztf-16(ok3028)lf* background were synchronized using the hypochlorite method and the L1 larvae were plated on NGM plates seeded with OP50 *E.coli*. The worms were grown at 25˚C. Starting at 11 hours post-plating and every three hours until 38 hours post-plating, worms from a subset of the plates were harvested by washing with M9. The worms were pelleted, the supernatant was removed, and the worms were flash-frozen in liquid nitrogen and stored at -80˚C until RNA extraction was performed.

RNA extraction and cDNA preparation: The frozen worm pellets were thawed and total RNA was extracted by the phenol/chloroform method using Ambion TRI-Reagent (Catalog Number AM9738). The RNA was quantified using the Invitrogen Quant-iT RiboGreen kit (Catalog

Number R11490) and 1µg of RNA was used to make complementary DNA using M-MLV Reverse Transcriptase (Promega, Catalog Number M1701).

qRT-PCR: Quantitative Real-Time PCR was performed using TaqMan chemistry (TaqMan Master Mix from ABI, Catalog Number 4440040) using specially designed probes (ABI) and primers for *lin-42A*, *lin-42B*, *lin-42C*, *ztf-16*, *GFP-PEST* and *ama-1*. The threshold values (Ct) for each *lin-42* isoform, GFP-PEST, and *ztf-16* were compared to the threshold value of *ama-1* at the same time point to calculate the relative difference between transcript levels. Primer and probe sequences for qRT-PCR are listed in **Appendix D**.

N2 adult staging: Staging of wildtype adult *C. elegans* was performed as described for the reporter strains with the following exceptions: The L1 larvae were plated on NGM plates supplemented with 1mM IPTG and 100mg/L of Ampicillin and seeded with *pos-1* RNAi bacteria to prevent eggs from hatching. See **Appendix C** for primer sequences used in the construction of *pos-1* RNAi vector. The adult harvests were performed once every 24 hours starting at 24 hours post-plating through 9 days post-plating. For the cDNA synthesis, 3µg of total RNA was used to increase the likelihood of detecting *ztf-16* transcripts. All other aspects of the harvest, RNA extraction, and qRT-PCR were performed as described for the *C. elegans* larval staging.

Lethargus Assays

Synchronized populations of N2 or *ztf-16(ok3028)* L1 larvae were obtained using the hypochlorite method. The worms were plated on NGM plates seeded with OP50 *E. coli* at a density of ~50 worms per plate. The worms were kept at 20˚C and scored each hour post-plating for the presence or absence of pharyngeal pumping, the lack of which is a hallmark of lethargus. The plates were scored up to 57 hours post-plating. Three plates were scored per time point.

High-temperature induced dauer (HID) Assays

Eggs were obtained from N2, *lin-42(mg152)*, *lin-42(n1089)*, *ztf-16(ok3028)*, and *ztf-16(ok1916)* using the hypochlorite method. The un-hatched eggs were immediately plated onto NGM plates seeded with *E. coli* OP50 at a density of approximately 50 eggs per plate, 3 plates per strain. The plates were placed at 27˚C for 48 hours, after which time they were scored for dauer and partial dauer formation. Partial dauer formation was classified as any worm approximately the same size as an L2 larva after 48 hours, with no visible vulva formation but an unconstructed, pumping, pharynx.

RESULTS

ztf-16(lf) results in down-regulation of *lin-42* isoforms

Genetic interaction screens have identified the zinc-finger transcription factor ZTF-16 as a candidate regulator of *lin-42* expression [33]. To verify that ZTF-16 is indeed a *lin-42* transcriptional regulator, mRNA levels of *lin-42* were measured using quantitative real-time polymerase chain reaction (qRT-PCR). Three isoforms (*lin-42A*, *lin-42B*, and *lin-42C*) are expressed from the *lin-42* genomic region and are controlled by different regulatory regions, suggesting that they may have variant expression patterns (**Figure 1**). We thus designed isoform specific qRT-PCR primers and probes to measure expression of all three *lin-42* isoforms (**Appendix D**). The *lin-42* transcript levels were measured by qRT-PCR from developmentally synchronized populations of larval worms grown at 25°C that were harvested every 3 hours over the time course of larval development. The *lin-42* transcripts were measured in N2 wildtype

worms (full *ztf-16* function), N2 worms in which *ztf-16* expression was knocked-down by RNA interference (RNAi), and the *ztf-16(ok3028)* loss-of function mutant strain (**Figure 2**).

In wildtype genetic background, *lin-42A* and *lin-42B* are expressed cyclically with peaks of expression during each larval stage and down-regulation at the molt between each stage (**Figure 2A)**. These expression patterns are consistent with published reports of *lin-42* expression that did not distinguish among the *lin-42* isoforms [1, 2]. However, our data show that the *lin-42C* isoform, which shares 5' regulatory sequences with *lin-42B*, is expressed only basally during larval development (**Figure 2A).**

When *ztf-16* expression was reduced using RNAi, *lin-42A* and *lin-42B* expression patterns were wildtype until the L4 larval stage, at which time the expression was down-regulated approximately 3-fold relative to *lin-42A* and *lin-42B* expression in wildtype at the equivalent stage [33] (**Figure 2B**). Down-regulation of *lin-42A/B* at the L4 stage suggests either that ZTF-16 is a stage-specific regulator that acts during late larval development or that *ztf-16* down-regulation by RNAi is not sufficient in the early larval stages. Therefore, to test whether or not the L4 *lin-42* down-regulation was the result of stage-specific interactions with ZTF-16, *lin-42* transcript levels were measured in *ztf-16(ok3028)* mutants [33]. The *ztf-16(ok3028)* mutation is a deletion that removes exons 8 to 10 of the coding region, but does not remove any zinc-finger domains. *lin-42A* and *lin-42B* expression were both down-regulated in the *ztf-16(ok3028)lf* mutant and cyclic expression at all larval stages was lost (**Figure 2C**). These data indicate that ZTF-16 function is required to regulate the wildtype cyclic expression patterns of *lin-42A* and *B* throughout larval development. Nevertheless, *lin-42A* and *B* expression is not completely abrogated in the *ztf-16(ok3028)* mutants, suggesting that the deletion mutation does not eliminate

all ZTF-16 transcriptional activity or that basal expression of *lin-42A* and *B* isoforms are regulated by additional factors.

ztf-16 is expressed cyclically and in anti-phase to *lin-42*

Our expression analysis of *lin-42* isoforms indicates that ZTF-16 is directly or indirectly responsible for the cyclic expression of *lin-42A* and *lin-42B* during larval development in *C. elegans*. Cyclic expression of the mammalian and Drosophila *lin-42* ortholog, *period*, is regulated by the transcription factors *Clock* and *Bmal*, which are themselves cyclically expressed in a complex feedback loop with *period* [Reviewed in [16, 17]]. To determine whether *ztf-16* exhibits cyclic expression similar to *Clock* and *Bmal*, *ztf-16* expression was measured in wildtype larvae using qRT-PCR (**Figure 3**). We found that *ztf-16* is expressed cyclically and in anti-phase to *lin-42A*/*B* expression pattern. Thus, *ztf-16* expression peaks during the lowest expression levels of *lin-42* and is lowest during the peaks of *lin-42* expression (**Figure 3A**). These data are consistent with the *ztf-16* and *lin-42* expression profiles generated from a temporal series of whole genome microarrays of synchronized larval stage N2/wildtype *C. elegans* (**Figure 3B**) [33]. The *ztf-16*/*lin-42* anti-phase expression pattern is similar to the *Clock/Bmal* expression pattern relative to *period*. Our data suggest that cyclic expression of *lin-42* may be driven by pulses of *ztf-16* expression and activity.

ztf-16 loss of function phenocopies *lin-42* loss of function

Since *lin-42A* and *lin-42B* expression is down-regulated but not completely abrogated in *ztf-16(lf)*, we sought to determine whether *ztf-16* regulation of *lin-42* is functionally relevant. We thus investigated whether *ztf-16(lf)* down-regulation resulted in phenotypes consistent with *lin-*

42A and *B* loss of function, such as precocious terminal differentiation, abnormal lethargus and molting and dauer sensitization.

The three *lin-42* isoforms vary in their exonic and protein structures, have different regulatory regions and are not redundant in their functions. For example, the *lin-42(n1089)* mutation, which affects only the *lin-42B* and *C* isoforms, exhibits precocious heterochronic phenotypes, low penetrance egg-laying defects and dauer induction phenotypes, but is not lethal [2, 9] (**Table 1, Figure 1**). These observations indicate that *lin-42B* functions in the heterochronic pathway to regulate the asymmetric division patterns of hypodermal seam cells. Loss of function of *lin-42B/C* causes the seam stem/progenitor cells to differentiate during the L3 or L4 larval stage of development instead of at the L4 to adult transition. In contrast, *lin-42(ok2385)* is a deletion mutation that eliminates both the *lin-42A* and *lin-42B* isoforms [2]. This deletion mutant has severe *lin-42(lf)* phenotypes, including precocious seam cell differentiation, a loss of synchronization in lethargus and molting, a higher penetrance of egg-laying defects, and lethality caused by molting defects [2, 8] (**Table 1**). The more severe phenotypes are due in part to the loss of *lin-42A* and the molting irregularities that result from that loss. Recent work has shown that *lin-42A* is required to maintain the synchronous patterns of lethargus, a sleep-like state during which *C. elegans* larvae synthesize a new cuticle in preparation to shed the old one [8]. A loss of synchrony in lethargus also results in molting defects and lethality. Taken together, the phenotypes of the different *lin-42* mutant strains indicate that loss of LIN-42B in the presence of LIN-42A activity primarily affects terminal differentiation during late larval development, while loss of LIN-42A and LIN-42B together result in more severe developmental defects including asynchronous lethargus and lethality. Loss of *lin-42A* and *lin-42B* also sensitizes larvae to enter dauer under normal environmental conditions, and this sensitization is

substantially more severe with LIN-42A loss in comparison to loss of only LIN-42B [9]. Our research group has previously shown that *ztf-16* loss or reduction of function results in *lin-42B(lf)* heterochronic phenotypes, including precocious seam cell terminal differentiation and precocious alae formation [33]. Therefore, to determine whether the *lin-42A* down-regulation observed in *ztf-16(lf)* mutants is developmentally relevant, we assayed lethargus and dauer sensitization phenotypes in *ztf-16(ok3028*) mutants.

Lethargus is a behaviorally quiescent state that occurs four times during larval development. During these quiescent periods, feeding behavior completely ceases, as observed by a lack of pharyngeal pumping. We found that that there are four distinct periods of lethargus, or time during which no pharyngeal pumping takes place in at least 20% of the population of wildtype worms (**Figure 4A**). At a population level, these periods of inactivity lasted for approximately 3 to 4 hours and were separated by 4 to 6 hours of activity during which less than 10% of the population was inactive. In the *ztf-16(ok3028)* mutants, periods of lethargus are extended and after the L1 stage, the population does not appear to fully exit lethargus (**Figure 4B**). In the wildtype worms, there is a short period of time during which 100% of the worms are active. In the *ztf-16(ok3028)* mutant strain, more than 10% of the population is inactive throughout larval development. These data are similar to reports of abnormal lethargus in *lin-42(ok2385)* mutants [8]. Loss of cyclic *lin-42A* expression in *lin-42(ok2385)* mutants results in prolonged periods of lethargus and an overall asynchrony in lethargus and molting behaviors across worm populations. It is worthwhile to note that exogenous overexpression of *lin-42A* also resulted in a loss of synchronization in molting and lethargus, suggesting that the cyclic expression of *lin-42A* is required to maintain synchrony [8]. In addition to lethargus arrhythmia, the *lin-42(ok2385)* mutants exhibited molting defects [8]. *ztf-16(lf)* mutants also exhibited

molting defects, although these defects were observed at a low penetrance of less than 10%. Our finding of abnormal lethargus and molting in *ztf-16(ok3028)* mutants, in which *lin-42A* and *lin-42B* are non-cyclically expressed at low levels, underscores that cyclic expression of *lin-42* is important for controlling the timing of developmental events in *C. elegans* larvae.

In addition to the lethargus assays, we performed a high-temperature induced dauer (HID) assay to assess whether *ztf-16(ok3028)* mutants are sensitized to dauer formation. HID phenotypes occur when worms inappropriately form dauer larvae under conditions of mild-temperature stress (27˚C) but in the presence of ample food and low population densities. In wildtype worms, temperature stress alone is not sufficient to induce dauer formation. Therefore, *C. elegans* mutants that exhibit this phenotype are considered to be sensitized to dauer formation. Two *lin-42* mutants have been previously tested for HID phenotypes: *lin-42(ve11),* a mutant strain with *lin-42A* and *B* loss of function, and *lin-42(n1089),* a strain in which only *lin-42B* is eliminated [9] (**Table 1**). The *lin-42(ve11)* exhibits a high rate of dauer formation at 71%, whereas the *lin-42(n1089)* exhibits 21% dauer formation according to published reports [9]. Our HID assays were performed using *ztf-16(ok3028), ztf-16(ok1916),* N2 wildtype worms, and *lin-42(n1089)* mutant strains. Both *ztf-16* mutant strains show statistically significant increase in dauer formation compared to wildtype (**Figure 5**). There is less dauer formation in the *ztf-16(ok1916)* mutant, however a previous study has shown that *lin-42(lf)* heterochronic phenotypes in the *ztf-16(ok1916)* mutant are less penetrant than in the *ok3028* strain [33]. Thus, this result is consistent with our expectations. Dauer induction in the *ztf-16(ok3028)* is consistent with the average dauer induction in *lin-42(n1089)* strain, as published in [9]. Interestingly, we were not able to replicate the *lin-42(n1089)* results from [9] in our own work.

Together, our data indicate that down-regulation of the developmentally important isoforms *lin-42A* and *lin-42B,* in combination with their loss of cyclic expression, is functionally relevant to *C. elegans* larval development. In particular, the asynchronous periods of lethargus in *ztf-16(ok3028)* larvae correlate with the loss of cyclic expression of *lin-4A,* the down-regulation of *lin-42B* expression in *ztf-16* mutant correlates with precocious heterochronic phenotypes, and the down-regulation of both *lin-42A* and *B* isoforms in *ztf-16(ok3028)* is sufficient to sensitize the worms to dauer formation.

ZTF-16 regulates both *lin-42A* and *lin-42BC* promoters

Our expression and phenotypic analyses indicate that both *lin-42A* and *lin-42B* are regulated by the zinc-finger transcription factor ZTF-16. However, *lin-42A* and *lin-42B* expression is thought to be controlled via different promoter regions (**Figure 1**). The *lin-42B* and *C* isoforms share a promoter region in addition to the first 5 exons of the *lin-42* coding sequence. The promoter for the *lin-42A* isoform, on the other hand, is within the 3kb of intronic sequence between exons 6 and 7. This 3 kb intragenic sequence has been shown to function as an independent promoter [8]. Therefore, using qualitative and quantitative analyses of *lin-42* transcriptional reporter constructs, we sought to determine whether ZTF-16 regulates one or both of the *lin-42* promoters.

Three transcriptional reporters were made to test ZTF-16 regulation of *lin-42*. Each transcriptional reporter contained GFP-PEST and the *C. elegans unc-54* 3'UTR sequence. The addition of the PEST sequence to the GFP gene reduces the half-life of the attached protein to approximately 1 hour, allowing for dynamic changes in protein expression over the course of *C. elegans* development to be observed using fluorescence microscopy [45]. The *unc-54* 3'UTR is a

myosin gene that is not post-transcriptionally regulated, allowing us to determine which aspects of the expression profiles are conferred through transcriptional regulation [58]. Three different promoter sequences were used to drive the GFP-PEST reporters: 1) The 3 kb intragenic sequence of *lin-42A* promoter (*lin-42AFLp*), 2) Approximately 2.0 kb of the proximal *lin-42BC* promoter upstream of the ATG translational start site of *lin-42* exon 1 (*lin-42BCFLp*), and 3) A deletion version of the *lin-42BC* 2.0 kb promoter minus a 65 bp region (BR2) that contains a putative ZTF-16 transcription factor binding site (**Figure 9A**). These transcriptional reporter constructs were biolistically transformed into wildtype and *ztf-16(ok3028)lf* genetic backgrounds. We used simultaneous RNAi of *glo-1* and *glo-3*, which positively control gut granule formation, in order to reduce granule auto-fluorescence in these strains that was obscuring *lin-42*-specific tissue expression patterns [56, 57] **Appendix E** illustrates the difference between the gut auto-fluorescence of strains raised on OP50 *E.coli* versus *glo-1;glo-3* double RNAi bacteria.

To evaluate the effect of *ztf-16(lf)* on *lin-42* expression patterns, we first characterized expression from *lin-42A* and *lin-42BC* full length transcriptional reporters in wildtype genetic background. Spatial expression patterns were nearly identical for both the *lin-42A* and *lin-42BC* promoter reporters. GFP was observed consistently in gland cells in the tail and pharynx, in hypodermal and seam cells, other pharyngeal cells, and the Hermaphrodite Specific Neuron (HSN). Less often, expression was observed in vulva cells, distal tip cells (DTCs), the ventral nerve cord (VNC), gonadal sheath cells and unidentified neurons in the mid-body and tail (**Figure 6 and 7**). Expression from the *lin-42A* and *lin-42BC* reporters in wildtype genetic background largely resembles previously reported *lin-42* expression patterns obtained using antibody staining, although neuronal and neuronal-associated cell expression were not previously reported [1, 2]. Additionally, we identified some of the specific cells in which *lin-42*

is expressed, including the rectal gland, g1 pharyngeal gland, and amphid socket cells (AMso) (**Figure 7**). It is possible that inconsistencies between the previously published data and our data are due to the different methods used (antibody staining versus transcriptional reporter expression) or, in cases where we did not observe reporter expression in a specific cell type, the promoter sequences in our reporters may lack particular cell/tissue-specific enhancers. However, the observed expression patterns from the *lin-42A* and *lin-42BC* reporters indicate that the promoter regions used in the reporters are sufficient to recapitulate wildtype *lin-42* spatial expression patterns in tissues that are known to be affected by *lin-42* mutation.

The *lin-42* transcriptional reporter strains were crossed into *ztf-16(ok3028)lf* genetic background to observe whether *ztf-16(lf)* resulted in any changes in expression. The *lin-42A/B reporter;ztf-16(lf)* expression patterns are summarized in **Figure 6**. Expression from the *lin-42BCFLp* reporter in *ztf-16(ok3028)* was not greatly altered in comparison to expression in wildtype background (**Figure 6**). Although the spatial expression pattern did not change, the frequency of expression was reduced such that GFP expression was observed in fewer worms at each larval stage and the intensity of the GFP signal in many cell types was diminished. Surprisingly, expression from the *lin-42AFLp* reporter was completely lost in the *ztf-16(lf)* background until the late L4 and adult stages, at which time only limited tail cell expression was observed (**Figure 6**). This lack of expression is in stark contrast to the extensive spatial expression patterns observed for the *lin-42AFLp* reporter in wildtype background. These data indicate that while expression from both *lin-42* promoters requires some degree of ZTF-16 function, expression from *lin-42A* promoter is more dependent upon ZTF-16.

GFP expression from *lin-42A* and *lin-42BC* reporter strains, in both wildtype and *ztf-16* mutant backgrounds, was temporally dynamic in a number of cell types, including the seam

cells. To quantify dynamic expression patterns, and to evaluate the effect of *ztf-16* loss of function on expression from *lin-42A* and *lin-42BC* promoters, we used qRT-PCR to measure GFP transcript levels in the reporter strains (**Figure 8**). In wildtype genetic background, GFP expression in the *lin-42BC* reporter cycles synchronously with the endogenous *lin-42B* transcript, although there are a greater number of GFP transcripts than *lin-42B* transcripts relative to *ama-1* (**Figure 8A**). In combination with the tissue specific expression patterns from the *lin-42BC* reporter, the cycling of reporter transcript in tandem with endogenous *lin42B* indicates that the selected 2.0 kb of *lin-42BC* promoter contains the regulatory sites that drive both spatial and temporal expression patterns of *lin-42B* during larval development. In *ztf-16(lf)* background, the cyclic expression patterns of both *GFP* and *lin-42B* transcripts are lost, and expression is down-regulated through most of larval development (**Figure 8A**). These results indicate that ZTF-16 regulates *lin-42* expression, either directly or indirectly, through sites in the 2.0 kb of *lin-42BC* promoter used in the reporter constructs.

The *lin-42A* reporter exhibits cyclic GFP expression in the wildtype background; however, the cyclic expression pattern does not coincide with *lin-42A* endogenous transcript expression, and during early larval stages is in anti-phase to the endogenous *lin-42A* transcript (**Figure 7B**). In the *ztf-16(lf)* genetic background, the GFP expression pattern is dramatically reduced and is non-cyclic in nature. These results are consistent with tissue expression data, and indicate that the 3 kb of the *lin-42A* promoter is not sufficient to drive *lin-42A* temporal expression patterns. However, the *lin-42A* transcriptional reporter recapitulates the spatial expression pattern of *lin-42* if not the temporal expression pattern. Furthermore, the complete loss of *lin-42A* tissue specific expression in the *ztf-16(lf)* background indicates that expression from the *lin-42A* promoter is dependent upon ZTF-16 expression.

lin-42BC promoter contains a putative ZTF-16 binding site that is required for gene expression

ZTF-16 was identified as a candidate *lin-42* transcriptional regulator based on the presence of a putative transcription factor binding site (TFBS) within the *lin-42BC* promoter [33]. The process of discovery was as follows: A 1.6 kb region of the *lin-42BC* promoter was systematically tested for binding by *C. elegans* larval nuclear extracts using Electrophoretic Mobility Shift Assays (EMSAs). The EMSAs revealed three regions of the promoter, designated Binding Regions 1-3 (BR1-3), that were specifically bound by nuclear proteins. These binding regions (BRs) were subsequently analyzed by bioinformatic analysis to identify putative transcription factor binding motifs. A GATA transcription factor binding site was identified within the 65bp BR2 that was conserved among other closely related nematode species, indicating that this was likely to be bona fide regulatory control site. A list of mammalian transcription factors that bind to GATA transcription factor binding sites was made, and *C. elegans* homologs of those transcription factors were identified. Top candidates for *lin-42* transcriptional regulators were identified by tissue specific and temporal expression patterns, if known. ZTF-16, which is expressed in the seam cells during larval development, was therefore identified as a candidate (**Figure 9**).

Our results indicate that ZTF-16 function is required for transcriptional reporter expression from the *lin-42BC* promoter. Thus, we sought to determine if deletion of the putative ZTF-16 binding site in the *lin-42BC* promoter affects expression from the *lin-42BC* reporter. A deletion reporter, lacking only the 65bp BR2 was created and transformed into wildtype genetic background. The presence of the GFP reporter in the generated worm strains was confirmed by

PCR using GFP specific primers. Expression analysis of three independently transformed strains revealed a complete lack of reporter expression at all stages of larval development and also in the adult (**Figure 9**). These results, together with our data showing that *lin-42A* and *B* expression is dependent on ZTF-16 function, strongly suggest that the 65 bp BR2 region of the *lin-42BC* promoter contains a conserved ZTF-16 binding site that is required for *lin-42* expression.

ztf-16 does not regulate *lin-42* during the adult stage

Our findings indicate that ZTF-16 regulates *lin-42* expression during larval development. In order to determine whether the regulatory relationship between ZTF-16 and *lin-42* continues in adult *C. elegans*, we measured endogenous *lin-42* and *ztf-16* transcript levels during the adult stage.

During the larval stages, *lin-42* has developmental roles in the regulation of somatic cell division and differentiation, but these roles cannot extend into the adult stage. In *C. elegans*, all somatic lineages terminally differentiate at the end of larval development, leaving the germline as the only actively dividing tissue in the adult worm, which typically lives for 10 to 12 days. Nevertheless, *lin-42* appears to function during the adult stage as a regulator of circadian behavior. Adult *C. elegans* exhibit circadian rhythms in locomotion in response to temperature and light/dark cycles [7, 35, 36]. Although little is known about the role of *lin-42* in adult stage circadian rhythms, there is a single report that *lin-42(n1089)* and *lin-42(mg152)* mutants have longer circadian periods than wildtype worms [6]. Therefore, there is a temporal separation of developmental and physiological roles for *lin-42* during larval versus adult stages. However, no studies have been performed to ascertain if the same *lin-42* isoforms that function during development, namely *lin-42A* and *B*, also function during the adult stage, whether *lin-42*

functions in the same cell types during both larval and adult stages, or whether *lin-42* is regulated by the same molecules in both stages.

Our adult stage expression analysis shows that, contrary to larval expression patterns, *lin-42A* and *B* isoform expression is repressed during the adult stage while *lin-42C* expression is up-regulated and appears to increase as the adult worms age (**Figure 10A**). The *lin-42A* and *lin-42BC* transcriptional reporter drives GFP expression in the HSN neurons and some vulva tissues during the adult stage (**Figure 10B**). Also, infrequently, *lin-42A* reporter expression was observed in the pharynx of *C. elegans* adults. Therefore, it is possible that LIN-42 function and expression is restricted to certain cell types, particularly neurons, during the adult stage in order to regulate physiology and behavior. The other possible explanation for these GFP expression patterns is that tissue-specific enhancer elements that regulate *lin-42* expression during the adult stage are absent from the *lin-42* promoter regions used in the reporter constructs.

Our qRT-PCR data indicate that *lin-42C*, but not *lin-42A* or *lin-42B*, regulates the circadian behaviors that are altered in *lin-42(n1089)* and *lin-42(mg152)* mutants [6]. Neither of these mutants produce functional LIN-42B or LIN-42C protein (**Table 1**). Since *lin-42A* and *B* are not expressed in the adult stage, the loss of *lin-42C* must be responsible for these circadian phenotypes. Therefore, our results suggest that there is isoform-specific division of *lin-42* function in which *lin-42A* and *B* regulate larval somatic development and *lin-42C* regulates circadian patterns of behavior in the adult stage. The experimental design of the adult qRT-PCR assays, which measured transcript levels at 24 hour intervals, could not to be used to ascertain whether *lin-42C* adult stage expression is cyclic, like that of *period*, which exhibits a 12 hour phase [17, 59]. However, a microarray study indicates that *lin-42* transcripts expressed during the adult stage do not show a circadian cyclic expression pattern [36]. The findings from the van der

Linden study suggest that the mechanism behind the non-cyclic *lin-42C* expression in the *C. elegans* circadian pathway may be different from the cyclic regulation of *period* expression in Drosophila and mammalian circadian pathways. However, we found that *ztf-16* expression is also repressed during the adult stage, indicating that ZTF-16 is not responsible for *lin-42C* expression in adult *C. elegans*.

DISCUSSION

This study shows that ZTF-16, a *Hunchback/Ikaros-like* family zinc finger transcription factor, controls transcription of the developmentally important *lin-42* isoforms during *C. elegans* larval development. We have shown that ZTF-16 regulates two of the three known *lin-42* isoforms, *lin-42A* and *lin-42B*, and is required to maintain the cyclic expression pattern of these two isoforms throughout larval development. We have also confirmed *ztf-16* transcript expression profiles from microarray data that indicate that *ztf-16* expression is cyclic and anti-phase to *lin-42* expression. Our data suggest that *lin-42* cyclic expression patterns may be driven by the periodic expression and activity of *ztf-16*. We found that the down-regulation of *lin-42* expression that results from *ztf-16(lf)* is of phenotypic relevance because *ztf-16(lf)* mutants exhibit the developmental phenotypes associated with *lin-42* loss of function mutations. Specifically, *ztf-16(lf)* mutants exhibit precocious terminal differentiation of epithelial cell lineages, are sensitized to dauer induction, and have asynchronous periods of lethargus and molting. Since expression driven by the *lin-42BC* 2 kb proximal promoter and the *lin-42A* 3 kb intragenic promoter are repressed in the *ztf-16* mutant background, we are able to conclude that ZTF-16 acts through both of these promoters. Additionally, a 65bp region within the *lin-42BC* promoter that contains a putative ZTF-16 transcription factor binding site was shown to be

essential for *lin-42* expression. We further found that only *lin-42C* is expressed during the adult stage, while *lin-42A*, *lin-42B*, and *ztf-16* are not expressed. These findings indicate that *lin-42C* must be the isoform responsible for regulating *lin-42*-dependent circadian patterns of behavior in adult worms. Since *ztf-16* is not expressed during the adult stage, a different set of factors must be regulating *lin-42C* expression with regard to the physiological circadian functions of *lin-42*.

ztf-16 regulation of *lin-42B*

In the *ztf-16(ok3028)* mutant, expression of *lin-42A* and *lin-42B* is down-regulated but not completely abrogated (**Figure 2C**). Since the *ztf-16(ok3028)* mutation is not null, a complete loss of *lin-42* expression was not expected, although the possibility remains that additional regulatory proteins function with ZTF-16 to control *lin-42* expression. However, the deletion of the 65 bp BR2 region containing the putative ZTF-16 transcription factor binding site within the *lin-42BC* promoter results in a complete loss of *lin-42* tissue-specific expression patterns (**Figure 9**). Since no other conserved TFBSs were identified within BR2, our results strongly suggest that ZTF-16 is both necessary and sufficient for transcriptional control of the *lin-42B* isoform. Nevertheless, cryptic TFBSs may exist within the BR that were not identified by our bioinformatic methodology. Transcription factors that bind to these novel sites could act independently or in combination with ZTF-16 to regulate *lin-42* spatio-temporal expression patterns.

Additional, albeit indirect, evidence that *lin-42B* expression is driven by ZTF-16 can be found in the cyclic expression pattern of ZTF-16, which occurs prior to *lin-42B* cyclic expression and may be driving *lin-42B* expression. Furthermore, the 2 kb *lin-42BC* promoter recapitulates the cyclic expression pattern of the endogenous *lin-42B* transcript. Cyclic expression of both

endogenous transcript and reporter are lost in the *ztf-16(lf)* genetic background, indicating that the cyclic expression pattern of *lin-42B* is driven through sites in the 2 kb of *lin-42BC* promoter and that ZTF-16 is required for cyclic expression.

Our results lead us to propose a transcriptional model of cyclic regulation in which ZTF-16 is periodically expressed and transiently binds to the GATA TFBS within the *lin-42BC* promoter to induce *lin-42B* transcription. Falling levels of ZTF-16 due to *ztf-16* transcriptional down-regulation would thus be followed by a decrease in *lin-42B* expression. Nevertheless, the data we have presented does not preclude the possibility that the cyclic expression pattern of *lin-42B* is not solely dependent on periodic transcription by ZTF-16. In fact, in mammals, cyclic *period* transcription is regulated in part by inhibition of the *period* transcription factors, *Clock* and *Bmal*, by the PER protein itself. While *ztf-16* expression follows the anti-phase expression pattern characteristic of *Clock* and *Bmal*, there is currently no indication that *lin-42* regulates its own transcription through negative feedback of *ztf-16*. This is supported by expression data from the *lin-42(mg152)* mutation, which is a frame-shift mutation that produces a truncated and non-functional version of LIN-42B protein. Levels of the *lin-42B* mRNA continue to cycle in this *lin-42* mutant, indicating that if there is a feedback loop regulating ZTF-16 transcription of *lin-42*, it is not controlled by the LIN-42B protein [1].

ztf-16* regulation of *lin-42A* and relationship to *daf-12

While our data support a direct transcriptional regulation model of *lin-42B* by ZTF-16 via the 65 bp TFBS within BR2 region of *lin-42BC* promoter, the relationship between ZTF-16 and *lin-42A* appears to be more complex.

Spatial and temporal expression from the *lin-42A* promoter transcriptional reporter is dramatically repressed in the *ztf-16(lf)* mutant background (**Figure 8**). This indicates that *lin-42A* expression is dependent upon ZTF-16 function. Nevertheless, qRT-PCR data shows that, unlike 2 kb of *lin-42BC* promoter, the *lin-42A* promoter sequence alone is not sufficient to drive wildtype *lin-42A* expression patterns (**Figure 8B**). This discrepancy suggests that ZTF-16 regulation of *lin-42A* is mechanistically different from ZTF-16 regulation of *lin-42B*. That is, ZTF-16 likely does not regulate *lin-42A* expression directly through a transcription factor binding site in the *lin-42A* promoter. Rather, ZTF-16 may regulate *lin-42A* expression indirectly, and there may be other transcription factors involved. Two lines of evidence support this model. First, bioinformatic analysis of the *lin-42A* promoter did not reveal any conserved binding sites for ZTF-16 or related transcription factors, indicating that ZTF-16 does not bind to the *lin-42A* promoter. Second, it has been shown that the transcription factor DAF-12, which is involved in the dauer decision pathway, directly regulates *lin-42A* through a site in the *lin-42A* promoter [43]. No such sites were found within the 2 kb of *lin-42BC* promoter used in the transcriptional reporter. Since this 2 kb segment of the promoter is sufficient to recapitulate the majority of the endogenous spatio-temporal expression patterns of *lin-42B,* DAF-12 likely does not regulate *lin-42B* expression directly (**Figure 8A**).

DAF-12 is a key component of the dauer induction pathway. In fact, *daf-12* mutants in which *lin-42* expression has also been knocked-down are more susceptible to dauer formation under mildly stressful conditions than wildtype worms [9]. Similarly, *ztf-16(lf)* mutants, in which *lin-42* expression is low, are also sensitive to dauer formation as shown in the HID phenotype of *ztf-16(lf)* (**Figure 5**). These results suggest that both ZTF-16 and DAF-12 are required for *lin-42A* expression. It is possible that ZTF-16 may interact with DAF-12 to indirectly modulate

transcription of *lin-42A*. Nevertheless, a model in which ZTF-16 interacts with DAF-12 bound to the *lin-42A* promoter to induce *lin-42A* expression does not explain the discrepancy between the expression patterns of the *lin-42A* transcriptional reporter and the endogenous *lin-42A* transcripts. The *lin-42A* transcripts cycle in tandem with *lin-42B* transcripts during larval development in wildtype genetic background, but *GFP* transcripts from the *lin-42A* reporter are not coordinately expressed with endogenous *lin-42A* transcripts (**Figure 8B**). The simplest explanation for this discrepancy is that cis-regulatory regions that are required for the cyclic temporal expression of *lin-42A* are not present in the *lin-42A* transcriptional reporter. For example, the native *lin-42A* 3'UTR, which may contain regulatory sites that are required for correct temporal expression patterns, is not utilized in our *lin-42A* reporter construct. Another explanation may be that *lin-42A* cyclic expression is dependent upon *lin-42B* expression. For example, there may be interaction between the *lin-42BC* and *lin-42A* promoter regions in order to coordinate *lin-42A* transcription with that of *lin-42B*. This could potentially be achieved through *lin-42BC* promoter regulatory complexes in which the *lin-42B* promoter, while bound perhaps by ZTF-16, must contact DAF-12 and the *lin-42A* promoter to induce transcription of *lin-42A*. In the *lin-42A* transcriptional reporter strain, unbound ZTF-16 may be able to partially substitute for ZTF-16 that is bound to *lin-42BC* promoter DNA; however, this regulation may be less efficient, resulting in the alteration of *lin-42A* cyclic expression.

CONCLUSIONS AND FUTURE DIRECTIONS

ztf-16 in *C. elegans* developmental pathways and beyond

ZTF-16 is a member of the *Hunchback/Ikaros-like* (HIL) family of proteins, which are characterized by four N-terminal, DNA binding zinc-fingers and two C-terminal zinc-fingers

responsible for protein-protein interactions [52]. The mammalian *Ikaros* functions in the hematopoietic system, specifically as a transcriptional activator responsible for the development/differentiation of lymphoid precursor cells into the B- and T-cells of the immune system [52]. *Hunchback*, the other founding member of the HIL family of proteins, is a zinc finger protein that is important for patterning during *Drosophila* embryonic development [51].

Two recent studies have shown that ZTF-16 regulates a number of important developmental events in *C. elegans*, including progenitor cell differentiation in reproductive development and morphological remodeling upon entry into dauer. More specifically, *ztf-16* is involved in the regulation of somatic gonadal precursor cell morphology in *C. elegans*. *ztf-16* loss of function affects both the somatic gonad development and gonad migration [50]. In addition to its role in the *C. elegans* reproductive system, ZTF-16 is also involved in sensory neuron remodeling upon entry into dauer [60]. In dauer larvae, the amphid sheath glia of the head fuse together at the tip of the nose to allow the AWC neurons to expand and overlap [60]. *ztf-16* is a transcriptional regulator of *ver-1*, a receptor tyrosine kinase that is required for dauer-induced neuronal remodeling in the amphid sheath glia [61]. Loss of function of *ztf-16* results in incomplete glial fusion in dauer larvae [60].

These *ztf-16* gonadal and dauer phenotypes are of particular significance in light of the known functions and expression patterns of *lin-42*. For example, *lin-42* is a regulator of dauer; down-regulation of *lin-42A* and *B* is required at the L2 stage to allow for dauer entry, and exogenous expression of *lin-42* can prohibit dauer formation in worms that would otherwise form dauers [9]. These previous reports did not identify the specific cell types in which *lin-42* may be regulating dauer formation. We present the first reports of *lin-42* expression in specific pharyngeal cells that may function in the regulation of dauer formation. This work shows that

lin-42 is expressed in the amphid socket glia (**Figure 7**). There are two types of neuronal support cells in the *C. elegans* head: the amphid sheath glia (AMsh) and the amphid socket glia (AMso). These two support cells are connected by adherens junctions near the nose of the animal. The amphid socket glia are epithelial derived cells that form a pore to the outside of the nose into which they secrete cuticle [Reviewed in [62, 63]]. The pore formed by the AMso allows the chemosensory amphid neurons to sense the environment, including the levels of dauer pheromone [63]. This suggests that *lin-42* expression in the AMso is required as part of the dauer induction pathway. Although no direct correlation has yet been made, it is interesting to note that *ztf-16* is also expressed in the AMso and in the AMsh and is required for the remodeling of the AMsh upon entry into dauer [60]. Our data show that ZTF-16 is a positive regulator of *lin-42* expression. Therefore, we would anticipate that *lin-42* and *ztf-16* would be expressed in the same tissues during continuous larval development. Prior to dauer entry, however, loss of *ztf-16* expression or inhibition of ZTF-16 function resulting in *lin-42* repression would be expected in tissues involved in dauer development.

Gonad development in *C. elegans* larvae is also regulated by *lin-42*. Expression of *lin-42* is present in the distal tip cells (DTCs), which are largely responsible for gonad migration [2]. Our observations of *lin-42* reporter expression support these findings, as we observed *lin-42* reporter expression in the DTCs and other reproductive tissues such as the gonadal sheath cells. Studies have found that *ztf-16* is also expressed in the DTCs and that *ztf-16(lf),* like *lin-42(lf),* results in gonad migration defects [2, 50]. These loss of function phenotypes are consistent with our findings that ZTF-16 is a positive regulator of *lin-42* expression during continuous larval development.

Finally, *ztf-16* and *lin-42* are both expressed in the hypodermis and seam cells of *C. elegans* larvae [1, 2, 50]. Work from our laboratory shows that *ztf-16* mutants exhibit a precocious seam cell differentiation phenotype that is consistent with the precocious phenotypes previously reported for *lin-42(lf)* mutants [1, 33]. Once again, these findings support that ZTF-16 is a transcriptional regulator of *lin-42* expression. Taken together, these results suggest that ZTF-16 is a master regulator of cell fate in a number of tissues in *C. elegans* larvae and acts through its regulation of *lin-42* expression. *Ikaros*, a HIL family transcription factor, and *period* are also involved in the regulation of stem cell differentiation in mammals. No connection has yet been made between *Ikaros* and the regulation of *period* expression during stem cell development. However, our findings that ZTF-16 regulates *lin-42* expression in *C. elegans* suggest that other HIL family proteins may regulate *period* transcription in mammals to control stem/progenitor cell differentiation. Ultimately, it will be important to define how ZTF-16 and HIL transcription factors involved in stem cell and other cell fate pathways are regulated in order to be able to define the different mechanisms through which *lin-42/period* expression and functions are controlled in developmental versus physiological pathways.

Testing models of ZTF-16 regulation of *lin-42*

This work has led us to form two different models to explain ZTF-16 regulation of *lin-42A* and *lin-42B*. Future studies will aim to test the validity of these models. First, we hypothesize that ZTF-16 directly binds to the 65bp transcription factor binding site within the *lin-42BC* promoter and drives the cyclic expression pattern of *lin-42B* though periodic expression or activity. This model can be tested by expressing *ztf-16* at a constant level from a constitutive promoter and assaying for the predicted loss of the cyclic pattern without total loss

or down-regulation of *lin-42B* expression. To determine whether ZTF-16 regulation of the *lin-42BC* promoter is direct, we could employ EMSA to test whether purified ZTF-16 protein is capable of binding to the 65 bp BR2 DNA from the *lin-42BC* promoter. Alternatively, One-Hybrid assay could be used to determine whether there is direct binding of ZTF-16 to the *lin-42BC* BR2 promoter region.

Our results suggest that a direct binding model of transcriptional regulation cannot be used to describe ZTF-16 regulation of *lin-42A*. Rather, we hypothesize that ZTF-16 regulates *lin-42A* indirectly through interactions with the known *lin-42A* regulator, DAF-12. Based on the coordinate expression pattern of *lin-42A* and *B*, we hypothesize that ZTF-16, while bound to the *lin-42B* promoter, interacts with DAF-12 as it is bound to the *lin-42A* promoter. These interactions function together to regulate the cyclic expression pattern of *lin-42A*. To test these hypotheses, we would first test whether ZTF-16 and DAF-12 are capable of interacting through *in vitro* formation of DAF-12:ZTF-16 protein complexes on the *lin-42A* promoter sequence using antibodies against the two transcription factors. Alternately, we could use Two-Hybrid assay to more generally test for direct interaction between ZTF-16 and DAF-12. If the results of these assays indicate that ZTF-16 and DAF-12 are capable of interacting, a hybrid transcriptional reporter strain containing both the *lin-42A* and *lin-42B/C* promoter regions would be generated to determine whether the presence of both promoter regions is required to recapitulate endogenous *lin-42A* transcript expression. These studies will allow us to more precisely determine the mechanism of ZTF-16 regulation of *lin-42A* and *lin-42B*, which is vital for a complete understanding of the developmental functions of *lin-42* and will provide a basis for defining the mechanisms of *period* regulation in mammals.

Table 1. *lin-42* and *ztf-16* mutants

Strain	Mutation	*lin-42A* mRNA/ Protein	*lin-42B* mRNA/ Protein	*lin-42C* mRNA/ Protein	Phenotypes					
					Seam Cell Division/ Differentiation	Lethargus and Molting	Dauer (HID)	Egg-laying abnormal (egl)	Lethality (let)	Circadian behavior
N2/wildtype	N/A	+/+	+/+	↓/↓	+	+	+	+	+	+
lin-42(n1089)	Deletion (Exons 1-6) [A]	+/+	0/0	0/0	M	+	HID	egl	+	M
lin-42(mg152)	Frameshift insertion/ deletion (Exon 1) [A]	+/+	+/0	+/0	M	+	HID	egl	+	ND
N2; lin-42B/C (RNAi)	N/A	+/+	↓/↓ [C]	↓/↓ [B]	M	+	+	egl (mild)	+	ND
lin-42(ok2385)	Deletion (Exons 7-12) [A]	0/0	0/0	+/+ [B]	M	M	ND	egl	let	ND
lin-42(ve11)	Nonsense (Exon 9)	?/0	?/0	+/+ [B]	M	ND	ND	egl	ND	ND
N2; lin-42A (RNAi)	N/A	↓/↓	↓/↓	+/+ [B]	M	+	+	egl (mild)	+	ND
ztf-16(ok3028)	Deletion (Exons 8-10)	↓/↓ (not cyclic)	↓/↓ (not cyclic)	+/+	M	M	HID	egl	let	ND
ztf-16(ok1916)	Deletion (Exon 6, zinc-fingers 3 and 4)	ND	ND	ND	+	ND	HID	+	+	ND
N2;ztf-16 (RNAi)	N/A	↓/↓ (cyclic) [C]	↓/↓(cyclic) [C]	+/+	+	+	+	+	+	ND

[A] Refer to **Figure 1**

[B] Predicted from mutation and/or phenotype, but not experimentally confirmed

[C] Reduced expression only in L4

M indicates Mutant Phenotype

+ indicates Wildtype Phenotype

↓ indicates that expression is reduced

ND: Not Determined

References for **Table 1** [1, 2, 8, 9, 50]

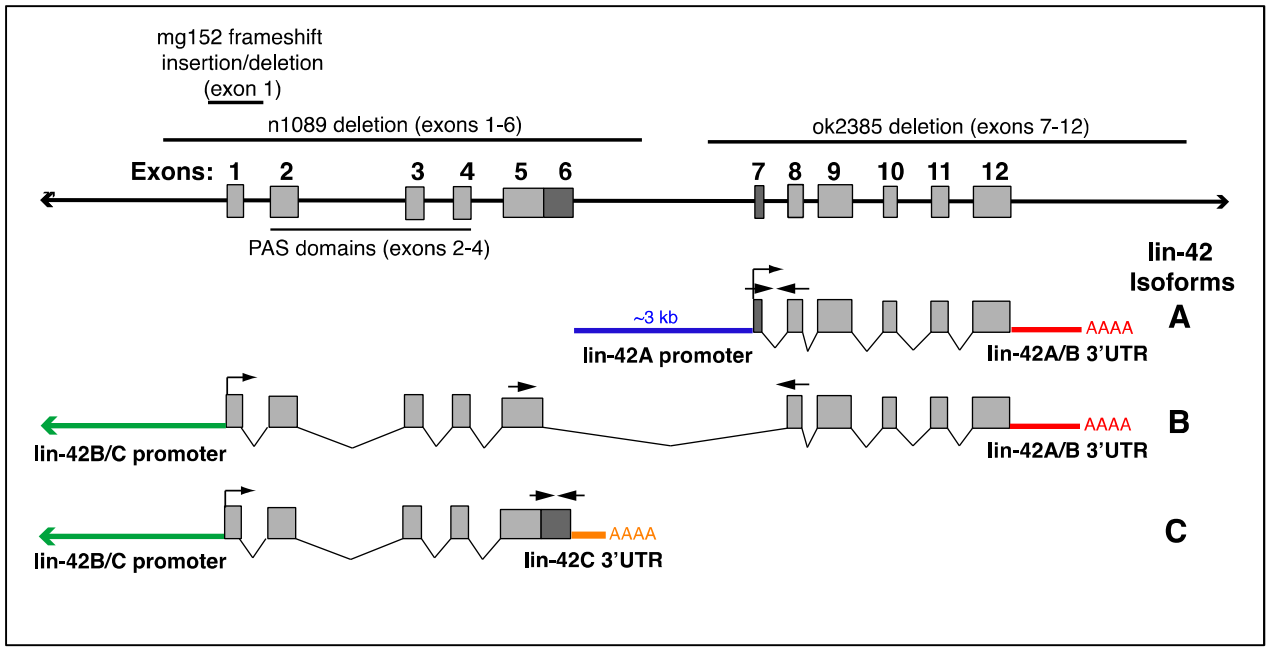

Figure 1. *C. elegans lin-42* isoforms and mutations.

Exons and regulatory regions of *lin-42A*, *B* and *C* isoforms. The positions of the deletions and mutations in *lin-42(n1089)lf*, *lin-42(ok2385)lf* and *lin-42(mg152)lf* strains are indicated. Arrows indicate the positions of primers for isoform-specific qRT-PCR. Figure from [33].

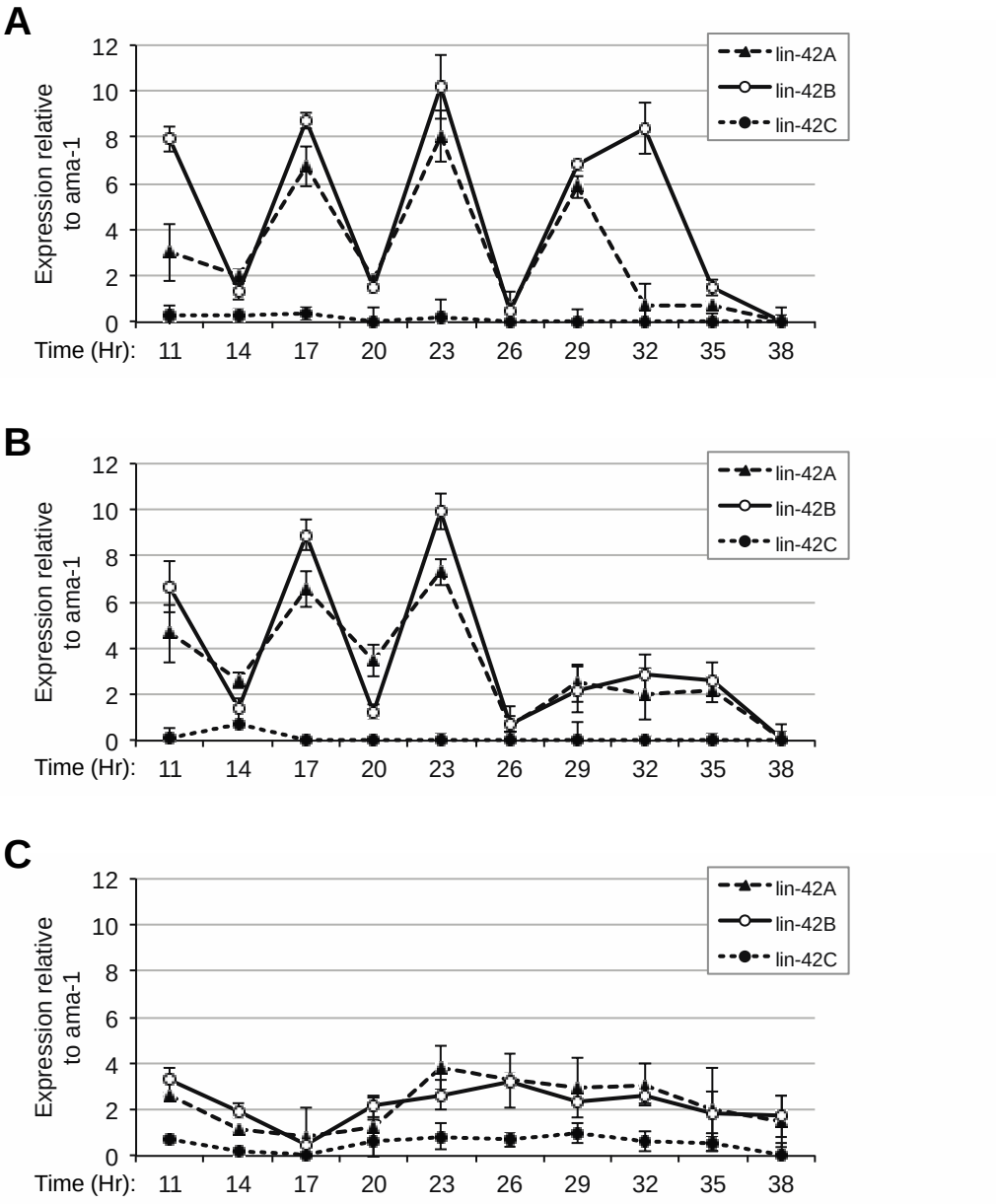

Figure 2. Expression of *lin-42* transcripts during larval development in (A) N2/wildtype, (B) *N2;ztf-16(RNAi)* and (C) *ztf-16(ok3028)lf* mutant.

(A) *lin-42A* and *B* isoforms exhibit oscillatory expression patterns in the wildtype genetic background while *lin-42C* is only expressed basally. (B) *lin-42A* and *B* isoform expression is down-regulated during L4 stage of larval development in *ztf-16(RNAi)* worms. (C) In the *ztf-16(ok3028)* strain, *lin-42* isoform expression is reduced and oscillatory patterns are abolished. Figure adapted from [33].

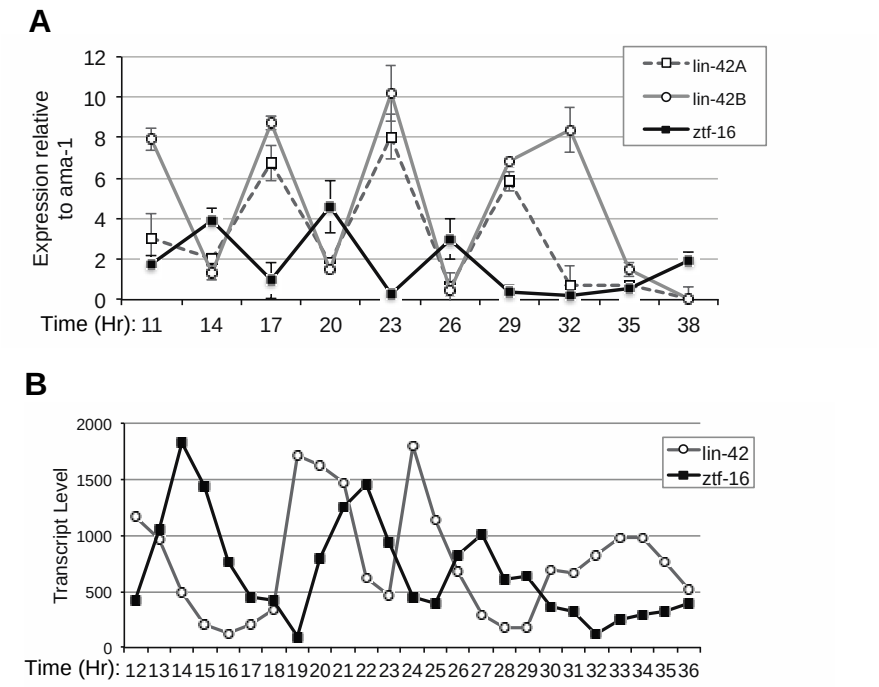

Figure 3. Expression of *ztf-16* and *lin-42* isoform transcripts during larval stages in N2/wildtype worms.

(A) Quantitative real-time PCR in wildtype *C. elegans* larvae shows that *ztf-16* is expressed in anti-phase to *lin-42A* and *B* during larval development, with peaks of *ztf-16* expression when *lin-42* levels are lowest. (B) Microarray data of *C. elegans* transcripts throughout larval development confirm the anti-phase expression pattern of *ztf-16* relative to *lin-42*.

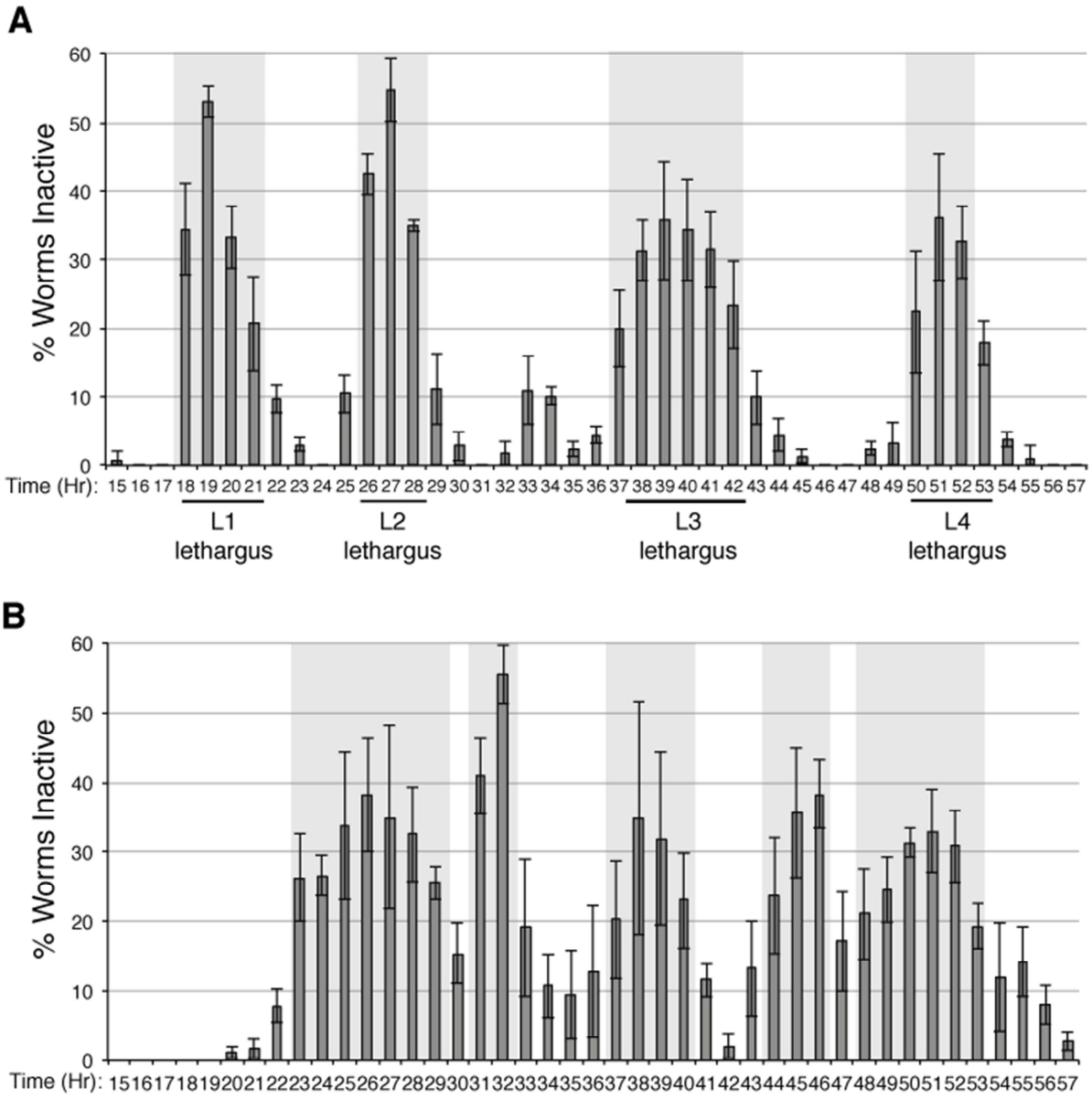

Figure 4. Lethargus in (A) N2/wildtype and (B) *ztf-16(ok3028)lf* strains.

(A) In wildtype *C. elegans* larvae, there are four periods of lethargus, one during each stage of larval development. (B) In the *ztf-16(ok3028)* mutant strain, larvae either do not exit lethargus or inappropriately reenter lethargus during larval stages. Periods of lethargus highlighted in gray are periods of time during which at least 20% of the population of worms were inactive, that is they did not exhibit pharyngeal pumping, a hallmark of lethargus.

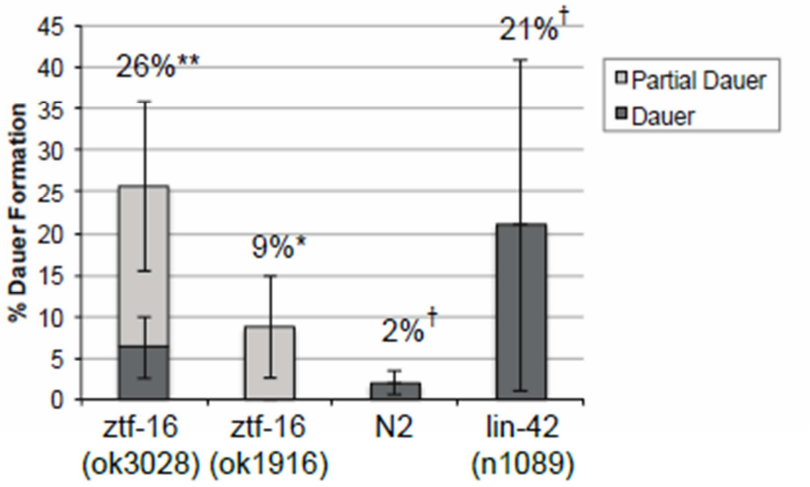

Figure 5: Dauer sensitization in *ztf-16(lf)* mutants.

ztf-16(ok3028) and *ztf-16(ok1916)* mutants exhibit a High-temperature induced dauer (HID) phenotype. In *ztf-16(ok3028)* mutants, the percentage of dauer induction is similar to dauer induction previously reported in *lin-42(n1089)* mutants [9]. We were unable to replicate the results reported for *lin-42(n1089)* mutants in our experiments.

† Data from [[9]]
**p-value ≤.0.01
*p-value≤0.05

		Hypodermis &/or Seam cells				Neurons			
Reporter	**Background**	**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**
lin-42Ap::gfp-pest::unc-54	wildtype								
lin-42Ap::gfp-pest::unc-54	ztf-16(ok3028)								
lin-42BCp::gfp-pest::unc-54	wildtype								
lin-42BCp::gfp-pest::unc-54	ztf-16(ok3028)								
lin-42BCpΔTFBS::gfp-pest::unc-54	wildtype								
αLIN-42 Ab *	wildtype								

		Pharynx				Tail			
Reporter	**Background**	**L1**	**L2**	**L3**	**L4**	**L1**	**L2**	**L3**	**L4**
lin-42Ap::gfp-pest::unc-54	wildtype								
lin-42Ap::gfp-pest::unc-54	ztf-16(ok3028)								
lin-42BCp::gfp-pest::unc-54	wildtype								
lin-42BCp::gfp-pest::unc-54	ztf-16(ok3028)								
lin-42BCpΔTFBS::gfp-pest::unc-54	wildtype								
αLIN-42 Ab *	wildtype								

Figure 6. Summary of larval expression patterns of *lin-42A* and *lin-42BC* transcriptional reporters.

lin-42 transcriptional reporter expression during larval development divided by reporter strain, genetic background, and tissue type. *lin-42* reporter expression is observed in the hypodermis/seam cells, neurons, pharynx and tail cells at various stages throughout larval development. The dark green bars indicate the presence of GFP expression. The orange bars indicate that expression was seen in the listed tissue type; however, the frequency of expression is reduced compared to other strains. * indicates previously published *lin-42* expression data using antibody staining [2].

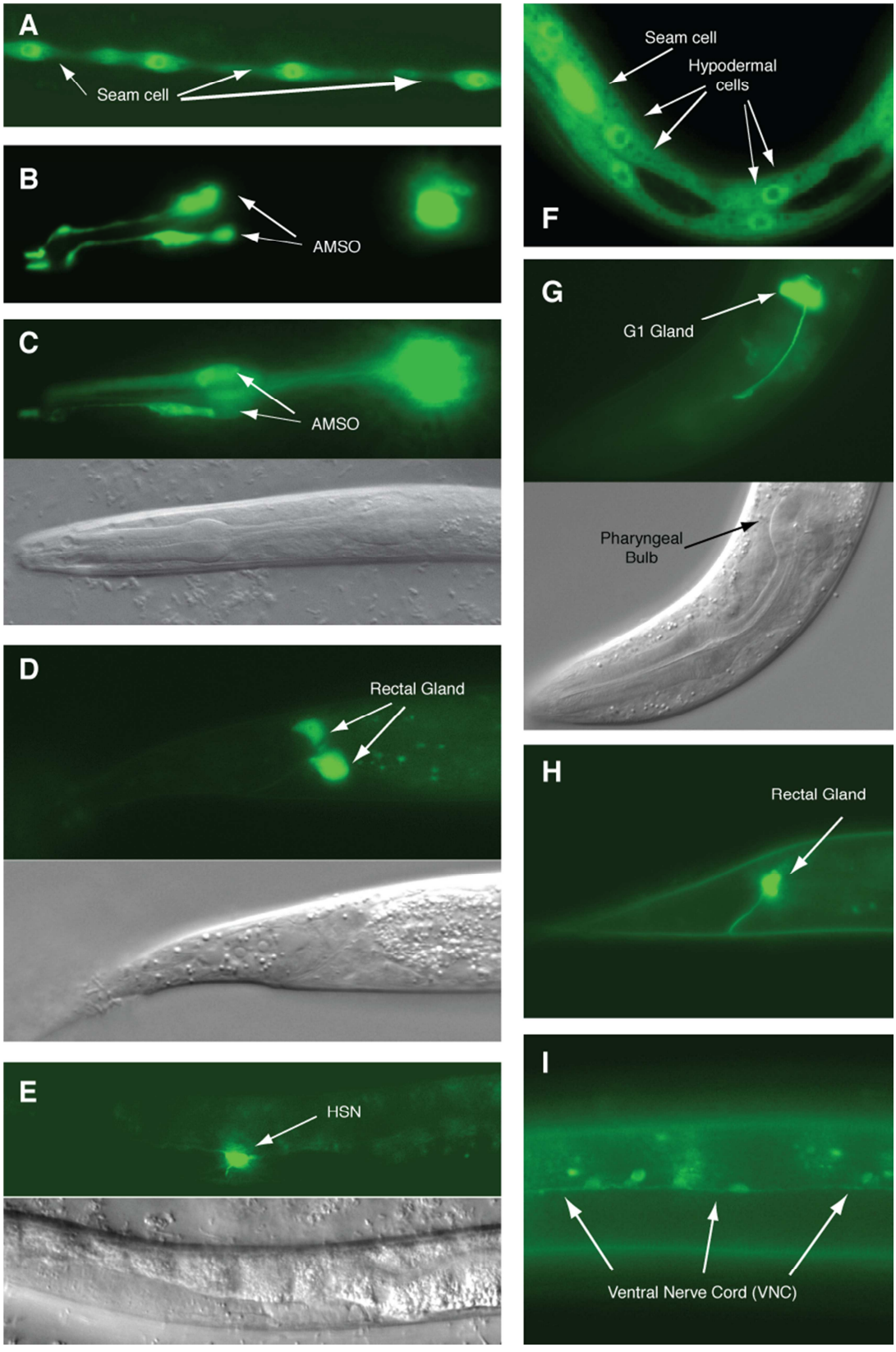
A
Seam cell
B
AMSO
C
AMSO
D
Rectal Gland
E
HSN
F
Seam cell
Hypodermal cells
G
G1 Gland
Pharyngeal Bulb
H
Rectal Gland
I
Ventral Nerve Cord (VNC)

Figure 7. Larval expression patterns of *lin-42A* and *lin-42BC* transcriptional reporters.

(A-I) Examples of *lin-42A* and *lin-42BC* transcriptional reporter expression patterns in *C. elegans* larvae, including expression in the seam cells (A&F), the amphid socket cells, AMSO (B&C), the rectal gland (D&H), the Hermaphrodite Specific Neuron, HSN (E), and the Ventral Nerve Cord, VNC (I).

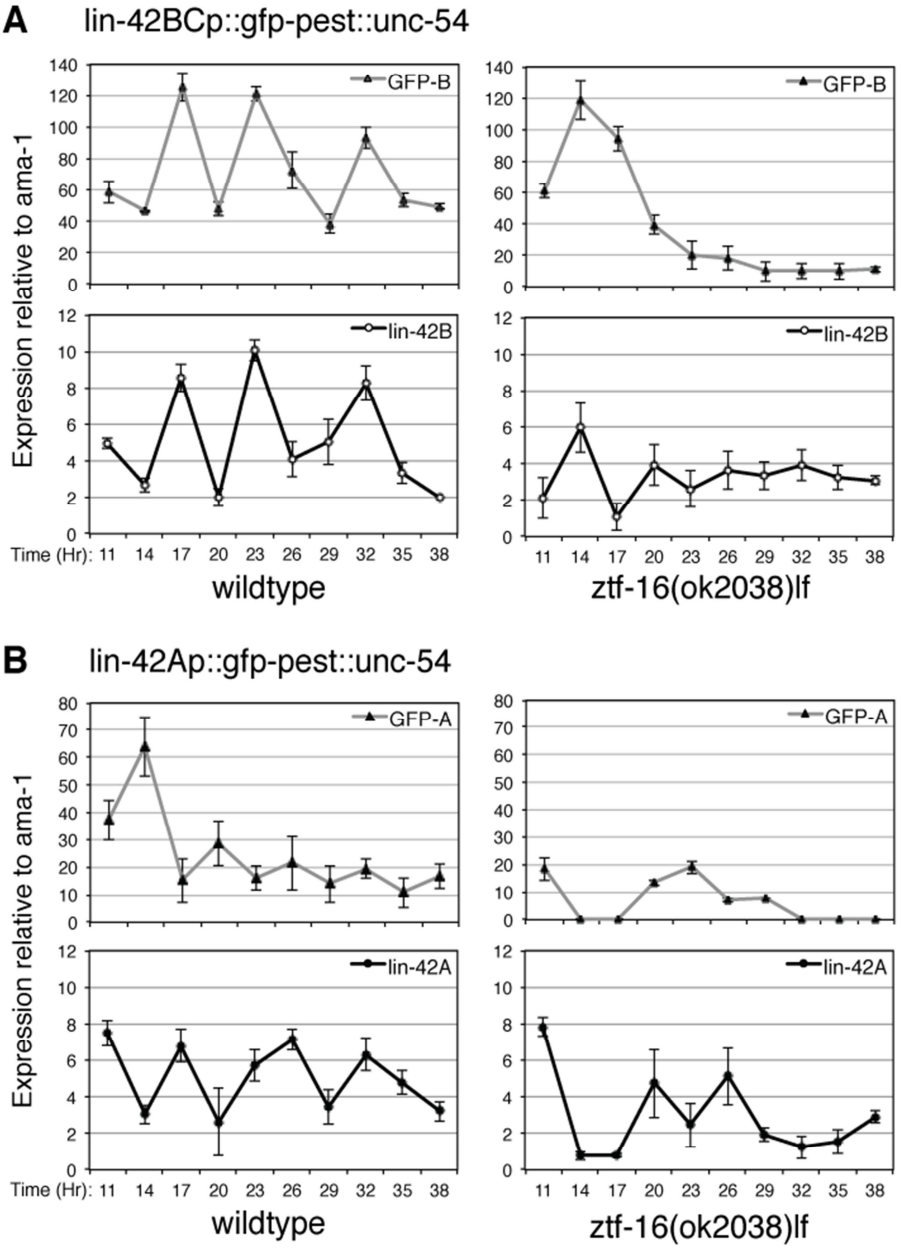

Figure 8. Reporter expression driven by *lin-42BC* and *lin-42A* promoters.

(A) GFP transcript expression profile from the *lin-42BC* transcriptional reporter closely mirrors the cyclic expression profile of endogenous *lin-42B* in wildtype genetic background (left panels). In the *ztf-16(ok3028)* mutant strain, cyclic expression of both GFP and *lin-42B* transcripts are lost (right panels). (B) GFP transcript expression profile from the *lin-42A* transcriptional reporter (left panels) does not cycle synchronously with endogenous *lin-42A* transcripts in the wildtype genetic background. In the *ztf-16(ok3028)* mutant strain, the cyclic behavior of the GFP and *lin-42A* transcripts is dampened and/or severely down-regulated (right panels).

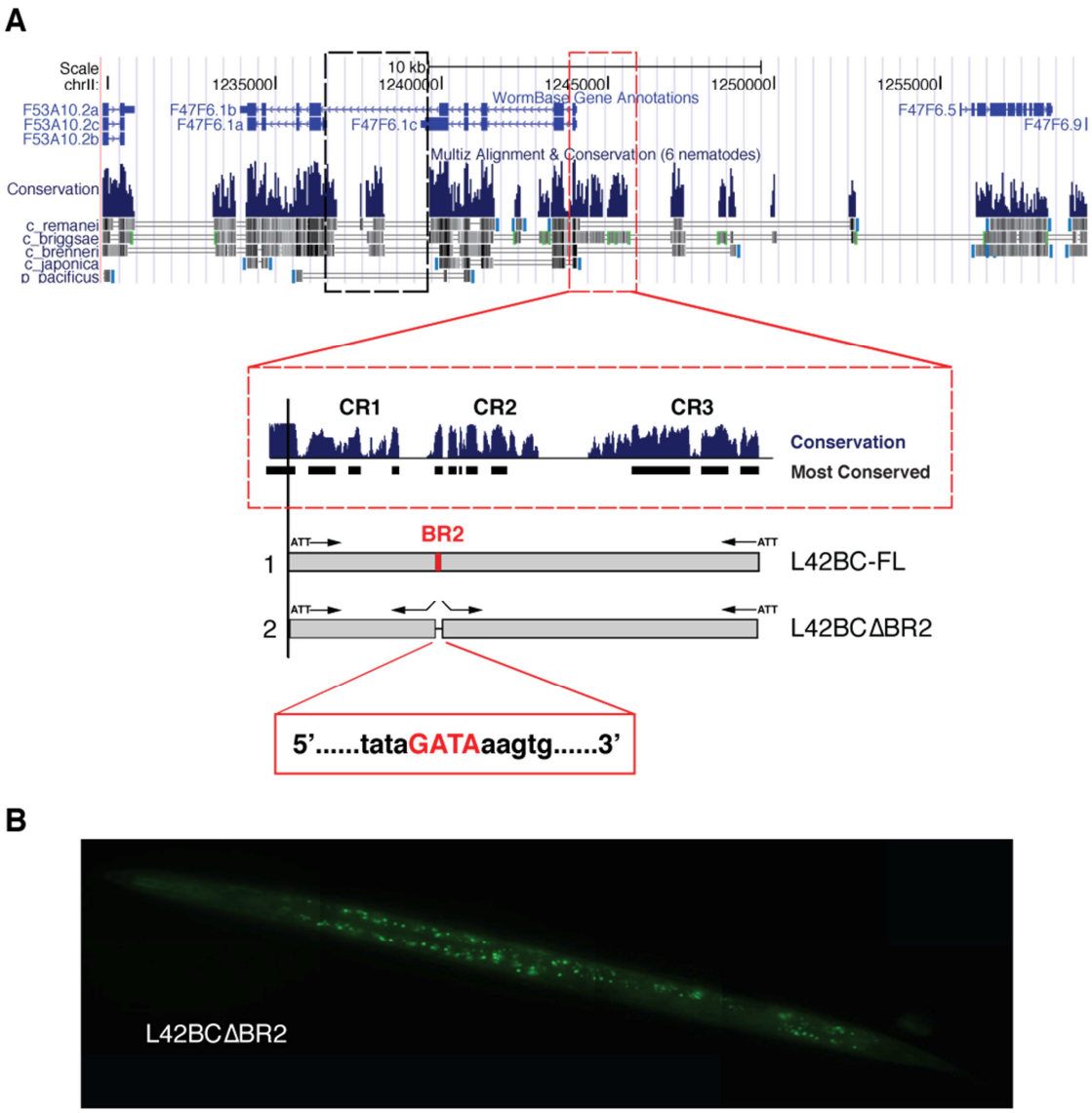

Figure 9. The putative ZTF-16 transcription factor binding site in *lin-42BC* promoter.

(A) *lin-42* (F47F6.1a, b, c) genomic region showing conservation of sequence between *C. elegans* and five other nematode species (screen capture from UCSC Genome Browser). The black dotted box represents the *lin-42A* intragenic promoter region. The GATA TFBS to which ZTF-16 may bind is within a 2 kb region of the *lin-42BC* promoter (red dotted box). Within the 2 kb promoter segment, there are three regions (Conserved Regions, CR1-3) that are the most conserved (magnified). The GATA transcription factor binding site is within the second conserved region and is designated Binding Region 2 (BR2). (B) Lack of cell-specific GFP expression in representative worm with *lin-42BCΔBR2* transcriptional reporter. Deletion of BR2 abolishes all *lin-42* tissue-specific expression from *lin-42BC* reporter strains.

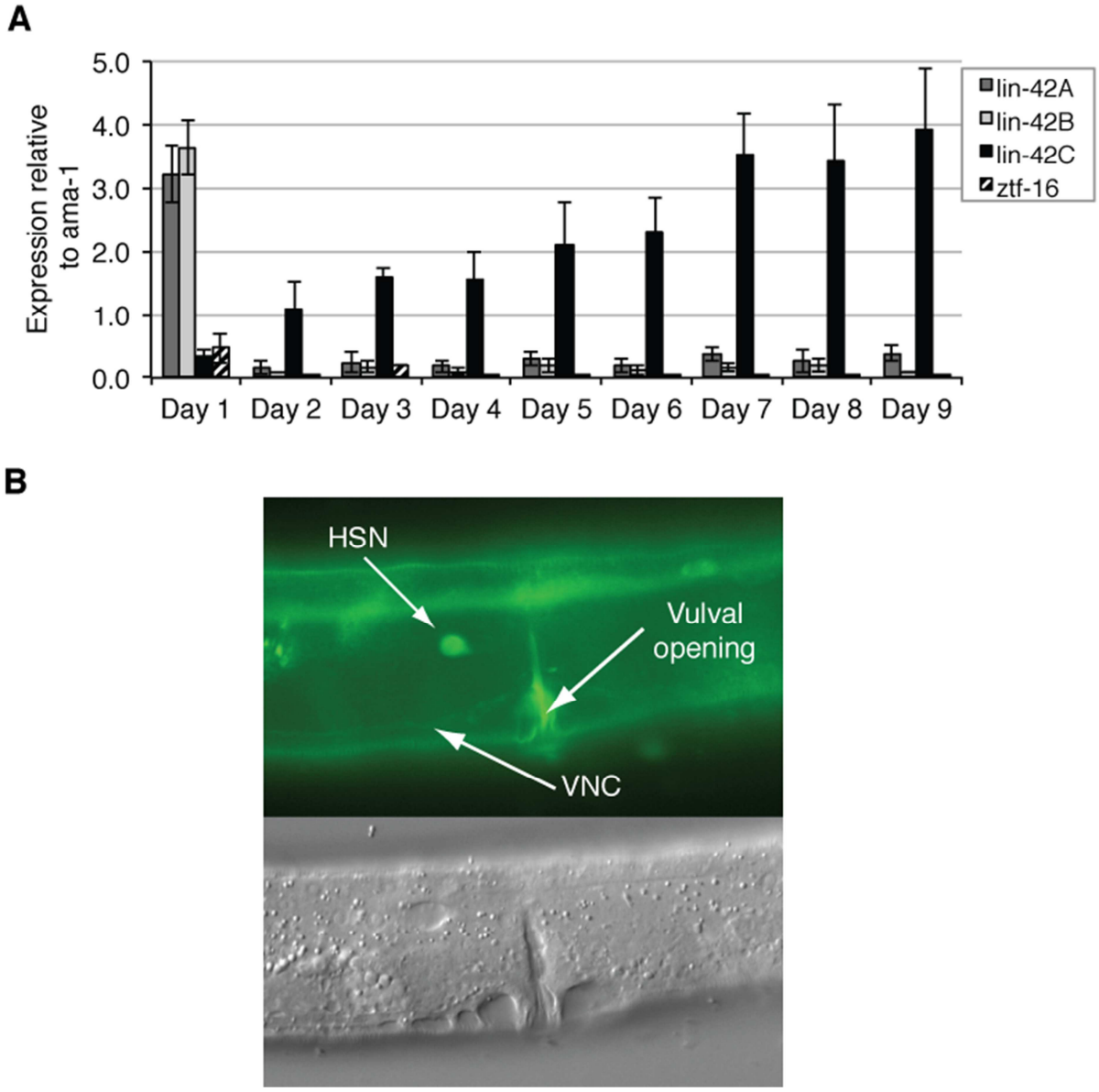

Figure 10. *ztf-16* and *lin-42* isoform specific expression in the adult stage

(A) *ztf-16* and *lin-42* isoform expression in synchronized populations of adult N2 *C. elegans*, as determined by qRT-PCR. *lin-42A* and *B* are down-regulated at the beginning of the adult stage. *lin-42C*, which is only expressed basally during larval development, is up-regulated throughout the adult stage. *ztf-16* is not appreciably expressed during the adult stage. (B) Representative adult expression pattern of *lin-42A* and *BC* transcriptional reporters. Most adult expression is confined to the HSN in all reporter strains.

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APPENDIX A

PCR Primers Used in the Construction of Reporter Genes

Promoter/Gene Amplified	Forward Primer	Primer Sequence (5'-3')	Reverse Primer	Primer Sequence (5'-3')
lin-42BC promoter 2kb	attB4-L42BC-P2	GGG GAC AAC TTT GTA TAG AAA AGT TGT CCA AAT TTC GAG TCG CTA CTC AGC	attB1r-L42BC-P2	GGG GAC TGC TTT TTT GTA CAA ACT TGT TTG GCT GAT GGT GCC ACG TTC
lin-42A promoter 3kb	attB4-L42A-P3	GGG GAC AAC TTT GTA TAG AAA AGT TGA ATT CGG GAA AAG GAC CAG AAG G	attB1r-L42A-P3	GGG GAC TGC TTT TTT GTA CAA ACT TGT AGG GTG GTA GGC TTA GGA GTT TC
GFP-PEST	attB1-gfppest-F	GGG ACA AGT TTG TAC AAA AAA GCA GGC TGG ATG AGT AAA GGA GAA GAA CTT TTC AC	attB2-gfppest-R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CTA CAC ATT GAT CCT AGC AGA AG
unc-54 3'UTR	attB2r-unc54-F	GGG GAC AGC TTT CTT GTA CAA AGT GGT TCA TTC GTA GAA TTC CAA CTG AGC	attB3-unc54-R	GGG GAC AAC TTT GTA TAA TAA AGT TGG GGA AAC AGT TAT GTT TGG TAT ATT GG
lin-42BCΔTFBS Overlap Extension Product 1	CR2_del_F1	TAA TAC GAC TCA CTA TAG AGA ATT TTA TAG GTT TGG AC	attB1r-L42BC-P2	GGG GAC TGC TTT TTT GTA CAA ACT TGT TTG GCT GAT GGT GCC ACG TTC
lin-42BCΔTFBS Overlap Extension Product 2	attB4-L42BC-P2	GGG GAC AAC TTT GTA TAG AAA AGT TGT CCA AAT TTC GAG TCG CTA CTC AGC	BR2_del_F2	CTA TAG TGA GTC GTA TTA CAG GCT CGG CTG GAG CTG TGT G

Appendix A. PCR primer sets used to construct *lin-42A, lin-42BC*, and *lin-42BCΔTFBS* reporter constructs. There are two primer sets for the *lin-42BCΔTFBS* in order to amplify the sequence before and after the deletion region, based on the Overlap Extension PCR method.

APPENDIX B

Biolistic Transformation of *C. elegans*

Adapted from G. Seydoux and M. Hengartner Laboratory Protocols

I. *C. elegans* Preparation

Required Materials:

- *unc-119(ed3)* animals
- 30 100mm NEP (Enhanced Peptone Plates) seeded with *E.coli* NA22
- 2 100mm NEP plates, unseeded
- M9 buffer

1. Chunk *unc-119(ed3)* worms onto 8 NEP plates seeded with NA22. Allow worms to starve until most animals are L1 larvae.

2. Wash worms off the starved plates using M9 buffer and place in 15 mL conical tubes. Place the tubes on ice for 5-10 minutes to allow the larger/adult worms to fall to the bottom of the tube. Transfer the supernatant containing the L1 larvae to a fresh 15mL tube and repeat gravity sedimentation twice more.

3. Spin the L1 larvae down and wash with 10 mL of M9 buffer to remove remaining bacteria and dead worms. Repeat once more.

4. Plate the L1 larvae onto 10 NEP/NA22 plates at a density of ~12,000 worms per plate. Place plates at 25˚C and grow for ~65 hours, or until most worms are gravid.

5. Wash the gravid adults of the plates using M9 buffer and split evenly into 4 15 mL conical tubes. Spin the worms down and wash them with M9 buffer 2-3 times or until supernatant is clear.

6. Perform large-scale egg prep:

- To 7 mL of worm suspension add 1 mL of 5M NaOH and 2 mL Bleach. Vortex the worms vigorously for 5 minutes. Wash the eggs 3 times in M9 buffer and allow them to hatch in the M9 overnight.

7. The following day, plate the synchronized L1 larvae onto 11 NEP/NA22 plates at a density of ~20,000 worms per plate. Allow the worms to grow at 25˚C for 52-54 hours or until all the worms have a single line of eggs in the uterus.

8. Wash the worms off the plates and pool into a single 15 mL conical tube. Spin down the worms and wash with M9 3-4 times or until supernatant is clear. There should be a loose pellet of ~2 mL of worms in the tube.

9. Resuspend the pellet in 2 mL of M9 buffer and plate 1 mL of the worm suspension onto one of the unseeded NEP plates. Repeat with the second milliliter of worms.

10. Place the plates on ice until ready to bombard.

II. Bombardment Procedure

Part 1 – DNA Preparation – at least 1 week before Bombardment

Required Materials:

- Qiagen Miniprep Kit (Catalog Number 27104)
- LB agar + Ampicillin (100mg/L)
- LB broth
- Restriction Enzymes
- Qiagen Qiaquick Gel Extraction Kit (Catalog Number 28704)
- Sterile Water

1. Streak out bacterial stocks containing desired construct for bombardment and pMM571 rescue construct onto LB Amp_{100} plates. Incubate overnight at 37˚C.

2. Inoculate 4-6 15 mL tubes containing 3 mL of LB Amp_{100} with a single colony from the streaked plate containing the construct for bombardment. Repeat procedure with colonies containing pMM571. Grow overnight at 37˚C in a shaker.

3. Mini-prep the bacterial cultures using the Qiagen Miniprep kit. Pool the mini-preps for each construct and determine the DNA concentrations by UV spectroscopy. ~20- 25µg of DNA from each construct will be needed.

4. Linearize the two constructs separately using appropriate restriction enzymes. Run out linearized DNA on an agarose gel and purify using gel-purification kit. To maximize DNA yield, add no more than 1.5µg of DNA to each gel-purification column. Elute DNA with 40 µL of sterile water.

5. Pool the gel-purified products for each construct separately and determine the DNA concentration of each by UV spectroscopy. If necessary, dilute or speed vacuum the DNA to obtain a concentration of ~0.5-1µg/µL. **For the bombardment, 5µg of each of the two constructs is required.**

Part 2- Microbead Preparation- 3 weeks to 1 day before Bombardment

Required Materials:

- Tungsten microbeads
- Sterile Water
- Sterile 50% glycerol
- Siliconized 1.5 mL microcentrifuge tubes
- Siliconized pipette tips
- 70% Ethanol

1. Weigh out 10 mg of beads per bombardment set (i.e. 10 mg of beads for 2 plates of 100,000 worms each) into a siliconized microcentrifuge tube.

2. Add 1 mL of 70% Ethanol to the tube of beads and vortex for 15 minutes. Allow the beads to settle for 15 minutes and then pellet the beads in a mini-centrifuge for 5-6 seconds. Pipette off and discard the supernatant.

3. Add 1 mL of sterile water, vortex the beads for 1 minute and then allow the beads to soak for 1 minute. Pellet the beads in a mini-centrifuge for 5-6 seconds, pipette off and discard the supernatant.

4. Repeat step 3, two additional times.

5. Resuspend the beads in sterile 50% glycerol. Use 100µL of 50% glycerol per 10 mg of beads.

6. Store beads at 4˚C until ready to use. The beads may be stored for up to 4 weeks.

Part 3- Autoclave Bombardment Materials- 1 day before Bombardment

Required Materials:

- Stopping screen
- Microcarriers (14 per bombardment set)
- Microcarrier Holder
- Hepta Adapter
- Forceps, 2 pairs

1. Wrap all items separately in aluminum foil and autoclave on a dry cycle.

Part 4 – Bead Preparation for Bombardment- Day of Bombardment

Required Materials:

- Linearized DNA to be bombarded (5µg of desired construct + 5µg rescue construct)
- 2.5M $CaCl_2$
- 0.1M Spermadine

- 70% Ethanol
- 100% Ethanol
- Bead Batch
- Siliconized 1.5 mL microcentrifuge tubes
- Siliconized pipette tips

1. Vortex the tube containing the bead batch for 5 minutes. Cut the end off of a siliconized pipette tip using a sterile razor blade. Remove 100 µL of beads to a new siliconized microcentrifuge tube using the siliconized pipette tip.

2. Add, in order, and vortex for 1 minute between each addition:

    - 10 µg total of DNA (5µg of desired construct + 5µgrescue construct)
    - 100 µL 2.5M $CaCl_2$ (add dropwise, vortex for 1 second between each drop)
    - 40 µL 0.1M Spermadine

3. Vortex for 3 minutes, let beads soak for 1 minute. Pellet the beads in a mini-centrifuge for 5-6 seconds and aspirate off supernatant.

4. Add 280 µL of 70% Ethanol and scrape across tube rack to resuspend the beads. Pellet the beads in a mini-centrifuge for 5-6 seconds, aspirate off supernatant.

5. Add 280 µL of 100% Ethanol and scrape across tube rack to resuspend the beads. Pellet the beads in a mini-centrifuge for 5-6 seconds, aspirate off supernatant.

6. Add 100 µL of 100% Ethanol and scrape across tube rack to resuspend the beads. Pellet the beads in a mini-centrifuge for 5-6 seconds, aspirate off supernatant.

7. Resuspend the beads in 100 µL of 100% Ethanol. Hold on ice until ready for bombardment.

Part 5 – Load Beads onto Microcarriers – Day of Bombardment

Required Materials:

- Microcarriers
- Forceps, sterile
- 100% Isopropanol
- Siliconized pipette tips, ends removed with sterile razor blade

1. Vortex 100 µL of DNA coated beads for 5 minutes to resuspend.

2. Transfer 7 µL of suspended bead solution onto each sterile microcarrier (14 microcarriers per bombardment set).

3. Allow ethanol to evaporate.

4. Using sterile forceps, place microcarriers into sterile microcarrier holder.

Part 6- Bombardment

Required Materials:

- Rupture discs (1/bombardment)
- Stopping Screen, sterile
- Microcarrier Holder with microcarriers
- Hepta Adapter
- Forceps, sterile
- PDS-1000/HE bombarder
- 100% Isopropanol
- 70% Ethanol
- M9 buffer
- Helium Tank
- Vacuum pump
- *unc-119(ed3)* synchronized adult *C. elegans* on unseeded NEP plates

1. Wipe down inside of PDS-1000/HE bombarder and sample holder with 70% Ethanol.

2. Soak rupture discs in 100% Isopropanol for 1 minute. Allow to air dry.

3. Place one rupture disc in retaining cap of Hepta Adapter and tighten. Place stopping screen in microcarrier holder. Place the microcarrier holder in the bombarder. Make sure that the microcarrier holder and hepta adapter are aligned.

4. Place the first uncovered worm plate in chamber on sample holder, bottom shelf. Use tape to secure the plate of worms to the sample holder.

5. Open helium tank valve and adjust the pressure to 1550 psi.

6. Start the vacuum pump.

7. Press VAC button on the PDS-1000/HE bombarder. When the vacuum gauge reaches 27 inches of Hg, switch VAC button to the Hold position.

8. Press the FIRE button until the rupture disc makes a loud sound. Release the FIRE button and switch the VAC button to the Vent position. When the vacuum drops to 0 inches of Hg, open the bombarder and remove the worm plate.

9. Immediately add 1 mL of M9 buffer to the plate of worms and transfer the worms to 15˚C for recovery.

10. Remove spent microcarriers and rupture disc from the apparatus. Replace with second set of prepared microcarriers and rupture disc. Repeat steps 3 through 9 with the second plate of worms.

11. To shut down bombardment equipment:

- Repeat bombardment without and discs or plates to release pressure.
- Turn off vacuum pump
- Close helium tank
- Turn power off on PDS-1000/HE bombarder.

III. Worm Recovery and Transformation Screening

Required Materials:

- M9 Buffer PLUS (M9 buffer with 10 µg/mL tetracyclin and 2.5 µg/mL Fungizone/Amphotericin B)
- 30 NEP plates seeded with NA22

1. Allow worms to recover for 1 hour at 15˚C

2. Resuspend worms on each bombarded plate in 13 mL of M9-PLUS in 15 mL conical tube.

3. Plate worm suspension onto 13 large NEP/NA22 plates, with 1 mL of worm suspension on each plate.

4. Allow plates to dry down at room temperature overnight. Place at 25˚C the following morning.

5. Allow the plates to starve out completely (7-10 days).

6. Reintroduce food to the starved plates by "top-chunking" or placing 5-6 chunks of NEP/NA22 agar right side up on the starved plates. Transformed worms will be able to climb up to the food.

7. After ~3 days, screen the worms for wild-type movement. Wild-type movement is an indication of a worm transformed with at least the rescue construct. If none are seen, check plates again in another 3 days.

8. Pick any suspected transformants to individual plates. Allow them to propagate.

9. To verify the transformation of the desired construct, take a small population of the transformed line and perform *C. elegans* genomic DNA prep. Use PCR to confirm the desired gene is present in the line.

APPENDIX C

Primer Sequences for RNAi Plasmid Construction

Gene	Forward Primer	Primer Sequence (5'-3')	Reverse Primer	Primer Sequence (5'-3')2
glo-1	glo-1F	GTG TTG GTA ATT GGT GAT CCA GGT G	glo-1R	CGA GTC GTA TCT AGA ATG AGT GG
glo-3	Glo-3F_2	GGA AGT TCT ACG TGT ACA GAA GAA CAA G	Glo-3R_2	GAA TCA ACG GCT TTG ACT GAG TTC GG
pos-1	pos-1 F	CCT GAG CGG TGA AGC AAT CAT GG	pos-1 R	GCC GTA GAA TTG TAT CCA GAT ACG TCC
ztf-16	ztf-16_F	ATG CAA GAG CAT TCA ATG ACA C	ztf-16_R	TTC GGG AGT CGG TGA TTT CTC

Appendix C. Primer sequences used to amplify spliced sequences from wild-type *C. elegans* cDNA for the construction of RNAi plasmids.

APPENDIX D

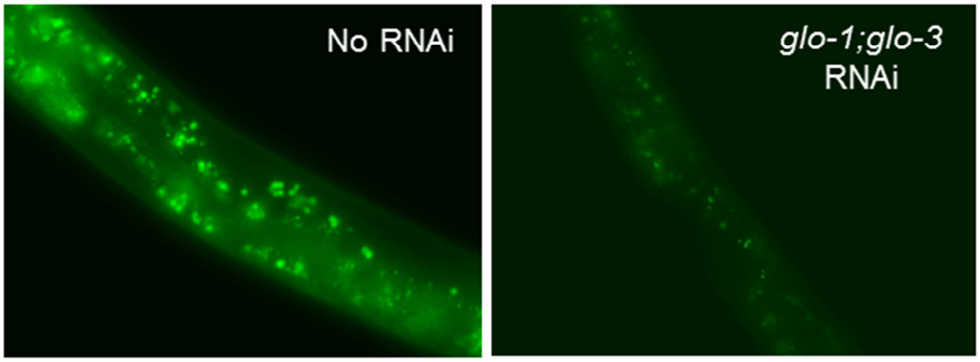
Primer and Probe Sequences for qRT-PCR

Target Gene	Primer/Probe	Primer/Probe Name	Sequence (5'-3')
lin-42A	Forward Primer	lin-42A-F1	ACG ATC TTG CAG AGC CAG TAC
lin-42A	Reverse Primer	lin-42A-R3	GCC CTG GTT CTG ATC CTT G
lin-42A	TaqMan Probe	lin-42-TM-A1	CAC AGC CAC CAC CAC CAT CAC TCA AGC
lin-42B	Forward Primer	lin-42B-F6	AAT CGG AGA CTC GGA TGT G
lin-42B	Reverse Primer	lin-42B-R6	TGA CGG GAG GTG GAT GG
lin-42B	TaqMan Probe	lin-42-TM-B1	ACC ACC ACC AGG CAT CCA GTC AA
lin-42C	Forward Primer	lin-42C-F5	TTC AGT AAC CTT CTT ATC ATC CG
lin-42C	Reverse Primer	lin-42C-R4	GCT GCT GAC CAC TCT CG
lin-42C	TaqMan Probe	lin-42-TM-C1	TGC CAG TCA CCG CTT ATC TTC TTC TTC AGT
ztf-16	Forward Primer	ztf16-F1	GGT GAA ATC AGA GTT TAG C
ztf-16	Reverse Primer	ztf16-R1	TCT CCG AAT CCA TTG AAC
ztf-16	TaqMan Probe	ztf16-TM1	CGA CAC CTC CGA TAA CTC CAC
GFP	Forward Primer	GFP-PEST_Specific_F	GTG AAG GTG ATG CAA CAT ACG G
GFP	Reverse Primer	GFP-PEST_Specific_R	GCA GCT GTT ACA AAC TCA AGA AGG ACC
GFP	TaqMan Probe	GFP-TM1	CGA GAA GCA TTG AAC ACC ATA ACA G
ama-1	Forward Primer	ama-1-F3	AAG GTC GCA GGT GGA TGC
ama-1	Reverse Primer	ama-1-R3	GTC CTC ATT CAC GTT CTT CTT CC
ama-1	TaqMan Probe	ama-1-TM1	TCG CTA TCA ACC ATC ATA CCG CCG TGT C

Appendix D. Primer sets and TaqMan Probe sequences used to measure mRNA transcript levels in *C. elegans* through quantitative real-time PCR.

APPENDIX E

Gut-granule auto-fluorescence reduction on *glo-1;glo-3* double RNAi



Appendix E. A representative image of gut-granule auto-fluorescence reduction in the *C. elegans lin-42BC* reporter strain. Both worms were raised at 15˚C. One worm was fed OP50 *E. coli* (left), while the other was fed *glo-1;glo-3* double RNAi (right).