Regulation of *C. elegans* developmental timing by the GATA transcription factor *elt-1*

by

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S.B., Massachusetts Institute of Technology, 2005

A thesis submitted to the

Faculty of the Graduate School at the

University of Colorado Boulder

in partial fulfillment of the

requirements for the degree

Doctor of Philosophy

Department of Molecular, Cellular, and Developmental Biology
This thesis entitled:

Regulation of *C. elegans* developmental timing by the GATA transcription factor *elt-1*

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Regulation of *C. elegans* developmental timing by the GATA transcription factor *elt-1*  

Thesis directed by Professor Min Han

The heterochronic gene network controls developmental timing in the nematode roundworm *Caenorhabditis elegans*. Bi-stable switch-like changes in gene expression occur during its development as stage-specific microRNAs are expressed and subsequently down-regulate other stage-specific factors, allowing for developmental progression. Key genes in this regulatory network are phylogenetically conserved and include the post-transcriptional microRNA repressor *lin-28*; the nuclear hormone receptor *daf-12*; and the microRNAs *lin-4*, *mir-48*, *mir-84*, *mir-241*, and *let-7*. *daf-12* is the only factor currently known to regulate transcription of the Let-7 microRNA family, but its contribution is insufficient to account for all of the transcriptional regulation observed. In this work, a forward genetic screen is successfully used to identify a pathway that is genetically redundant with *daf-12* in the regulation of developmental timing. This pathway is determined to require post-embryonic activity of the GATA-family transcription factor *elt-1* and shown to likely act by directly regulating transcription of the Let-7 microRNA family during late larval developmental stages.

In a separate effort, the activated *let-60/Ras* Suppressor *sur-4* is positionally cloned and shown to encode an allele of *cnk-1*, the *C. elegans* ortholog of the *Drosophila* gene *connector enhancer of ksr*. The suppressor allele of *cnk-1* has a mutation in a conserved residue in its PH-domain and is predicted to have defects in plasma membrane localization.
This thesis is dedicated to the memory of my grandfather, Leslie Cowan
Acknowledgments

I would like to thank Min Han, my advisor, for his generosity and mentorship while I have been a graduate student in his lab. Min has patiently encouraged my development, taught me to never back down from a research challenge, and shown me how to think critically about science. He believed in me enough to let me figure things out for myself, even when it was more difficult; in doing so, I’ve developed skills and confidence for which I will always be grateful.

Many thanks to my thesis committee, including Professors Han, Tom Blumenthal, Michael Klymkowsky, Jinshi Shen, and Angie Ribera. Their willingness to carve time from busy schedules to talk science and support my career development continues to amaze me. It has been a pleasure to be part of the Department of Molecular, Cellular and Developmental Biology and CU-Boulder communities; the MCDB leadership and administration has been wonderful, especially Karen Brown, who has patiently answered countless questions. I would like to thank all the members of the Han lab with whom I have been lucky to share some time in the lab. In particular, huge thanks Aileen Sewell for keeping the whole operation running; to Mingxue Cui, for countless late-night discussions of the intricacies of experimental biology; to Rebecca Zabinsky, for her friendship; and to Xiaochang Zhang, for his contagious enthusiasm for science.

I would also like to thank the leadership of the Medical Scientist Training Program at the University of Colorado School of Medicine for the opportunity to study in this superb program. Dr. Mark Earnest of the University of Colorado Hospital and Dr. Kerry Broderick of the Denver Health Medical Center have generously and graciously helped me to maintain
a connection to the School of Medicine and its core mission of service, for which I am very grateful. Caring for patients with them always kept my perspective straight and re-energized me to continue to try my best in the lab.

This thesis wouldn’t have been imaginable without the company of many friends. In particular, thanks to Brian Harry, Ben St Clair, Samantha Friend, Qinghua Zhu, Jim Mapes, Jordan Rubinstein, Eric Fellheimer, and Shirali Pandya, who have all shared many highs and lows over the years, including too much coffee, shows that were way too loud, epic camping trips, great meals at dodgy restaurants, inappropriate laughter at the movies, weddings, and now, wonderfully, the arrival of the next generation.

Last, but not least, I would like to thank my family: my father, for his example of how rewarding a career of scholarship can be, and my mother, whose love, support, and encouragement has been the foundation upon which everything has been built.
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CHAPTER 1: INTRODUCTION AND BACKGROUND

Introduction to genetics and developmental biology

Multicellular animals all begin life as a single cell, the fertilized egg. A new genome is created following fertilization; along with some inherited non-genomic factors such as maternal messenger RNAs, it is able to fully direct the development of the new animal. This complex process has been the topic of extensive study for many decades, but remains incompletely understood. Research using many different model systems has been the basis for the formation of the general principles of developmental biology, such as how gene expression switches, cell fate specification, and pattern formation occur at the molecular level. Several famous examples include the regulation of the life-cycle decision in Enterobacteria Phage λ, mating-type switching in the yeast Saccharomyces cerevisiae, vulval formation in the nematode worm Caenorhabditis elegans, and larval imaginal wing disk formation in the fruit fly Drosophila melanogaster, to name just a few. Discoveries made during the study of these systems’ specific characteristics have in many cases been generalizable and had tremendous impact on all aspects of biology, and, in some cases, medicine. For example, the notch gene was initially identified from Drosophila mutants with “notched” wings and is now understood to be an important mechanism by which cell-cell signaling occurs during development in a wide range of organisms and tissues (Borggrefe and Oswald, 2009). Each organism is the result of different evolutionary pressures and responses, so findings from any one organism are not necessarily generalizable to other model organisms.
Among the experimental approaches used, the study for the inherited basis of phenotypes – genetics – has been particularly prominent in developmental studies, which have been primarily concerned with the establishment of the principles by which organs and whole organisms form and function. In all of the experimental systems already mentioned, isolation of mutants with altered phenotypes, including mutants that suppress or enhance primary phenotypes, has, following determination of the genetic basis for the variation, identified many or even most of the biological molecules involved and assigned specific functions to many of these genes. Even with the complete genome sequence now available for all common model organisms, often with extensive annotation, a major challenge in biology remains to understand how individual molecules collectively create functioning cells, tissues, and organisms (Evans, 2000; Kirschner et al., 2011).

This is complicated, in part, by extensive genetic redundancy. Genetic redundancy is when parallel genetic pathways buffer against phenotypic change in response to mutations (Thomas, 1993). Indeed, genetic redundancy has been reported in diverse settings (Rudnicki et al., 1992) and has been proposed as a cause for the observation that mutation of a surprisingly large percentage of the genes in most multicellular organisms’ genomes does not result in an observable phenotype (Bouché and Bouchez, 2001). Redundancy may be at the level of a gene function, such as when two paralogs continue to have the same function, masking the phenotype from inactivation of either, or at the level of gene regulatory networks, where collections of genes create complex information processing circuits. In the latter case, the structure of the network itself can make it robust to mutation of any but the few genes that form critical nodes, creating functional redundancy among structurally and
even functionally unrelated genes. Indeed, genetic regulatory networks have been proposed to be one of the fundamental functional units for cells (Barabási and Oltvai, 2004).

**The nematode *C. elegans* is a tractable experimental model organism**

*C. elegans* is a free-living soil roundworm approximately 1mm in size and found throughout the world. The species is sexually dimorphic, with a hermaphroditic sex capable of self-fertilization and a male sex capable of cross-fertilizing hermaphrodites. Its genome contains approximately one-hundred-million base-pairs of DNA encoding approximately twenty-thousand protein-coding genes and over thirteen-hundred non-coding RNAs (Hillier et al., 2005). *C. elegans* has become a staple model organism since its introduction to contemporary experimental biology in the early 1970s due to its unique combination of attractive experimental features (Brenner, 1974). First, the animal is readily cultured in the laboratory. Second, it has a rapid life cycle, reaching reproductive maturity approximately two days after hatching and with the capability of producing up to 1000 progeny per animal. Third, the animals are transparent and have a cellular lineage that is fully described and largely invariant, with, for example, 959 somatic nuclei per hermaphrodite (Sulston and Horvitz, 1977). Finally, this combination of factors has allowed for the isolation, mapping, and cloning of many mutations that affect cell lineages (Sulston and Horvitz, 1981), which has led to a tremendous contribution to our knowledge of the mechanisms through which genes specify cell fate during development.

Indeed, *C. elegans* has remained a tremendously useful system for studying biology even in the post-genomic era. This is in part because much research using the worm has involved forward genetic screens for suppressors and enhancers of basic phenotypes, and
the information identified from such screens is thus inherently functional in nature. For example, the synthetic multivulval (SynMuv) gene network is an excellent example of functional genetic redundancy. Mutation of genes in any one class of SynMuv genes does not result in a multivulval (Muv) phenotype, but simultaneous mutation of genes in multiple SynMuv gene classes results in extra precursor cells adopting vulval fates and the Muv phenotype (Cui et al., 2006; Ferguson and Horvitz, 1989). In another excellent example of *C. elegans* forward genetics identifying functionally-important genetic redundancy, an enhancer screen was used to identify mutations that only caused a phenotype when the Retinoblastoma ortholog *lin-35/Rb* was mutated, unmasking diverse functions for the *lin-35/Rb* gene (Fay et al., 2002). In many cases, genetic redundancy is among structurally unrelated genes, so without such studies this type of functional relationship would be

*Figure 1.1: Post-embryonic cell lineage diagram for some hypodermal lineages of C. elegans, demonstrating vulval formation during the L3-L4 stages*

*Figure from Sulston and Horvitz, 1977*
unlikely to be uncovered.

**Developmental timing is a regulated process**

The heterochronic gene regulatory network controls developmental timing in *C. elegans*. Mutants that cause temporal shifts in cell fates were among the earliest genes identified from screens for cell lineage abnormalities in *C. elegans* (Chalfie et al., 1981) and their study has proven to be of enduring broad interest. This gene regulatory network controls the timing of certain cell division and differentiation events, with some mutations causing stage-specific events to occur a stage earlier or later than normal (Ambros, 1989; Ambros and Horvitz, 1984). While most studies have focused on the abnormalities in heterochronic mutants’ lineages in the hypodermal tissue, recent studies have shown at least some of the heterochronic gene relationships have similar functions in neurons as well (Olsson-Carter and Slack, 2010; Zou et al., 2012).

Genes have been identified that control nearly each developmental stage transition (Figure 1.2). During the first larval stage (L1), the transcription factor *lin-14* is widely expressed, but the microRNA *lin-4* becomes expressed during the stage, causing a down regulation of the transcription factor *lin-14* during the stage to allow for the transition to the L2 stage. Loss-of-function mutation of *lin-14* causes premature adoption of L2-stage-specific cell fates, termed a precocious phenotype, while loss-of-function mutation of *lin-4* causes reiteration of L1-stage-specific fates, termed a retarded mutation. Widely expressed during embryonic development through the L2 stage, the microRNA-regulator *lin-28* is down-regulated at the L2-L3 molt by Lin-4 and the newly-expressed Let-7 family microRNAs Mir-48, Mir-84, and Mir-241; reduced activity of *lin-28* causes precocious
adoption of L3 stage fates while persistent activity of lin-28 or decreased activity of mir-48/84/241 causes reiteration of L2-specific fates. During the L3 stage, mir-48, mir-84, and mir-241 expression causes down-regulation of the hunchback-homolog transcription factor hbl-1, which specifies temporal fates during mid-larval development. The let-7 microRNA is initially expressed in late L3, down regulating its target lin-41 during L4 and allowing for L4-to-young adult transitions, including de-repression of the transcription factor lin-29, which promotes terminal differentiation of certain cell types during adulthood. Figure 1.2 summarizes the current model for the heterochronic gene network (Ambros, 2011; Resnick et al., 2010).

One of the key regulated steps in this process is the expression of the microRNAs
mir-48, mir-84, and mir-241 at the L2 molt (Li et al., 2005). This is regulated in part by the nuclear hormone receptor daf-12, which promotes their expression in response to the production of the steroid hormone dafachronic acid (Bethke et al., 2009; Hammell et al., 2009a), which is produced by well-fed worms exposed to favorable developmental conditions. In the absence of dafachronic acid, the un-ligated DAF-12/NHR protein blocks developmental progression and instead promotes the alternative stress-resistant dauer larval stage. In this way, the nuclear-hormone receptor DAF-12 acts as a key switch in regulating developmental cell fate.

Many of the core genes in the heterochronic gene network are widely conserved in metazoans, where they have important functions. For example, the lin-28::let-7 regulatory axis is broadly conserved, with mutual antagonistic regulation (Hagan et al., 2009; Nam et al., 2011; Piskounova et al., 2011; Viswanathan et al., 2008; Wilbert et al., 2012). Human Lin28 is a stem-cell factor that is able to promote resistance to cellular differentiation (Thornton and Gregory, 2012) and is able to function as an alternative factor in induced pluripotent stem cell induction (Buganim et al., 2012; Shyh-Chang and Daley, 2013; Yuan et al., 2012); misregulation of Lin28 causes abnormalities in glucose metabolism (Zhu et al., 2011). Human Let-7 has been shown to regulate differentiation and tumorigenicity of breast cancer cells (Yu et al., 2007) and its reduced expression is strongly associated with shortened survival in lung cancer (Takamizawa et al., 2004). While other heterochronic genes’ function in species other than C. elegans is not understood at the same level of detail, they are generally conserved and believed to have analogous functions.

lin-4 and let-7 were the first two microRNAs discovered in any organism, both from mutagenesis screens for heterochronic genes in C. elegans (Lee et al., 1993; Reinhart et al.,
While initially considered an oddity of nematode biology, 21-24 nucleotide-long so-called microRNAs have since been found to be a large class of regulatory small RNAs that are present through metazoans and essential for many biological processes (Bartel, 2004, 2009). Understanding the function of microRNAs is a major focus of current experimental biology research. Research with *C. elegans* has continued to make significant contributions to microRNA research, identifying, for example, several new components of the microRNA-induced silencing complex (Ding et al., 2005; Hammell et al., 2009b), which is required for microRNA::mRNA interactions and silencing of target genes’ expression.

While the identity of the core components of the heterochronic gene network in *C. elegans* appears to now be known, the full regulation of developmental timing by this gene regulatory network is still incomplete. In particular, the “upstream” mechanisms that act to initiate transcription of microRNAs remain incomplete, as described further in Chapter II.
CHAPTER II: IDENTIFICATION OF A MUTATION THAT ENHANCES THE DEVELOPMENTAL TIMING PHENOTYPE OF ANIMALS MUTANT FOR THE NUCLEAR-HORMONE RECEPTOR DAF-12

Introduction

As described in Chapter I, the nuclear-hormone receptor daf-12 has a central role in the regulation of C. elegans developmental timing (Antebi et al., 1998, 2000). It promotes gene expression changes required for progression through larval developmental stages in response to sterol-based hormones, which signal the presence of favorable growth conditions (Motola et al., 2006); several recent studies have determined that it does so, at least in part, by regulating the transcription of the Let-7 family of microRNAs (Bethke et al., 2009; Hammell et al., 2009a; Hochbaum et al., 2011). The Let-7 family of microRNAs contains seven genes in C. elegans: let-7, mir-48, mir-241, mir-84, mir-793, mir-794, and mir-795. Let-7 is expressed beginning late in the third larval stage, and during the L4-stage it targets and down-regulates, among other targets, mRNA encoding the E3 ligase LIN-41 (Ecsedi and Grosshans, 2013; Slack et al., 2000); mutation of the let-7 gene results in a fully-penetrant L4-stage-specific bursting vulva phenotype that is lethal (Reinhart et al., 2000). Mir-48, -241, and -84 are important for down-regulation of the hunchback-like transcription factor hbl-1 during the L3 stage, and in a startingly clear example of genetic redundancy, mutation of any two of the three genes causes only a mild phenotype, while loss of all three results in a high-penetrance lethal L4-bursting vulva phenotype as well as proliferation of seam cells and defective alae formation (Abbott et al., 2005). Daf-12 is the only gene currently known to regulate the transcription of let-7-family microRNAs, but daf-12-null mutant animals have a markedly distinct and weaker phenotype than that of either let-7(If) single-mutant animals or of mir-48,241,84 triple loss-of-function mutant animals (Abbott et al., 2005). Additionally, not all let-7 family members were dysregulated in a daf-
12-null strain when measured by quantitative real-time reverse-transcription PCR (Hammell et al., 2009a). This collectively implies that *daf-12* is likely to have other targets in addition to *let-7* family genes and, more importantly for the present studies, that genes in addition to *daf-12* are likely to regulate transcription of the *let-7* family of microRNAs. A previous study determined that animals double-mutant for *lin-66* and *daf-12* have a strong seam-cell proliferation defect, indicating *lin-66* is likely to act in parallel to *daf-12* (Morita and Han, 2006). This demonstrates that there is redundancy with *daf-12*, but unfortunately the biochemical functions of LIN-66 remain unknown.

As described earlier, *C. elegans* have been amenable to genetic screens, some of which have been to identify redundant genetic regulatory networks that control cell fate. In this chapter, a screen for a *daf-12*-enhancer mutation is described; the goal of the screen was to identify upstream regulation of the Let-7 family that is in parallel to *daf-12*, either at the level of transcriptional, or post-transcriptional, regulation, such as that provided by *lin-28*. The logical basis for this is that the increased robustness of genetically redundant pathways will often mask the phenotype of a particular gene mutation, and the identification of enhancer mutations may unmask these phenotypes by disabling a compensatory pathway (Guarente, 1993). Past genetic screens in *C. elegans* have identified genetic redundancy in the regulation of, for example, vulval precursor cell fate specification (Ferguson and Horvitz, 1989; Sieburth et al., 1998), transcription by the

![Figure 2.1 Proposed model for genetic redundancy in the regulation of the Let-7 family of microRNA](image-url)
Retinoblastoma ortholog lin-35 (Fay et al., 2002), and signaling by the PTEN tumor-suppressor ortholog daf-18 (Suzuki and Han, 2006).

Materials and Methods

A screen for daf-12 enhancers

A strain was constructed to optimize screening for enhancers of the daf-12-null phenotype: dpy-20(e1282) IV; wIs51(promoter*scm::gfp) V; daf-12(rh61rh411). The dpy-20(e1282) allele on Chromosome IV was present in the mutagenesis strain to allow for rapid analysis of candidate mutations’ linkage to Chromosome IV, as the lin-66 gene on the right arm of chromosome IV has previously been shown to enhance the daf-12(rh61rh411) mutation (Morita and Han, 2006). The allele daf-12(rh61rh411) was chosen because it encodes for a protein with a premature stop codon that has previously been shown to be a genetic null allele (Antebi et al., 1998, 2000). Mutagenesis was performed using ethylmethylsulfonylic acid as previously described (Brenner, 1974). Approximately 10 enhancer mutations were identified, and one allele was selected for further study based upon its phenotype (described below) and failure to map to a region with a gene with a known relationship with daf-12. This allele was designated daf-12 enhancer 1, dfe-1(ku491); “ku491” is the allele designation, keeping with standard C. elegans genetic nomenclature. Kiyokazu Morita, Ph.D., a former postdoctoral fellow in the Han Lab, performed the screening and isolation of daf-12 enhancer alleles and the initial mapping of the candidate mutation to chromosome IV; from this point forward I took over the project and performed all experiments, except where specifically noted.
**Positional Cloning**

The *dfe-1(ku491)* mutant allele was mapped to Chromosome 4 by linkage to the *dpy-20* phenotype by K. Morita upon isolation of it in the enhancer screen; he generated a three-point-mapping strain consisting of *unc-24(e138), dfe-1(ku491),dpy-20(e1282)* IV to allow for quantitative linkage mapping of the association of the *dfe-1(ku491)* phenotype with the *unc-24* and *dpy-20* genes, located at genetic positions +3.51 and +5.22, respectively. The strategy for isolating recombinants for three-point mapping is described in figure 2.2 and is similar to previously described approaches (Fay, 2006). Initially, all animals isolated with a recombination un-linking *unc-24* and *dpy-20* also had the *scm::GFP V; daf-12(rh61rh411)* X genotype. Additional mapping was performed where animals’ genotype was *scm::GFP V; daf-12(+) X* during isolation of recombinants, and the *daf-12(rh61rh411)* mutation was subsequently re-crossed into those recombinants to assess the presence or absence of *dfe-1(ku491)*. This was done because when the *daf-12(rh61rh411)* allele was present, there was markedly strong selection against the presence of the *dfe-1(ku491)* mutation, but isolation of recombinants in the presence of *daf-12(+)* overcame that problem. The seam-cell phenotype of *dfe-1(ku491) IV; daf-12(rh61rh411) X* mutants (described below) was exclusively used to map the *dfe-1(ku491)* mutant gene allele.

Whole-genome shotgun sequencing was also performed to identify candidate mutations in parallel to traditional three-point mapping. For this, DNA was isolated with a genomic DNA isolation kit (Qiagen) from a strain with the genotype *unc-24, dfe-1(ku491), dpy-20* IV and from the parental mutagenesis *dpy-20* IV; *daf-12(rh61rh411)* strain. DNA was then fragmented by sonication and libraries for sequencing were prepared using 300-500bp DNA inserts using an Illumina DNA sequencing sample preparation kit (Illumina
Inc.; San Diego, CA). Sequencing was performed with an Illumina HiSeq 2000 machine with a single read direction and read length of 100bp (1x100). Data was analyzed with the assistance of Philip Richmond and Robin Dowell (MCDB, University of Colorado Boulder) by adapting a pre-existing sequencing analysis pipeline as follows. Briefly, raw data set quality was analyzed with FastQ and reads were mapped to the *C. elegans* genome, downloaded from WormBase.org, with both Bowtie2 and BWA software packages; post-alignment variant-calling was performed with SAM tools, and the results were visualized with Integrated Genome Viewer (Broad Institute; Cambridge, MA). The region between *unc-24* and *dpy-20* on Chromosome IV was manually examined for both strains and all sequence variations between the two strains were noted and confirmed by Sanger sequencing of PCR amplicons.
Once the \textit{dfe-1(ku491)} mutation was mapped to a single candidate mutation within the \textit{elt-1} gene, microinjection-mediated transformation was performed with the fosmid WRM0619bE05, which contains the \textit{elt-1} gene, and with a sub-cloned fragment of that fosmid that contains only the \textit{elt-1} gene, including 2.75kb of the putative promoter. Microinjections were performed into the \textit{wls51 V; daf-12(rh61rh411)} X strain, with the fosmid or plasmid at 10-25 ng per μL using \textit{sur-5} \textit{promoter::dsRed} as a co-injection marker and empty pBSK as carrier DNA, and crossed into the \textit{dfe-1(ku491) IV; wls51 V; daf-12(rh61rh411)} X strain using the nT1 (IV:V) balancer. The phenotype of animals that lost the extrachromosomal array was scored to confirm no error had been made during generation of transgenic strains to assess complementation.

\textit{Assessment of Phenotypes}

The phenotype of strains was scored as follows. For all strains scored for seam-cell number, the seam-cell-specific nuclear-localized GFP reporter \textit{wls51}, which is integrated into chromosome V, was crossed into the strain and scored at 64x magnification using GFP filters on a Zeiss AxioPlan 2 Microscope configured for Differential Interference Contrast (DIC) imaging with epifluorescence capability. Strains were scored in the period between completion of the previous molt and the initiation of the following round of seam-cell mitosis (Morita and Han, 2006). If the timing of an animal could not unambiguously be assigned to this time within the molting cycle, the animal was not scored; additionally, GFP-positive nuclei were assessed for characteristic nucleolar morphology prior to categorization as true seam-cells. The absence or presence of alae was scored by Nomarski optics at 64x magnification. The \textit{ajm::gfp} adherens junction GFP marker \textit{kuls47} (Chromosome II) was crossed with the \textit{dfe-1(ku491)} strain and wild-type- and \textit{dfe-1(ku491)}-homozygous progeny
were isolated and scored for GFP expression using DIC microscopy at 100x magnification (Smith et al., 2005). To determine the L4 bursting-vulva rate, fed L1 animals were picked to fresh plates and allowed to develop to L4 and adulthood, and the number of animals picked at L1 and with the described L4 phenotypes was recorded. The L4 bursting vulva rate was calculated as the number of animals with a burst vulva during the L4 period divided by the sum of that number and the number of animals that survived to past the L4 molt without a burst vulva. Feeding RNAi was performed as described (Kamath et al., 2001).

Results

Isolation of the daf-12-enhancing allele dfe-1(ku491)

Kiyokazu Morita, a previous member of the laboratory, performed an ethylmethylsulfoxide (EMS) mutagenesis screen for mutations that enhance the defect seen in the number of seam-cells in daf-12-null animals. Among others, he isolated a recessive mutation subsequently named dfe-1(ku491). As described in detail later in this chapter, double-mutant animals with both the dfe-1(ku491) allele and the daf-12(rh61rh411) loss-of-function allele accumulate abnormally large numbers of seam-cells, have a high incidence of a bursting vulva during the L4 developmental stage, and fail to form adult alae at the L4 molt (table 2.1 and figure 2.5).

Positional cloning of gene containing the dfe-1(ku491) mutant lesion

The dfe-1(ku491) allele was mapped within the C. elegans genome using both standard genetic techniques and whole-genome shotgun sequencing to identify candidate mutations. As shown in figure 2.3, a total of 65 animals were identified with reifications separating unc-24 and dpy-20 from the parental mapping strain unc-24, dfe-1(ku491), dpy-
of these, 5 showed the ku491 phenotype when the \textit{daf-12(rh61rh411)} mutant allele was also present. Whole-genome sequencing identified several sequence variations induced during the EMS-mutagenesis (represented as “dots” in figure 2.3), but of these, only one was both present in all strains with the mutant phenotype and absent from all strains without the mutant phenotype. This mutation is a C-to-T substitution at physical position 9,617,289 and genetic position +4.34 of Chromosome IV, in the GATA-transcription factor \textit{elt-1}. This mutation is predicted to cause a proline to serine substitution mutation at amino acid residue 298 (P298S) of the ELT-1/GATA protein, in one of two conserved Zn-finger DNA binding domains.

Transgenic lines expressing a fosmid containing the \textit{elt-1} gene (2/2 lines) or of a plasmid containing a subcloned 7.9kb AgeI-PmeI fragment containing only the \textit{elt-1} gene (1/1 lines) could each complement the seam-cell phenotype of \textit{dfe-1(ku491);daf-}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.3.png}
\caption{Summary of mapping data for \textit{dfe-1(ku491)}}
\end{figure}

\textit{Filled circles on the top schematic represent candidate mutations identified whole-genome sequencing and orange-blue junctions demonstrate the location of informative recombinations isolated during three-point mapping.}
12(rh61rh411) animals (figure 2.4) and bursting-vulva phenotype (to ~1%, data not shown); the difference in the gross appearance of the animals with a ‘rescuing’ transgene is markedly healthier looking than those without, even at low-magnification. Interestingly, only the one fosmid transgenic line that could rescue the embryonic lethality of an elt-1(null) allele could also rescue the alae phenotype (15/17 young adult dfe-1(ku491);daf-12(rh61rh411) animals had complete adult alae and 2/17 had gapped adult alae). These results establish that ku491 is an allele of elt-1 and that dfe-1 is elt-1.

*Figure 2.4: Complementation of dfe-1(ku491);daf-12(rh61rh411) animals' phenotype by transgenic elt-1*
**Phenotype of elt-1(ku491);daf-12(rh61rh411) double-mutation animals**

During the process of positional cloning, the *elt-1(ku491)* mutation on Chromosome IV was separated from *daf-12(rh61rh411)* on Chromosome X, outcrossed over six times, and a recombinant was isolated where *elt-1(ku491)* not linked to either *unc-24* or *dpy-20*. Alleles of *scm::gfp V* and *daf-12(rh61rh411)* X that had not been mutagenized in this screen were then re-crossed with the strain carrying the *elt-1(ku491)* allele; strains derived from this cross were used for all subsequent work. For all phenotypes, *elt-1(ku491)* was found to be fully recessive.

Animals double-mutant for *elt-1(ku491)* and *daf-12(rh61rh411)* have strong heterochronic phenotypes: lethality during the L4 stage from bursting vulvae, accumulation of stem-cell-like seam cells during late larval development, and defective formation of alae at the L4 molt (figures 2.5 to 2.7 and tables 2.1 and 2.2). Notably, seam-cell accumulation and L4 lethality phenotypes are only present when animals with the *elt-1(ku491)* mutation also have a null mutation in *daf-12*, whereas the defect in adult alae formation is independent of *daf-12* genotype. Seam cells terminally differentiate normally following the L4 molt in *elt-1(ku491)* animals, regardless of genotype at *daf-12*. No supernumerary molts were observed. Similar to previous studies that used *elt-1* RNAi during postembryonic development, *elt-1(ku491)* has a subtle, weak Uncoordinated phenotype. Male animals with the *elt-1(ku491)* allele do not, however, have any grossly notable tail ray abnormalities and are competent for crossing. Animals with the *elt-1(ku491)* allele do not appear to have an embryonic lethality rate that is different from wild-type animals, as dead eggs are not seen on plates and animals picked clonally consume their *E. coli* food source in a normal amount of time.
As shown in table 2.1, *elt-1(ku491)* single mutant animals hatch with a normal number of seam cells, and have near-normal proliferation of seam-cells during early larval stages, going from 9.83 after hatching to 16.38 after the L1 molt and 18.49 seam cells after the L2 molt. During the L3 stage, *elt-1(ku491)* single-mutant animals’ seam cell numbers declined, to 14.76 after the L3 molt and further to 14.45 after the L4 molt. The p-value for the number of seam cells post-L2 molt vs post-L3 molt and for post-L2 molt vs post-L4 molt are each <0.01. Seam cells maintain a connection to each other through an adherens junction protein, and a transgenic marker for this was used to examine seam-cell-seam-cell interactions. When the fluorescent marker *kuIs47* (AJM::GFP) was crossed into *elt-1(ku491)* single mutant animals, gaps were see in the normally continuous border of seam cells at the L4-stage; this indicates that there is premature differentiation and loss of seam-cells in the *elt-1(ku491)* mutants (Figure 2.8). Following the L4 molt and the onset of terminal differentiation, *elt-1(ku491)* animals’ seam-cells were capable of terminal differentiation to form the seam syncytium, as they do in wild type animals.
Figure 2.5: Gross seam cell phenotype of elt-1(ku491) mutants

<table>
<thead>
<tr>
<th></th>
<th>DIC</th>
<th>GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td><img src="image1" alt="DIC" /></td>
<td><img src="image2" alt="GFP" /></td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td><img src="image3" alt="DIC" /></td>
<td><img src="image4" alt="GFP" /></td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td><img src="image5" alt="DIC" /></td>
<td><img src="image6" alt="GFP" /></td>
</tr>
<tr>
<td>elt-1(ku491); daf-12(rh61rh411)</td>
<td><img src="image7" alt="DIC" /></td>
<td><img src="image8" alt="GFP" /></td>
</tr>
</tbody>
</table>

Images were taken at 64x magnification using a DIC Microscope. As described in Materials and Methods, all animals have the scm::gfp reporter wls51
Seam cells were scored per lateral side using the SCM::GFP marker. At L4 and Young Adult stages, p-value for comparison of elt-1(ku491); daf-12(rh61rh411) strain with each other strain is < 0.0001.
**Table 2.1: Summary of elt-1(ku491) mutants’ seam cell phenotypes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>L1 Stage</th>
<th>L2 Stage</th>
<th>L3 Stage</th>
<th>L4 Stage</th>
<th>Young Adult Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCM</td>
<td>Std Dev</td>
<td>N</td>
<td>SCM</td>
<td>Std Dev</td>
</tr>
<tr>
<td>Wild-type</td>
<td>9.71</td>
<td>0.47</td>
<td>14</td>
<td>15.81</td>
<td>0.75</td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td>9.65</td>
<td>0.61</td>
<td>17</td>
<td>16.19</td>
<td>0.91</td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td>9.83</td>
<td>0.39</td>
<td>12</td>
<td>16.38</td>
<td>0.65</td>
</tr>
<tr>
<td>elt-1(ku491); daf-12(rh61rh411)</td>
<td>9.83</td>
<td>0.39</td>
<td>12</td>
<td>16.39</td>
<td>1.38</td>
</tr>
</tbody>
</table>

**Figure 2.7: Gross Phenotypes of elt-1(ku491)**

elt-1(ku491) animals form normal vulval structures during L4 (top row), but they have a high penetrance bursting vulva phenotype (middle row). elt-1(ku491) animals have defects in forming adult alae at the L4 molt (bottom row).
Table 2.2: Bursting Vulva and Alae Phenotypes of elt-1(ku491) animals

<table>
<thead>
<tr>
<th>Strain</th>
<th>L4 Bursting Vulva</th>
<th>Alae after L4 Molt</th>
<th>Egl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (n)</td>
<td>Absent</td>
<td>Gapped</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0 (571)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td>0 (497)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td>0 (551)</td>
<td>88.4</td>
<td>3.8</td>
</tr>
<tr>
<td>elt-1(ku491); daf-12(rh61rh411)</td>
<td>44.0 (200)</td>
<td>88.8</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.8: AJM::GFP reporter defects in elt-1(ku491) mutants

Animals with the ajm::gfp reporter kuIs47 and the labeled genotypes were examined under a DIC Microscope with white light (left panels) or GFP optics (right panels), as described in the Materials and Methods section.
The elt-1(ku491) allele enhances the phenotype of alg-1-mutants

Many of the key genes in the heterochronic gene regulatory network regulate the activity of microRNAs, through both direct and indirect mechanisms. To examine a potential role for elt-1(ku491) in Argonaute-like gene (Alg)-mediated microRNA pathways, a double-mutant was constructed consisting of the elt-1(ku491) allele with the alg-1(214) allele, a genetic null. alg-1 is one of two Argonaute-like genes in C. elegans. alg-1 mutation has previously been shown to cause L4 burst vulva, adult alae formation, germline abnormalities, and slow growth phenotypes when singly mutant, and embryonic lethality when the sole other alg gene (alg-2) is also mutant (Grishok et al., 2001). Table 2.3 summarizes the results: there is a slight interaction that promotes seam-cell proliferation, but there is an extremely strong enhancement of the bursting vulva phenotype, with 59% of double-mutants having that phenotype. Germline defects were not examined in the elt-1(ku491);alg-1(gk214) animals as those animals displayed profound gross developmental abnormality (growth defects and L4 lethality).

Table 2.3: Genetic interaction between elt-1(ku491) and alg-1

<table>
<thead>
<tr>
<th>Strain</th>
<th>L4 Bursting Vulva (%)</th>
<th>L4 Molt Alae</th>
<th>Young Adult Seam Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent (%)</td>
<td>Gapped (%)</td>
<td>Present (%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td>0</td>
<td>88.4</td>
<td>3.8</td>
</tr>
<tr>
<td>alg-1(gk214)</td>
<td>7.7</td>
<td>95.0</td>
<td>5.0</td>
</tr>
<tr>
<td>elt-1(ku491);alg-1(gk214)</td>
<td>59</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Genetic classification of the elt-1(ku491) mutation

elt-1(ku491) can not encode for a null allele of elt-1, as wild-type elt-1 alleles have previously been shown to be required for early stages of hypodermal specification during embryonic development (Page et al., 1997). To examine the genetic nature of elt-1(ku491), the phenotype of the elt-1(ku491)-over-null transheterozygote animals was examined. To do
this, the \textit{unc-24,elt-1(ku491),dpy-20} allele generated during three-point mapping was placed in trans to a recessive null allele, \textit{elt-1(ok1002)}. The transheterozygote animals were identified by their non-dpy, non-unc phenotype and post-embryonic viability, and scored for seam-cell numbers, bursting vulva rates, and alae formation. The results are presented in table 2.4. In \textit{elt-1(ku491)-over-elt-1(ok1002); daf-12(rh61rh411)} transheterozygote double-mutant animals there was a substantial decrease in the number of seam cells, a mild to moderate increase in the rate of L4 bursting vulva, and little change in the already low rate at which animals make L4 molt alae. In \textit{elt-1(ku491)-over-elt-1(ok1002)} transheterozygote animals with wild-type \textit{daf-12}, there was a decrease in seam cells compared to \textit{elt-1(ku491)} homozygous animals (also with wild-type \textit{daf-12}), suggesting that the \textit{elt-1(ku491)} allele may have allelic haploinsufficiency for the maintenance of seam cell lineages. Overall, these data are consistent the interpretation that \textit{elt-1(ku491)} is a partial loss-of-function allele.

Additionally, the phenotype of \textit{elt-1(ku491);daf-12(rh61rh411)} animals was examined when \textit{elt-1} was knocked-down by feeding RNAi during post-embryonic development. It has recently been shown that RNAi of \textit{elt-1} during post-embryonic development causes loss of seam cell fates with premature differentiation (Brabin et al., 2011; Smith et al., 2005); the results are presented in table 2.4. Knock-down of \textit{elt-1} by feeding RNAi resulted in a loss of seam-cells throughout larval development, as previously published (Brabin et al., 2011); had little effect on the alae phenotype of \textit{elt-1(ku491)}, and increased the penetrance of the L4 bursting vulva phenotype.
Table 2.4: Relationship of elt-1(ku491) to elt-1(null)

<table>
<thead>
<tr>
<th>Strain</th>
<th>RNAi</th>
<th>L4 Bursting Vulva</th>
<th>L4 Molt Alae</th>
<th>Young Adult Seam Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>n</td>
<td>Absent (%)</td>
</tr>
<tr>
<td>wild-type</td>
<td>Empty vector</td>
<td>0.0</td>
<td>245</td>
<td>0.0</td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td>Empty vector</td>
<td>0.0</td>
<td>183</td>
<td>0.0</td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td>Empty vector</td>
<td>3.6</td>
<td>169</td>
<td>88.4</td>
</tr>
<tr>
<td>elt-1(ku491); daf-12(rh61rh411)</td>
<td>Empty vector</td>
<td>55.1</td>
<td>198</td>
<td>88.8</td>
</tr>
<tr>
<td>wild-type</td>
<td>elt-1</td>
<td>48.7</td>
<td>117</td>
<td>100.0</td>
</tr>
<tr>
<td>daf-12(rh61rh411)</td>
<td>elt-1</td>
<td>56.7</td>
<td>90</td>
<td>90.0</td>
</tr>
<tr>
<td>elt-1(ku491)</td>
<td>elt-1</td>
<td>76.3</td>
<td>114</td>
<td>100.0</td>
</tr>
<tr>
<td>elt-1(ku491); daf-12(rh61rh411)</td>
<td>elt-1</td>
<td>94.8</td>
<td>231</td>
<td>96.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>daf-12 genotype</th>
<th>elt-1 genotype</th>
<th>L4 Bursting Vulva</th>
<th>L4 Molt Alae</th>
<th>Young Adult Seam Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>%</td>
<td>n</td>
<td>Absent (%)</td>
</tr>
<tr>
<td>ku491</td>
<td>Wild-type</td>
<td>0.0</td>
<td>60</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>+</td>
<td>rh61rh411</td>
<td>0.0</td>
<td>57</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ok1002</td>
<td>Wild-type</td>
<td>0.0</td>
<td>92</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>+</td>
<td>rh61rh411</td>
<td>0.0</td>
<td>78</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ku491</td>
<td>Wild-type</td>
<td>1.9</td>
<td>52</td>
<td>84.0</td>
<td>16.0</td>
</tr>
<tr>
<td>ok1002</td>
<td>rh61rh411</td>
<td>65.9</td>
<td>41</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The p-value for the comparison of the Young Adult Seam Cell number for elt-1(ku491)-over-elt-1(ok1002) transheterozygote animals with wild-type daf-12 compared to elt-1(ku491) homozygotes was 0.0001 and while the comparison of elt-1(ok1002)-over-wild-type transheterozygotes to wild-type homozygotes was non-significant. Both comparisons done with Uncorrected Fisher’s LSD with Prism 6 software.
**Mutants of elt-1 isolated from mutagenesis without selection do not have a meaningful phenotype**

A comprehensive project was recently initiated to generate saturation-levels of mutations in the *C. elegans* genome (Thompson et al., 2013). Termed the million-mutation project, strains are mutagenized and sequenced without any selection for a phenotype other than viability. 5 such mutations in the region of the *elt-1* gene near the *elt-1(ku491)* mutation were obtained; double-mutants between these alleles were generated with *daf-12*; a minimum of 3 outcrosses were performed. None have an informative phenotype.

**Table 2.5: Million Mutation Project elt-1 alleles**

<table>
<thead>
<tr>
<th>ELT-1 Protein</th>
<th><em>elt-1</em> genotype</th>
<th><em>daf-12</em> genotype</th>
<th>Bursting Vulva %</th>
<th>L4 Molt Alae Absent (%)</th>
<th>Gapped (%)</th>
<th>Present (%)</th>
<th>n</th>
<th>SCM</th>
<th>Std Dev</th>
<th>N</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>wild-type</td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>24</td>
<td>19.2</td>
<td>2.2</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>G387E</td>
<td><em>gk636276</em></td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>13</td>
<td>18.5</td>
<td>1.6</td>
<td>13</td>
<td>0.3147</td>
</tr>
<tr>
<td>T410I</td>
<td><em>gk540167</em></td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>13</td>
<td>23.7</td>
<td>2.3</td>
<td>13</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G341E</td>
<td><em>gk805317</em></td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>6.7</td>
<td>93.3</td>
<td>15</td>
<td>23.1</td>
<td>2.1</td>
<td>15</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>G414R</td>
<td><em>gk678013</em></td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>13</td>
<td>19.5</td>
<td>1.8</td>
<td>13</td>
<td>0.6758</td>
</tr>
<tr>
<td>S275L</td>
<td><em>gk914718</em></td>
<td><em>rh61rh411</em></td>
<td>&lt; 1% many</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>12</td>
<td>17.6</td>
<td>1.8</td>
<td>12</td>
<td>0.0356</td>
</tr>
</tbody>
</table>
Discussion

Identification of a genetic pathway parallel to daf-12 in the regulation of developmental timing

The nuclear hormone receptor *daf-12* has previously been shown to be a central regulator of developmental timing in *C. elegans*, in part, but not exclusively, through transcriptional action on the Let-7 microRNA family. The mild phenotype of *daf-12*-null animals compared to *mir-48*, *mir-84*, *mir-241* triple-mutant animals implies that other factors must also act “upstream” within the heterochronic gene network to promote developmental progression, as mutation of downstream factors causes a stronger phenotype.

The characteristics of the *dfe-1(ku491)* mutant strain isolated from the EMS-mutagenesis screen described here, including the genetic relationship with *daf-12* and the strong heterochronic phenotype, imply that the mutation disrupts a genetic pathway that promotes developmental progression in parallel to *daf-12* and that can normally compensate for *daf-12* mutation. The severity of the newly isolated mutant phenotype further implies that *daf-12* and *elt-1* together provide critical regulation of the heterochronic gene network. An alternative interpretation would be that the phenotypes present in the *elt-1(ku491); daf-12(rh61rh411)* double-mutant animals are caused by a non-specific interaction. The specificity of timing and type of phenotypes (L4 and young adult stages, seam cell proliferation and bursting vulva phenotypes) plus the analysis presented later in this thesis, such as genetic epistasis analysis and dysregulation of a key heterochronic gene, suggest that the synthetic/enhancer phenotype seen in *elt-1(ku491); daf-12(rh61rh411)* double-
mutant animals is likely due to disruption of parallel (genetically-redundant) pathways that regulate late-larval developmental timing.

Traditional 3-point recombination mapping combined with whole-genome sequencing was a powerful tool to positionally clone the \textit{elt-1(ku491)} mutation. Three-point mapping is reliable but labor intensive; sequencing identified many variations induced during mutagenesis, which were able to serve as mapping-SNPs and accelerate the process of positional cloning.

\textit{The daf-12-enhancer mutation is in elt-1}

The \textit{dfe-1(ku491)} mutation is in \textit{elt-1}, which was the first GATA transcription factor identified in \textit{C. elegans} (Page et al., 1997; Spieth et al., 1991). \textit{elt-1/GATA} is now known to specify hypodermal cell fates during early embryonic development (Page et al., 1997) and to be required during post-embryonic development for the maintenance of the stem-cell-like seam cell lineage (Brabin et al., 2011; Smith et al., 2005). \textit{elt-1(ku491)} is the first viable mutant allele of \textit{elt-1} that has an abnormal phenotype, likely because most mutations of the \textit{elt-1} gene would either not affect the activity of the ELT-1 protein or would have an effect more severe than compatible with survival; compare to the mutants isolated by the Million Mutation Project, which have a normal/near-normal phenotype (Figure 2.5) or to the \textit{elt-1(zu180)} loss-of-function allele with embryonic lethality (Page et al., 1997). In this sense, the \textit{elt-1(ku491)} is a novel mutation. From a practical standpoint, the viability and phenotype of the \textit{elt-1(ku491)} demonstrates that the genetic basis for the post-embryonic role of \textit{elt-1} is separable from its role in embryonic development; the isolation of this allele uncovered a function for \textit{elt-1} that was previously masked by pleiotropism and genetic redundancy. \textit{elt-1}, like other GATA-family transcription factors, has two Zinc-finger DNA
binding domain; the \textit{elt-1(ku491)} mutation is in the N-terminal domain and affects \textit{elt-1}’s function in post-embryonic development, while the \textit{elt-1(zu180)} mutation, which has a premature stop codon mid-way through the C-terminal domain, has embryonic lethality as a result. This suggests a possible model where the N-terminal domain is responsible for post-embryonic functions of \textit{elt-1} while the C-terminal domain is responsible for the function during embryonic development, although there are other possibilities. The embryonic lethality seen with disruption of the C-terminal domain in the \textit{elt-1(zu180)} allele (Page et al., 1997) makes formal testing difficult.

\textit{The phenotype of elt-1(ku491);daf-12 animals indicates that elt-1 is a heterochronic gene}

Animals with the \textit{elt-1(ku491)} mutation have four phenotypes identified so far: a \textit{daf-12(null)}-dependent L4-stage-specific bursting-vulva (1) and accumulation of stem-cell-like seam cells (2) and \textit{daf-12}-independent defect in seam-cell identity maintenance (3) and formation of alae at the L4 molt. The two \textit{elt-1(ku491)} phenotypes unmasked by \textit{daf-12} mutation are classic heterochronic phenotypes, both of which are generally thought to arise from either defective expression of the microRNAs that down regulate stem-cell-promoting factors as developmental progression occurs or from a decreased ability of the animal to utilize those microRNAs (such as by genetic ablation of core proteins of the microRNA-induced silencing complex). The \textit{elt-1} phenotypes described here that are independent of \textit{daf-12} genotype are consistent with previous findings from work done using \textit{elt-1(RNAi)} (Brabin et al., 2011; Gillear and McGhee, 2001; Smith et al., 2005). It is interesting to note that the retarded heterochronic phenotype of \textit{elt-1(ku491); daf-12(rh61rh411)} double-mutant animals is quite similar to that seen with simultaneous loss-of-function of several Let-7 family members (compare data from here to that presented in, for example, Abbott et
The implication is the \textit{elt-1} and \textit{daf-12} may redundantly regulate a family of microRNAs that is itself redundant.

\textbf{\textit{elt-1(ku491)} has \textit{daf-12}-independent seam cell maintenance defects}

Interestingly, the \textit{elt-1(ku491)} mutation does cause a phenotype independent of \textit{daf-12}. \textit{elt-1(ku491)} have a near-complete defect in formation of adult alae at the L4 molt, the transition from larval stages to adulthood. Alae are normally secreted by the terminally differentiated daughters of the final seam-cells division, indicating some degree of abnormal function in seam-cells that are present in the \textit{elt-1(ku491)} animals. Additionally, while the seam-cell number is normal to slightly elevated at early to mid stages of larval development, beginning in the third larval stage, \textit{elt-1(ku491)} animals lose seam-cells, ultimately finishing development with fewer seam cells than wild-type animals. This is likely a contributor to the alae formation defects, and examination using the adherens junction reporter AJM::GFP, which fluorescently labels the seam-cell/seam-cell intercellular communications, clearly illustrated that the \textit{elt-1(ku491)} single-mutant animals consistently had gaps in the line of seam cells during the L4 stage; the gaps were formed by premature differentiation of the seam-cell that formerly occupied that location. This demonstrates that the \textit{elt-1(ku491)} animals have mild defects in the post-embryonic maintenance of seam-cell cell fate and in the synthesis of alae at the L4 molt, in addition to the \textit{daf-12}-dependent heterochronic phenotypes.
**Figure 2.9**: Alignment of ELT-1 Protein N-terminal Zn-finger DNA Binding Domain

*Red asterisk marks C. elegans ELT-1 Proline²⁹⁸*

<table>
<thead>
<tr>
<th>D. melanogaster</th>
<th>FBpp0303221</th>
<th>D. rerio</th>
<th>gata3</th>
<th>H. sapiens</th>
<th>GATA1</th>
<th>N. musculus</th>
<th>gata1</th>
<th>C. elegans</th>
<th>elt-1</th>
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</thead>
<tbody>
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<td>LPLPPCAEREVCCATAPFRGRDGCSLYAGCNCRCNRPLPKRRLTVS</td>
<td>LPLPPCAEREVCCATAPFRGRDGCSLYAGCNCRCNRPLPKRRLTVS</td>
<td>LPLPPCAEREVCCATAPFRGRDGCSLYAGCNCRCNRPLPKRRLTVS</td>
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<td>LPLPPCAEREVCCATAPFRGRDGCSLYAGCNCRCNRPLPKRRLTVS</td>
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</tr>
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<td>: ; * * * * ;</td>
<td>* * * ;</td>
<td>* ; ; ; ; *</td>
<td>* * * ;</td>
<td>* * * ;</td>
<td>* * * ;</td>
<td>* * * ;</td>
<td>* * * *</td>
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</tr>
</tbody>
</table>

---

**Effect of the elt-1(ku491) mutation on the function of the ELT-1 protein**

The *elt-1(ku491)* mutation causes a proline-to-serine missense mutation at amino acid residue 298 of the full-length ELT-1 isoform A. *elt-1/GATA* contains two Zinc-finger DNA binding domains, each of which has a C-X₂-C-X₁₇-C-X₂-C motif type of Zn-finger domain; there is a very high degree of sequence identity to a GATA factor in each of *H. sapiens*, *M. musculus*, and *D. rerio* in the region of the protein containing the two Zn-finger domains (figure 2.9). The proline²⁹⁸ residue is in the N-terminal Zn-finger binding in a conserved residue 6 amino acids C-terminal to the second cysteine of the Zn-finger DNA binding domain. Zn-finger DNA binding domains bind cognate DNA sequences when the four Cysteine residues coordinate interact with a Zn molecule; for this to occur, a specific and precise secondary structure must be maintained. Figure 2.10 shows a crystal structure of the Zn-finger DNA binding domains of Human GATA1 bound to DNA, highlighting the amino acid residue Proline 213, which is conserved with *C. elegans* proline 298. The *elt-1(ku491)* mutation, by causing the P298S missense substitution, introduces an amino acid capable of adopting flexible structures in place of one that provides structural rigidity at a hairpin turn within the Zn-finger DNA binding domain. In doing so, the P298S substitution likely interferes with the secondary structure required for this domain to properly interact with both the Zn-molecule and target DNA sequences that are needed for it to fulfill it’s normal
physiological functions. Thanks to excellent genetics previously done on *C. elegans elt-1*, it is known that *elt-1(null)* causes early embryonic lethality; this implies that the *elt-1(ku491)* allele could only partially compromise the function the ELT-1 protein, because the single-mutant *elt-1(ku491)* animals grow well. One reasonably parsimonious model, which will be further discussed in light of data from Chapter III, is that the *elt-1(ku491)* allele retains the full function of its C-terminal Zn-finger DNA binding domain while the P298S substitution results in functional inactivation of the N-terminal Zn-finger DNA binding domain. The presumed consequence of this would be an apparent loss-of-function for targets of the C-terminal Zn-finger DNA binding domain and either no effect or a weak gain-of-function effect on targets of the wild-type N-terminal Zn-finger DNA binding domain (the partial gain-of-function result could potentially occur through titration of ELT-1 to more targets of the N-terminal Zn-finger DNA binding domain because of increased free nucleoplasmic ELT-1; essentially, an application of Le Chatelier’s principle).

Interpretation of the results of the phenotype of the mutant-over-null transheterozygote phenotypes and *elt-1(RNAi)* phenotypes is complicated by the superimposition of two contrasting phenotypes. For the each test, the alae defects and bursting vulva rates either remained comparable or became more severe phenotypes than in the *elt-1(ku491); daf-12(rh61rh411)* and *elt-1(ku491)* strains, respectively; this is what would classically be expected by a partial loss-of-function allele. For both assays, however, the number of seam cells decreased tremendously (figure 2.4). With *elt-1(RNAi)*, this can be understood easily if the *elt-1(ku491)* gene product remains capable of maintaining cell lineages at near-normal levels, but decreasing the total amount of ELT-1 protein by RNAi causes a profound lineage defect as the ELT-1 protein becomes completely knocked-down.
In short, there couldn’t be excessive seam cell proliferation when the seam cells all terminally differentiate because they have lost a critical factor for the maintenance of cell identity. In *elt-1(ku491)-over-elt-1(null); daf-12(rh61rh411)* animals, the weaker seam cell phenotype could be caused by a similar lineage maintenance defect in the transheterozygote, which prevents the seam cells proliferation phenotype because the cells have prematurely differentiated. Indeed, the finding of decreased seam cells in the *elt-1(ku491)-over-elt-1(null)* with wild-type *daf-12* when compared to *elt-1(ku491)* homozygotes with wild-type *daf-12* is consistent with some degree of allelic insufficiency for the *elt-1(ku491)* allele in the post-embryonic maintenance of the identity of seam-cell lineage. An alternative hypothesis is that the seam cell proliferation seen in *elt-1(ku491); daf-12(rh61rh411)* mutants is due to a weak gain-of-function activity of ELT-1, which is lessened by a decreased ELT-1 protein in either of these experiments. While the first model cannot be definitively proven, it seems to be the simplest that can accommodate all the relevant observations, and will be revisited after a fuller picture of the role of *elt-1* in developmental timing is presented in Chapter III.
The crystal structure of the two Zn-finger domains of Human GATA1 bound to DNA was visualized with DeepView. The top image is a over-view with the C-terminal Zn-finger domain on the left side and the N-terminal domain on the right side. The lower panel is zoomed-in and rotated, with Proline 213 highlighted in orange in the middle of the panel highlighted by a red arrow. Structure is PDB #3VD6. Proline 213 in Human GATA1 is the equivalent residue to C. elegans ELT-1 Proline 298.
CHAPTER III: MECHANISM FOR REGULATION OF DEVELOPMENTAL TIMING BY THE GATA TRANSCRIPTION FACTOR ELT-1

Introduction

Beginning with the early discovery of *C. elegans* mutants with abnormal developmental timing, the heterochronic genetic regulatory network that controls developmental timing has been studied extensively (Ambros, 1989; Chalfie et al., 1981). At this time there is a widely agreed upon model in which a small number of transcription factors regulate stage-specific developmental programs (cell division, differentiation, fusion, etc); these transcription factors are expressed in a stage-specific manner largely due to negative regulation at their 3’UTRs by stage-specific microRNAs (Ambros, 2000, 2011; Resnick et al., 2010). While microRNAs are both transcriptionally and post-transcriptionally regulated, the mechanisms for transcriptional regulation are poorly described and remain a significant gap in the current understanding of *C. elegans* developmental timing. A current model that summarizes the hierarchical structure of the heterochronic gene network is summarized in Figure 3.1. For many of these genetic relationships, the mechanism is understood at the biochemical level, but in a few cases, such as with the *lin-66* gene, even the potential mechanism for regulation remains unclear.

The phenotype of the *daf-12(rh61rh411)*-enhancing mutation *elt-1(ku491)* was described in chapter II. In this chapter, experiments are described that (1) order the action of *elt-1* within the heterochronic gene network and (2) examine the regulation of downstream genes in *elt-1(ku491)* mutant animals. Interpretation of these types of data requires inference and can not always exclude all alternative interpretations (Ambros, 1989; Avery and Wasserman, 1992; Blinder et al., 1989; Phillips, 2008), but is nonetheless able to clearly suggest a specific role for *elt-1* in the regulation of developmental timing.
Materials and Methods

Genetic Epistasis Analysis

To determine the order of action of the products of the *elt-1* and *daf-12* genes in the heterochronic regulatory network, genetic epistasis experiments were performed using RNAi by feeding. Phenotypes were scored as described in Chapter II. Statistical comparisons of seam-cell phenotypes were performed with Prism 6 as one-way ANOVAs with p-values for each RNAi vs empty vector comparison calculated post-hoc with
Bonferroni’s multiple comparisons method. For L4 bursting vulva and alae formation, data was analyzed with Prism 6 and p-values were calculated with Fischer’s exact test.

Quantitative measurement of abundance of key heterochronic genes mRNA abundance

To measure the relative abundance of mRNA for key microRNA target genes, quantitative real-time polymerase chain reaction was performed. For each genotype and stage examined, 50 animals were picked off of mixed-stage plates into 25µL of M9 buffer based on gross morphology. Total RNA was extracted with TRIZOL, precipitated with isopropanol, washed with 75% ethanol, and suspended in Nuclease-free water. Contaminant DNA was digested with DNasel (Invitrogen), which was then heat-inactivated according to the manufacturers instructions. For quantification of mRNA, first-strand cDNA templates were prepared using the reverse transcription enzyme SuperScript III (Invitrogen). Input cDNA was quantified with a Rotor-Gene 3000 instrument using the Rotor-Gene SYBR Green PCR Kit (Qiagen). Relative gene expression was calculated as previously described (Applied Biosystems, 1997; Livak and Schmittgen, 2001); eef-2 was used as a loading control and targets dCts were normalized to the L1 mean dCt for that gene. Amplification of a single target for primer sets was validated by gel-electrophoresis and melting-curve analysis, and standard curves for all primer sets had consistent amplification efficiencies and a slope-fitting least-squares regression r^2 of >0.98. The qPCR primers had the following sequences: TCTTCTCGAGCCAGTCTACTT and AACGTGTCCTCTTCTTCTGTTC for eef-2, AGGTAGAGAAGCTTATGCGGTA and CCTTCTTACGACCCAATGGATG for lin-28, TTGCACAAGCTTCCCTACTC and GTCCAATACTTGCTGGGTATCA for hbl-1, and TGGCTCATTGGAGCTTTGAC and GGACCACCGAGAGACGAATA for lin-41. The fold-change value of each data point was used for graphing and statistical analysis with
Prism 6 software (GraphPad). Statistical analysis was performed using two-way ANOVA and p-values were calculated for pre-planned comparisons among L4 samples using an uncorrected Fischer’s Least Squares Difference test. Data is shown as mean plus standard deviation.

Expression of an in vivo reporter of regulation on the lin-41 3’ Untranslated Region

An integrated reporter for the lin-41 3’ Untranslated Region (UTR) has previously been generated (Denli et al., 2004; Reinhart et al., 2000). It contains the integrated transgenic allele pkIs2084, which is promoter$_{col-10}$::beta-galactosidase::lin-41 3’UTR, including approximately 1.4kb of the lin-41 3’UTR. This transgene is expressed in hypodermal lineages and is negatively regulated by the Let-7 microRNA; mutation of either let-7 or microRNA-induced silencing complex co-factors (e.g., Argonaute) causes a failure to down-regulate expression of beta-galactosidase after the L4 molt is completed (Denli et al., 2004; Reinhart et al., 2000). To generate strains with the reporter, nT1/elt-1(ku491) IV; daf-12(rh61rh411) X male animals were crossed with a strain containing pkIs2084, and pkIs2084+ homozygotic progeny of each possible genotype were isolated and confirmed by direct sequencing of PCR amplicons. These animals were stained for beta-galactosidase activity with X-gal as previously described (Fire, 1992) with the following adaption: 2-3 crowded mixed-stage 6cm plates of animals were washed 3 times in M9 buffer and pipetted into 1.5 mL epindorf microcentrifuge tubes, and the supernatant was aspirated to <100 μL. Animals were flash-frozen in liquid nitrogen, desiccated at low heat in a rotating DNA vacuum for 45 minutes, fixed in cold acetone for 3 minutes at -20 degrees centigrade, and stained for 6 hours. Following three washes in phosphate-buffered saline, an aliquot was visualized with Nomarski optics at 64 times magnification. Approximately 40 animals were
scored per genotype per stage. All four strains were prepared in parallel with minimal gaps in processing times between strains. Data was analyzed with Prism 6 and p-values calculated with Fischer’s exact test.

**Results**

**Genetic epistasis analysis of elt-1 in developmental timing**

The phenotype of *elt-1(ku491);daf-12(rh61rh411)* animals was examined when key genes in the heterochronic gene regulatory network were knocked-down using RNAi. As shown in table 3.1 and 3.2, *lin-28, hbl-1, lin-14, and lin-41* are each epistatic to *elt-1(ku491);daf-12(rh61rh411)* for the *daf-12*-dependent phenotypes, L4 bursting vulva and seam-cell proliferation, but not for the *daf-12*-independent alae formation defect. Knock-down of *lin-46*, conversely, enhanced the *daf-12*-dependent phenotypes of *elt-1(ku491);daf-12(rh61rh411)* but did not affect the *daf-12*-independent alae formation defect. Interestingly, knock-down of *lin-29* did not have an effect on the seam-cell proliferation phenotype of *elt-1(ku491);daf-12(rh61rh411)* mutants. For seam-cell phenotypes, the p-value is <0.0001 for comparisons of *lin-28(RNAi), hbl-1(RNAi), lin-14(RNAi), lin-41(RNAi)* and *lin-46(RNAi)* to empty-vector control, but >0.99 for *lin-29(RNAi)* vs empty-vector control. For the L4 bursting-vulva phenotype, p-values for empty-vector vs *lin-28(RNAi), hbl-1(RNAi), lin-14(RNAi) and lin-41(RNAi)* are < 0.0001 and for empty-vector vs *lin-46(RNAi)* it is 0.0012. For all comparisons of the rate of alae formation, empty-vector vs RNAi p-values were non-significant.

These data show that the core heterochronic genes *lin-14, lin-28, hbl-1, and lin-41* are all required for the heterochronic phenotypes (seam-cell proliferation and L4 bursting vulva) of *elt-1(ku491);daf-12(rh61rh411)* animals, but that the *daf-12*-independent alae
defect of *elt-1(ku491)* single mutant animals is independent of those genes’ status.

Interestingly, knock-down of *lin-46*, a positive regulator of the heterochronic gene network that acts downstream of *lin-28*, can enhance the heterochronic phenotypes of *elt-1(ku491);daf-12(rh61rh411)*. *lin-29(RNAi)* had no effect on either class of phenotypes, consistent with its known role as a terminal differentiation factor after the L4 molt.
Table 3.1: Epistasis Results for L4 bursting vulva and seam cell proliferation phenotypes

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<tr>
<th>Strain</th>
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<th>L4 Bursting Vulva %</th>
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<td></td>
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Quantitative measurement of abundance of key heterochronic genes mRNA abundance

The genetic epistasis analysis presented above suggests elt-1 may act upstream of the core heterochronic genes lin-14, lin-28, hbl-1, and lin-41. To examine which genes are possible targets of elt-1, quantitative PCR was performed with first-strand cDNA to measure the expression level of possible target mRNAs. The results of quantitative RT-PCR are shown in figure 3.2; note that error bars are standard error. The daf-12(rh61) allele is a dominant-negative allele with strong heterochronic phenotypes at the L4 stage and was included as a positive control for changed expression. These data show that lin-28 is down-regulated to normal levels by L4 in elt-1(ku491);daf-12(rh61rh411) mutants, as compared to wild-type and both single-mutants. For hbl-1, the data show a trend towards increased expression of hbl-1 in elt-1(ku491);daf-12(rh61rh411) animals that does not approach statistical significance. However, the change in level of hbl-1 in wild-type animals from L1 to L4 was not statistically significant (p-value, 0.2692), indicating that the magnitude of change of hbl-1 mRNA is small compared to the noise in the measurement. For lin-41, the mRNA level in elt-1(ku491);daf-12(rh61rh411) animals at L4 was similar to that at L1 for all animals, while for wild-type animals it had decreased by approximately 40%. The differences at the L4 stage between elt-1(ku491);daf-12(rh61rh411) and wild-type, daf-12(rh61rh411), and elt-1(ku491) are unlikely to have occurred due to chance, as the p-values are 0.0205, 0.0345, and 0.0003, respectively. This data shows that in elt-1(ku491);daf-12(rh61rh411) animals there is a failure to down-regulate lin-41, which has previously been shown to normally be down-regulated during L4 and at the L4 molt by the action of the let-7 microRNA (Reinhart et al., 2000).
The staged samples used in this analysis were identified based on stage-specific morphology, including size and vulval development. The animals used are therefore from throughout their respective developmental stage. This was done because the *elt-1(ku491);daf-12(rh61rh411)* double-mutant animals have poor survival of standard synchronization methods (such as the alkaline hypochlorite method). While the animals are all from the same stage, they are not tightly synchronized within the stage, which lasts approximately 13 hours for L1 and 8 hours for L4. Because some genes’ mRNA levels normally change during the time period of the measurement, the main effect expected from the synchronization method is an increase in the noise in the measured level of targets.
Figure 3.2: Relative mRNA abundance of key microRNA target genes

Error bars represent standard error. For lin-28 and hbl-1, p-values for all L4 strain comparisons are non-significant. For lin-41, p-values at L4 stage for wild-type, daf-12(rh61rh411), and elt-1(ku491) vs elt-1(ku491);daf-12(rh61rh411) comparisons, respectively, are 0.0205, 0.0345, and 0.0003.
Assessment of expression of a lin-41 3' Untranslated Region Reporter

The expression of lin-41 was examined by an independent method to both validate the findings of RT-qPCR and to further define the mechanism by which it might be misregulated in elt-1(ku491);daf-12(rh61rh411) animals. For this assay, a previously developed integrated reporter was obtained, which expresses the E. coli beta-galactosidase gene in C. elegans under the control of the pan-hypodermal col-10 promoter and the stage-specific lin-41 3’ untranslated region (UTR). The results are shown in Figure 3.3. These data show that elt-1(ku491);daf-12(rh61rh411) animals do not down regulate the lin-41 3’UTR as do wild-type animals and daf-12(rh61rh411) animals. Interestingly, elt-1(ku491) single-mutant animals consistently showed expression of the reporter near the anterior pharynx at the young-adult stage (compare photos in figure 3.3 A-D). The p-value for the difference between daf-12(rh61rh411) and elt-1(ku491);daf-12(rh61rh411) strains at the young adult stage is < 0.0001, indicting that this difference is highly unlikely to have occurred by chance.

An alternative possibility is that there is altered regulation of the col-10 promoter in elt-1(ku491) mutant animals. There are 7 potential binding sites in the ~800 bp region between the the col-10 gene and the next gene. The elt-1(ku491) allele is a genetic partial loss-of-function allele, though, and the over-expression of the lacZ reporter would therefore require ELT-1 to normally negatively regulate col-10, but it appears to act primarily as a transcriptional activator (Gilleard and McGhee, 2001; Smith et al., 2005). This suggests that the over-expression of the lacZ reporter observed is likely due to decreased repression of the lin-41 3’UTR rather than due to increased transcription.
Animals with the described genotypes and the transgene plIs2084 were stained for lacZ activity. At the L4 stage, animals of each genotype stained positive (red arrows), while at the Young Adult stage elt-1(ku491) single-mutants and elt-1(ku491); daf-12(rh61rh411) double-mutants continued to stain positive (purple arrows) past the time wild-type and daf-12(rh61rh411) animals no longer did so (yellow arrows). Representative images are shown for the L4 stage (A-D) and the Young Adult stage (E-H) and summary stacked-column graphs in I-L. In I-L, the number scored is shown above each bar.
Discussion

**Epistasis places elt-1 upstream of the L4 stage within the heterochronic gene network**

Animals with the \textit{elt-1(ku491); daf-12(rh61rh411)} genotype have two kinds of phenotypes. First, there are phenotypes that can be classified as heterochronic (seam-cell proliferation and L4 bursting-vulva); these are only present when \textit{daf-12} is also mutated. Second, there are phenotypes that can be called seam-cell identity maintenance defects (larval seam-cell loss and adult alae formation defects); these occur independent of \textit{daf-12} genotype. The \textit{daf-12} nuclear hormone receptor is already known to promote the expression of the \textit{let-7} family microRNAs \textit{mir-48, mir-84, and mir-241} during the L2/L3 stages and of \textit{let-7} beginning at late L3. The phenotype data presented in Chapter II suggests the that \textit{daf-12}-enhancing mutation \textit{elt-1(ku491)} may disrupt a parallel pathway that also regulates the expression of those microRNAs. If that is the case, knock-down of certain microRNA targets (\textit{lin-14, lin-28, hbl-1}, and \textit{lin-41}) should be epistatic to the \textit{elt-1(ku491); daf-12(rh61rh411)} heterochronic phenotypes, while knock-down of downstream positive regulators of developmental cell fates (\textit{lin-46}) should enhance the phenotypes and knockdown of downstream effectors (\textit{lin-29}) should not have a further effect, as they are may already be un- or under-expressed.

This is exactly what was observed. Epistasis experiments show that \textit{lin-14, lin-28, hbl-1}, and \textit{lin-41} are all required for the heterochronic phenotypes of \textit{elt-1(ku491); daf-12(rh61rh411)} mutant animals, but that \textit{lin-46} knock-down enhanced the phenotypes and that \textit{lin-29} knockdown had no effect on the phenotypes. Strict logical analysis of this result says that the four required genes may either act downstream of the \textit{elt-1} and \textit{daf-12} genes or that they may act through a required parallel pathway; epistasis alone cannot distinguish
between those interpretations. Fortunately, there is convincing evidence available to say that daf-12 does in fact act upstream of both hbl-1 and lin-41, through the let-7 family of microRNAs, which suggests that the epistatic relationships identified for those two genes is likely upstream-downstream. For lin-14 and lin-28, it is a reasonable inference that knockdown of those genes at early stages of larval development causes precocious adoption of larval cell fates that render late-larval animals no longer sensitive to the defect of elt-1(ku491); daf-12(rh61rh411) double-mutation; this would be a parallel pathway interpretation of the epistasis results for those genes. Consistent with this model, knockdown of the early-larval-stage factors lin-14 or lin-28 could suppress the elt-1(ku491); daf-12(rh61rh411) L4 bursting vulva phenotype less completely than could knockdown of the late-larval-stage factors hbl-1 or lin-41, which both showed very strong suppression. For suppression of seam-cell proliferation, there was not a clean segregation of magnitude of suppression by the time the knocked-down gene is normally expressed, possibly because that phenotype is regulated continuously rather than just at the L4 stage, as it is the L4 bursting vulva phenotype.

The results with lin-46 and lin-29 are quite interesting and further support the interpretation that the defect in elt-1(ku491); daf-12(rh61rh411) animals is at the let-7 activation step. lin-46 negatively regulates developmental timing downstream of lin-28; it is proposed to form a scaffold for a developmental timing complex, but the mechanism is currently unknown. Interestingly, its mutation can enhance the phenotype of let-7 family mutants (Abbott et al., 2005), similar to the result seen here for elt-1(ku491); daf-12(rh61rh411) animals. lin-29 is well known to be negatively regulated by lin-41; its protein is normally made at the L4 molt and promotes alae formation and terminal
differentiation of seam cells. Were it to be unexpressed in \textit{elt-1(ku491); daf-12(rh61rh411)} animals, further knockdown by RNAi would not be expected to affect the phenotype, consistent with the observed result.

\textit{A key microRNA target gene is over expressed in elt-1(ku491); daf-12(rh61rh411) animals}

Measurement of mRNA levels for \textit{lin-28, hbl-1, and lin-41} showed that the \textit{elt-1(ku491); daf-12(rh61rh411)} animals were able to down-regulate the microRNA targets normally down-regulated at mid-larval stages (\textit{lin-28} and \textit{hbl-1}) but that \textit{lin-41}, which is normally down-regulated most strongly during L4, remained elevated at its L1 level. Interestingly, there is a not-statistically-significant trend to increased expression of \textit{hbl-1}, consistent with a minor contribution of L4-stage specific factors to its temporal regulation. These gene expression data suggest that the defect in \textit{elt-1(ku491); daf-12(rh61rh411)} animals is at a stage upstream of \textit{lin-41}.

An increase in the level of \textit{lin-41} mRNA could be due to increased production of it through a transcriptional mechanism or due to increased stability of the mRNA, as abundance level is a function of production minus removal. To further examine the forces acting on \textit{lin-41}, a gene expression reporter than measures the regulation of the \textit{lin-41} 3’ untranslated region was crossed into \textit{elt-1(ku491); daf-12(rh61rh411)} animals. Down regulated of this reporter, as described earlier, normally occurs during the L4 and young adult stages and because of the action of the Let-7 microRNA. In \textit{elt-1(ku491); daf-12(rh61rh411)} animals, there was increased expression of the reporter as detected by lacZ staining for the activity of \textit{beta-galactosidase}. This result indicates that the increased expression of \textit{lin-41} detected by RT-qPCR can be explained largely in terms of decreased action of the \textit{let-7} microRNA on the \textit{lin-41} 3’UTR. Stated in another way, under-expression
of let-7 in elt-1(ku491); daf-12(rh61rh411) animals can explain the results described. This may be due to a decreased ability of the elt-1(ku491) mutant to directly promote transcription of let-7. There are many GATA and GATC sites within a 2kb genomic region that contains the let-7 gene, as shown in figure 3.4; the ELT-1 protein has previously been shown capable of regulating transcription through those sequences (Shim et al., 1995). Two alternative interpretations that the current data can not exclude are that elt-1(ku491) allele interrupts positive regulation of let-7 through an intermediate gene or that the elt-1(ku491) mutation causes increased expression of another gene that represses let-7. All would be interesting results.

A model of elt-1’s role in the heterochronic gene network is proposed in Figure 3.5.
Figure 3.4: 2kb genomic sequence surrounding the \textit{let}-7 gene

GATA and GATC sites are in bold and the \textit{let}-7 gene is shown underlined from 5’ to 3’
Figure 3.5: Proposed model for role of elt-1/GATA in developmental timing

Figure adapted from Resnick, McCulloch, and Rougvie (2010)
CHAPTER IV: POSITIONAL CLONING OF SUR-4, A SUPPRESSOR OF THE ACTIVATED RAS ONCOGENE

Introduction

During the third stage of larval development, *C. elegans* animals begin formation of the vulva, which connects the uterus to the external space and provides a passage through which eggs are laid. Six equipotent vulval precursor cells (VPCs) are formed during early postembryonic development as daughters of the Pn ventral neuro-ectoblasts, but during the third larval stage the gonad anchor cell secretes the epidermal growth factor ortholog LIN-3 to three VPCs, instructing them to adopt vulval fates. The cell closest to the Anchor Cell, P6.p, is induced to adopt a primary vulval cell fate and the two adjacent VPCs are instructed to adopt secondary fates largely by lateral signaling from the primary VPC. The three uninstructed VPCs adopt the default tertiary fate. Induction of the primary fate in the VPC that will adopt it is triggered by interaction of EGF from the anchor cell with the EGF Receptor Tyrosine-Kinase LET-23 on the target VPC, which in turn activates the GTPase LET-60/Ras pathway, including Raf, MAPK, and ERK. Lateral signaling from the cell induced to adopt the primary fate is transmitted to the two adjacent VPCs by the Notch ortholog LIN-12 to instruct them to adopt secondary fates. Following induction and proliferation during the L3 stage, differentiation and morphogenesis are completed during L4 to create a functional vulva prior to the

*Figure 4.1: VPC induction during L3*

*Figure from P. W. Sternberg, WormBook, 2005*
attainment of reproducing maturity at the L4 molt (Ferguson et al., 1987; Sternberg, 2005; Sulston and Horvitz, 1977).

Interestingly, increased activity of the Ras pathway causes extra VPCs to adopt primary and secondary fates, resulting in the formation of ectopic pseudovulvae (the Muv phenotype), while decreased activity of let-60/Ras causes a vulvaless (Vul) phenotype (Han et al., 1990; Sundaram, 2006). Vulval formation has been an incredibly informative model for tissue development in large part because of its reliance on conserved signaling modules to control the formation of a non-essential organ. let-60/Ras-mutant-based genetic suppressor screens have been extremely powerful at identifying genes that act in the Ras signaling pathway. Genes whose products negatively regulate the Ras pathway were discovered through the identification of lesions that revert the let-60/Ras partial-loss-of-function mutants’ vulvaless phenotype to the wild-type phenotype; genes that positively regulate the let-60/Ras pathway were discovered through the identification of mutations that suppress the multivulval phenotype of a let-60/Ras gain-of-function mutant. Using this approach, mutants in nearly all conserved components of the Ras signaling module have been identified, including Raf, ksr, mapk, erk, sur-8, sur-2, sur-8.
sur-5, sur-6/PP2Ab, sur-7/zinc transporter and cbp-1 (Gu et al., 1998; Han et al., 1993; Kornfeld et al., 1995; Li et al., 2000; Sieburth et al., 1998, 1999; Singh and Han, 1995; Sundaram and Han, 1995; Wu and Han, 1994; Yoder et al., 2004); several of these genes, such as ksr-1 and sur-8, were originally identified from C. elegans mutagenesis screens and subsequently determined to have conserved roles in other organisms, such as flies, mice, and humans.

One such mutant identified in our lab has been remarkably resistant to positional cloning efforts. A strain carrying the ku23 allelic mutation in the suppressor of ras (sur)-4 gene was isolated in approximately 1992 as a suppressor of the Muv phenotype caused by the let-60/Ras gain-of-function allele n1046. let-60/Ras(n1046gf) contains a C-to-T missense mutation causing a substitution of glutamate for glycine at amino acid residue 13 of the protein product of the gene. This interferes with the intrinsic GTPase activity of the LET-60/RAS protein, resulting in the protein being stuck in the “on” position, continually activating Raf and downstream signaling even in the absence of an input from the receptor tyrosine kinase LET-23. As described in table 4.1, wild-type animals are less than 1% Muv, let-60/Ras(n1046gf) animals are approximately 70% Muv, and sur-4; let-60/Ras(n1046gf) animals are 2.4% Muv. After isolation of a strain with the sur-4 suppressor mutation, several graduate students, postdoctoral associates, and visiting scientists have sequentially attempted to identify the molecular lesion.

<table>
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<th>Strain</th>
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<tr>
<td>sur-4(ku23) III;let-60(n1046) IV</td>
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<tr>
<td>hT2/sur-4(ku23) III;let-60(n1046) IV</td>
<td>52.0 (123)</td>
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Table 4.1: Phenotype of the Ras Suppressor sur-4
responsible for the Sur-4 phenotype, all unsuccessful despite significant effort over several years.

Previous 3-point mapping had estimated that sur-4 might be in the region immediately to the right of the vab-7 gene at position 10,920,336 of Chromosome III, however that data was suspect as it had failed to identify the molecular lesion of sur-4. With the development of affordable high-throughput sequencing technology, a new attempt was made to identify sur-4 using shotgun sequencing of whole genomes. That successful effort is described in this chapter.

Materials and Methods

Mutagenesis Screen and Phenotype Analysis

Mutagenesis screening was performed to find suppressor mutations that revert the Muv phenotype of let-60/Ras(n1046gf) to a non-Muv phenotype; EMS was used as previously described (Brenner, 1974). The sur-4 suppressor was isolated prior to my involvement in the project. Animals were examined on a bench-top low-powered dissecting microscope for the presence of absence of the multiple ventral protrusions indicative of the Muv phenotype.

Positional Cloning

To identify the molecular lesion responsible for the sur-4 phenotype, whole-genome sequencing technology was used as described in Chapter II. Briefly, the parental strain MT2124, with the genotype let-60/Ras(n1046gf) IV, and the minimally-outcrossed suppressor strain MH60, with the genotype sur-4 III; let-60/Ras(n1046gf) IV, each had shotgun sequencing of their genomes performed. Raw sequencing results were mapped to
the *C. elegans* genome using both Bowtie2 and BWA and visualized in Integrated Genome Viewer (Whitehead Institute, Cambridge, MA). Chromosome III was manually inspected for each strain to identify all variations between the two strains. Sanger-based sequencing of PCR amplicons containing candidate mutations was performed to confirm the presence of the mutation and to determine their presence or absence in the highly outcrossed suppressor strain MH408, with the genotype *sur*-4 III; *let*-60/*Ras*(n1046gf) IV. Finally, the strain MH408 was outcrossed with the GFP-marked balancer nT1 (which balanced *let*-60/*Ras*(n1046gf) on Chromosome IV but does not balance any of Chromosome III) and *let*-60/*Ras*(n1046gf)-homozygous F2 progeny were examined for the presence or absence of Muv phenotype and were genotyped for several Chromosome III variants to identify recombinant chromosomes.

To assess phenotype complementation, transgenic strains were generated using standard microinjection-mediated transformation. Only stably transmitting lines were examined.

**Results**

Chromosome III was visually inspected with the IGV software package for strain MH60, a minimally-outcrossed strain with the genotype *sur*-4 III; *let*-60/*Ras*(n1046gf) IV and the parental Muv strain MT2124, with genotype *let*-60/*Ras*(n1046gf) IV. 60 variations between the two strains that affect coding regions of genes were identified throughout the chromosome; variations not associated with genes were not counted, as were mutations associated with a gene but not predicted to affect it (e.g., substitutions in introns not near splice sites).
Next, Sanger sequencing of PCR amplicons was used to assess the strain MH408 for the presence or absence of a subset of these mutations. MH408 has the same genotype as MH60, *sur-4* III; *let-60/Ras(n1046gf)* IV, but has been extensively outcrossed and thus may no longer contain candidate variations that are not the *sur-4* lesion, allowing for their exclusion from the list of candidates. The results of this are presented in Table 4.2. This allowed for the exclusion of all candidates left of physical position 5,258,108 or to the right of 10,873,283.

**Table 4.2: Mutagenesis-induced sequence variations between parental strain, original suppressor strain, and outcrossed suppressor strain**

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Next, the strain MH408 was outcrossed, using the balancer nT1 to ensure that strains continued to have the *let-60/Ras(n1046gf)* mutation. Over 100 *let-60/Ras(n1046gf)*-homozygous F$_2$ progeny were examined for recombination between markers chosen to represent the outer region potentially containing *sur-4*. Several were identified; the results are summarized in Table 4.3. These data exclude all variations to the left of 10,276,407 or to the right of 10,477,360 as candidates for *sur-4*. One candidate gene, *cnk-1*, had
previously been shown to be involved with Ras-pathway signaling and so was a likely candidate to be sur-4 (Rocheleau et al., 2005; Therrien et al., 1998). For further analysis, a candidate approach was taken to examine complementation of the Sur-4 suppressor phenotype by a transgene containing wild-type copies of theses genes.

Table 4.3: Association of candidate variations with outcrossed sur-4 strains that had a recombination event in candidate region

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<td>F40F12.3</td>
<td>erk-1</td>
<td>D2045.2</td>
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<td>#3-8</td>
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<td>A A G A A G</td>
<td>A A G G</td>
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Microinjection-mediated DNA transformation was performed with the sur-4 III; let-60/Ras(n1046gf) IV strain using the fosmid WRM0629dG10. This fosmid contains 31,713 bases of C. elegans genomic DNA covering four genes, including cnk-1 and 13kb of potential upstream regulatory region. Empty pBSK plasmid was co-injected as carrier DNA and promoter,sur-4::dsRed was co-injected as selectable marker for transformation. Of the four independent transmitting transgenic lines obtained, 1 showed strong reversion to the
Muv phenotype, 1 showed weak reversion to the Muv phenotype, and two remained non-Muv. Extrachromosomal transgenes result in mosaic animals with variable expression of the transgene, so complementation by ¼ lines was interpreted as complementation of the sur-4(ku23) suppressor phenotype by wild-type cnk-1.

**Discussion**

The Sur-4 suppressor mutation cnk-1(ku23) described in this study is the first cnk-1 mutation isolated from genetic screen in *C. elegans*, and its identification ends a 20+ year mystery in Min Han’s laboratory. The screen for suppressors of the Muv phenotype of let-60/Ras(n1046gf) was extraordinarily successful, identifying alleles of MAP kinase (*mpk-1; sur-1*), the MEK kinase (*mek-2*), the Mediator subunit 23 (*sur-2*), kinase suppressor of Ras (*ksr-1; sur-3*), the serine/threonine phosphatase 2A (*PP2A-B; sur-6*), a cation diffusion facilitator protein (*sur-7*), a novel leucine-rich repeat protein (*soc-2; sur-8*), and the transcriptional co-activator p300/CBP (*cbp-1; sur-9*), among others. For several of these, such as *ksr-1, sur-6*, and *sur-8*, these studies were the first to identify the genes and their roles in the Ras-MAPK signaling pathway. Based on its strong suppression of the Muv phenotype from *let-60/Ras(n1046gf)*, *sur-4* was predicted to encode a gene intimately associated with the Ras pathway. This turned out to be true.

It is unclear why *sur-4* was extensively resistant to prior efforts to positionally clone it. One possibility is that of all the Muv suppressors isolated, suppression of *let-60/Ras(n1046gf) by sur-4(ku23)* is the most susceptible to subtle variations in the genetic background, rendering the mapping through linkage to known genes unreliable. This may have been made worse by the positions of hot-spots or other variations in chromosomal recombination rates, which are known to interfere with mapping for positional cloning.
Drosophila cnk was originally discovered from a screen for enhancers of the ksr phenotype, using tissue-specific gene expression methods and screening for altered eye morphology (Therrien et al., 1998); this was approximately six years after the Sur-4 Ras suppressor strain had been isolated in C. elegans in our lab. cnk-1 proteins contain conserved SAM-, CRIC-, PDZ-, and PH-domains and have been studied in Drosophila, mammals and C. elegans (Douziech et al., 2006; Laberge et al., 2005; Rajakulendran et al., 2008; Rocheleau et al., 2005), showing a conserved role in the Ras pathway. In the study using C. elegans, Rocheleau et al use two cnk-1 deletion alleles to show that cnk-1 is required for the manifestation of the Muv phenotype of let-60/Ras(n1046gf). However these cnk-1 mutations do not have the phenotypes caused by elimination of let-60/Ras activity (such as L1-stage lethality). Interestingly, they demonstrated that cnk-1 is required for some, but not all, Raf gain-of-function mutants’ phenotypes, suggesting that CNK-1 may not only help bridge Ras and Raf but may also regulate Raf. As shown in figure 4.3, CNK-1 is currently believed to be part of the KSR-containing multiprotein complex that activates Raf, MAPK, and ERK in response to Ras (Clapéron and Therrien, 2007), but it remains much less studied than other Ras pathway genes. The cnk-1(ku23) allele contains a single nucleic acid substitution (GAA=>AAA) causing a glutamate to lysine missense substitution at amino acid residue 647 of the protein (Figure 4.4). This occurs at a conserved residue early in the membrane-binding PH-domain, and thus may interfere with the subcellular localization of CNK-1 and CNK-1-containing complexes. Interestingly, the cnk-1(ku23) allele isolated as Sur-4 causes stronger suppression of Muv from let-60/Ras(n1046) than the alleles examined by Rocheleau et al.; it may potentially do so by preventing the CNK-1-containing Ksr scaffolding complex from properly interacting with Ras at the inner leaflet.
of the plasma membrane, sequestering Raf, MAPK-1, and/or ERK-1 from Ras. Additional experiments would be needed to determine the molecular mechanism by which the *ku23* mutant allele of *cnk-1* suppresses activated *let-60/Ras*.

There have been efforts to develop Ras-pathway targeted therapies for human cancer because more than 30% contain Ras-activating mutations. Inhibition of the post-translational processing required for Ras membrane localization using Farnesyltransferase inhibitors has proven to be an ineffective strategy for the treatment of human cancers. Inhibition of upstream receptor tyrosine kinases with monoclonal antibodies has improved outcomes, but only for patients who do not have common downstream Ras-activating mutations. Recently, drugs have been developed that block activated Raf; one such drug, vemurafenib, has shown success in the treatment of specific malignancies, such as
melanoma (Chapman et al., 2011). While it is effective at inducing remission for some patients, there is a high rate of relapse. Recent studies have examined the detailed structural mechanisms by which the downstream target Erk is activated by Ras-Raf activating complexes (Jameson et al., 2013) and how Raf auto-activation/auto-inhibition is balanced (Holderfield et al., 2013), encouraging further research into these mechanisms and raising hopes for eventual improvement in therapies for the treatment of cancer.

Figure 4.4: Alignment CNK protein region flanking the cnk-1(ku23) mutation in Humans, Mouse, and C. elegans

C. elegans cnk-1(ku23) mutation is E=>K at the conserved alignment position 775
CHAPTER V: CONCLUDING COMMENTS AND FUTURE DIRECTIONS

Discussion

In this thesis, two examples of genetic control of cell fate during *C. elegans* development have been shown. While involvement of *cnk-1* in the widely conserved EGFR-Ras-Raf-MAPK signaling pathway was previously known, identification of the *sur-4* gene brings to a close a longstanding mystery in our laboratory. Furthermore, the biochemical nature of this very strong suppressor mutation, altering a conserved residue in the membrane-binding PH-domain, suggests that the positional cloning of *sur-4* may have identified an absolutely required function of *cnk-1* – direct plasma membrane binding – that had previously been unappreciated. Additional research into the functional and structural consequences of the intra- and inter-molecular interactions that regulate Raf-activation within cnk-containing complexes is warranted given the intense focus Raf-activation is currently being given.

In the major effort of this thesis, the GATA transcription factor *elt-1* was identified as a heterochronic gene that is required for a genetic pathway functionally redundant with the nuclear hormone receptor *daf-12*. It was shown to act “upstream” of late-larval fates within the heterochronic gene network, but significant questions remain. Most importantly, these data do not establish whether the ELT-1 protein directly regulates transcription of the Let-7 family microRNAs or whether it acts further upstream through other intermediates. Additionally, the mechanism by which ELT-1 promotes stage-specific cell fates remains unclear, particularly given that it has relatively stable expression during different developmental stages. This is related to the largest general gap in our understanding of developmental timing, which is that we have little knowledge of upstream factors that initiate changes in gene expression through the heterochronic gene network.
The *elt-1(ku491)* allele is interesting in that it appears to largely separate its post-embryonic functions in developmental timing and in cell lineage specification. *elt-1* has previously been shown to be required for post-embryonic development and to also have a continued role in the maintenance of the stem-cell-like seam cells during post-embryonic development. The evidence provided here is the first to show a role for *elt-1* in post-embryonic developmental timing. Animals with *elt-1(ku491)* are viable, indicating that the allele is competent for *elt-1*’s normal role during embryonic development, but *elt-1(ku491)* single-mutant animals do have cell lineage defects: they have a slight loss of seam-cells during late post-embryonic development and failure to form adult alae at the L4 molt. The P298S mutation of *elt-1(ku491)* is likely to have its effect by interfering with the secondary structure of the N-terminal Zn-finger DNA binding domain. This likely reduces the ability of the ELT-1 protein to regulate targets where the N-terminal domain is required for interaction with their promoters or enhancer elements. One consequence of this could be a slight increase in regulation of targets of the C-terminal Zn-finger domain as increased free nucleoplasmic ELT-1. The simplest interpretation of the sum of the epistasis and gene expression studies described in Chapter III is that *elt-1* promotes the expression of one or more Let-7 family microRNAs, particularly Let-7, and that this activity is partly compromised by the *elt-1(ku491)* mutation; while this regulation is proposed to be direct, the data presented here does not prove that to be correct. An alternative possibility would be that the *elt-1* negatively regulates a repressor of a Let-7 microRNA and that the *elt-1(ku491)* mutation increases this repression. The most outstanding remaining question is to identify whether ELT-1 directly interacts with one or more of the regulatory regions controlling...
expression of the Let-7 family of microRNA. Additionally, identification of protein co-
factors for ELT-1’s role in developmental timing would be significant.

One question raised by this work is what evolutionary advantage genetic redundancy
in the regulation of developmental timing would offer *C. elegans*. The causes and
consequences of genetic redundancy remain incompletely understood, but there are potential
benefits. Redundancy can increase robustness, making the normal developmental outcome
more likely even under varying conditions, including gene mutation (McAdams and Arkin,
1999; Nowak et al., 1997). Additionally, the extensive negative feedback present in the
heterochronic gene network may serve a useful purpose, as it has in general been proposed
to increase the sharpness in cell state shifting, such as from a progenitor cell to a fully-
differentiated cell (Ferrell, 2002; Hart and Alon, 2013; Kudlow et al., 2012). Robustness in
cell fate outcomes combined with sharp switching between different fates may be sufficient
to explain the apparent evolutionary advantages of extensive use of redundancy in gene
regulatory networks.

**Genetics and Contemporary Medicine**

Beginning with Linus Pauling and colleagues’ 1949 discovery of the hemoglobin
mutation responsible for sickle-cell anemia (Pauling and Itano, 1949), the modern era of
molecular medicine has generated tremendous knowledge of the biological basis for human
diseases. In some cases, a detailed molecular understanding of diseases has led to the
development of effective treatments, such as with imatinib for chronic mylogenous
leukemia (Kantarjian et al., 2002; Sawyers et al., 2002) and statins for familial
hypercholesterolema (Basu et al., 1976; Goldstein and Brown, 1974; Kane et al., 1990).
However, our understanding of the underlying biology remains incomplete for the majority
of human diseases, and our therapies are all too often insufficient. In the current post-genomic era, there is tremendous information available both about diseases in general and about individual patients, including at the physiologic, molecular, and genetic levels, including now even whole-genome sequences. Turning this extensive, at times overwhelming, amount of information into actionable knowledge requires context and general principles, which can be developed, in part, by research with basic model organisms such as the nematode round worm *Caenorhabditis elegans.*
REFERENCES


Appendix 1: List of *C. elegans* strains

Chromosome II: *kuIs47*

Chromosome III: *cnk-1(ku23)*

Chromosome IV: *elt-1(ku491), elt-1(ok1002), unc-24(e138), dpy-20(e1282), let-60(n1046)*

Chromosome V: *wIs51*

Chromosome X: *daf-12(rh61), daf-12(rh61rh411), alg-1(gk2114)*