The *C. elegans* heterochronic pathway controls the timing of NAB/EGR-mediated terminal differentiation and the onset of adulthood

by

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Abstract

Most animals pass through a series of juvenile stages on their way from embryo to adult. These stages represent periods of time in which stage-specific developmental processes occur. At the end of development, the organism transitions from a juvenile state to a mature adult. The failure to undergo a specific developmental stage, or the failure to transition to an adult can lead to specific developmental deficits or the retention of juvenile characteristics throughout the remainder of the organism's life.

This thesis focuses on the study of the mechanisms controlling the larval-to-adult transition in *C. elegans*. In Chapter two, I perform a screen to identify mutants that are defective in a specific aspect of the larval-to-adult transition, exit from the molting cycle. From this screen I identify new alleles of the previously identified gene *mab-10*. I then clone *mab-10*, showing that it encodes the only *C. elegans* NAB family transcriptional cofactor. I go on to show that MAB-10 acts with LIN-29 to control the larval-to-adult transition and that LIN-29 shares homology with mammalian EGR proteins. These observations highlight the similarity between the mechanisms controlling the onset of adulthood in *C. elegans* and mammals as LIN-29 is required for the larval-to-adult transition in *C. elegans* and EGR1 is required for the onset of puberty in mice.

Chapter three is a further analysis of both *mab-10* and *lin-29*, focusing on their expression patterns and roles in the terminal differentiation of tissues outside of the hypoderm. We use a *mab-10* translational reporter to show that MAB-10 accumulates precociously in precocious heterochronic mutants and that *mab-10* transcription does not require *lin-29*, suggesting that the heterochronic pathway controls the timing of *mab-10* transcription in parallel to *lin-29*. We use single molecule fluorescence *in situ* hybridization to establish the wild-type transcription patterns of *mab-10* and *lin-29* and show that both undergo dynamic changes in expression throughout development. We show that *mab-10* is required for terminal differentiation in at least one other tissue outside of the hypoderm and that *mab-10* prevents seam cell overproliferation by antagonizing the activities of the *C. elegans* Runx and CBF-Beta homologs *rnt-1* and *bro-1*.

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Chapter One

Introduction

Introduction

The development of a multicellular organism is no small task. The mechanisms controlling the fate decisions of specific cell lineages, from stem cell to differentiated state, are of central importance to stem cell biology and metazoan biology in general. Together with the question of which specific fate a cell should take, is the question of *when* a cell should take that fate. Proper cell-fate decisions require that tissues throughout the organism develop within tight temporal constraints. Altering the timing of these precise developmental events can have dramatic consequences ranging from inviability to speciation. The work presented here is aimed at providing a better understanding of the mechanisms that control developmental timing.

Developmental timing

Life stages

When discussing the developmental timing of an organism, we often refer to its life cycle. The life cycle can be said to begin at fertilization when the organism is a zygote. The zygote proceeds through embryogenesis, birth, postembryonic development, maturation to adult (when the cycle can be re-initiated), senescence and finally death.

While embryogenesis proceeds as a more-or-less continuous process from the first cell division to the completion of organogenesis, post-embryonic development in most organisms proceeds through a series of larval/juvenile stages. In arthropods like *Drosophila melanogaster*, these larval stages represent discrete developmental programs that are demarcated by a molting cycle. In this case, the transition from the juvenile stage to the adult stage is accompanied by a metamorphosis that completely reshapes the body plan of the organism.

By contrast, in humans, post-embryonic development lacks the obvious demarcation that a molting cycle provides, and the transition from juvenile to adult appears superficially to be a continuous process. However, humans do undergo a juvenile-to-adult transition during puberty that involves sexual maturation, the development of adult physical characteristics, and the adoption of adult behaviors. While it is clear that this transition is controlled by genetic and environmental factors, it is unclear if the mechanisms controlling the timing of this transition are conserved across the animal kingdom.

Heterochrony

What is heterochrony?

The term "heterochrony," which comes from latin for "different time," can be defined as a change in the timing of one developmental event relative to another. Historically, the term heterochrony was first used when comparing a particular developmental process between two closely related species. If the onset time or offset time of the process differs, or if the rate of the process differs between the two species, then the process is heterochronic. More recently, heterochrony has been used to describe a change in the timing of one developmental process with respect to another developmental process within the same organism.

A classic example of heterochrony comes from the Mexican salamander, the axolotl (*Ambystoma mexicanum*), and the closely related tiger salamander (*Ambystoma tigrinum*). Salamanders typically undergo metamorphosis from egg to larva (an aquatic tadpole form) and then again from larva to a terrestrial adult form. The axolotl displays a specific form of heterochrony known as neoteny. Neotenic animals retain juvenile characteristics throughout their

adult lives. Instead of transforming from a larval form to an adult form, the larval axolotl grows in size, sexually matures, and remains aquatic for the duration of its life. This life cycle is in contrast to the life cycle of the closely related tiger salamander, which proceeds from its aquatic larval state to a terrestrial adult. Molecularly, this adaptation is caused by the failure to release thyrotropin, the thyroid stimulating hormone[1-3].

Heterochrony in evolution

Heterochrony, neoteny specifically, has been proposed to be a driving force in human evolution. Early comparisons between humans and chimpanzees revealed that infant chimpanzees and infant humans have a very similar facial bone structure. Juvenile chimpanzees appear to have a bone structure that resembles the human adult bone structure. As juvenile chimpanzees age, their facial bones continue to grow and as adults are morphologically distinct from the adult human. These observations drove the idea that humans may have evolved in a neotenic fashion[4,5]. More specifically, humans evolved to retain the juvenile form of the chimpanzee-human common ancestor. As a result, adult humans have retained the physical attributes and cognitive plasticity found in juvenile chimpanzees. By prolonging the juvenile state, humans have more time to develop and learn.

Similar observations have been made with respect to the domestication of dogs. Domesticated dogs are descendants of the Gray wolf (*Canis lupus*)[6]. The gray wolf is a predatory canine that was commonly found throughout Eurasia and North America. Present day domesticated dogs are the result of multiple generations of selective breeding for traits that were deemed desirable by human companions. It is hypothesized that domesticated dogs have been

selected for a juvenile temperament that is more compatible with human-canine relationships. This selection strategy has had the interesting side-effect of also selecting for the retention of other juvenile wolf characteristics. Therefore, current domesticated dogs more closely resemble the juvenile wolf with respect to behavior, temperament, and bone structure than they do adult wolves[7,8].

These observations have been mirrored in a more directed experiment involving the domestication of the silver fox (*Vulpes vulpes*). After over 40 generations of breeding and selection for "tameness," the domesticated fox has lost many of its wild traits and has in both behavior and physical appearance, retained many of its juvenile qualities[9].

The grouping of these juvenile traits via selection suggests that there are specific loci that can affect multiple aspects of developmental timing[4]. Understanding the mechanisms that govern developmental timing will give us a better understanding of our own development and might give us insight into our own evolution.

Developmental timing in *C. elegans*

The power of *C. elegans*

The nematode *C. elegans* is an excellent organism with which to study the genetic control of developmental timing. Adult *C. elegans* are approximately 1 mm in length and exist as either self-fertilizing hermaphrodites or males (Figure 1a). *C. elegans* has an extremely well characterized life cycle in which the animal begins as an embryo and proceeds through four larval stages (L1-L4) before becoming a sexually mature adult (Figure 1b). Each stage of development is defined by a characteristic pattern of gene expression, cell division and tissue

morphogenesis and is demarcated by a period of molting during which the animal generates a new cuticle and sheds its old cuticle[10]. *C. elegans* is atypical in that its cell lineage is essentially invariant and has been fully described[11]. As such, it is the only organism in which the timing and position of every cell division and cell death from zygote to adult is known. This knowledge makes it possible to study alterations of specific cell lineages at single-cell resolution at specific times during development.

Cell lineages that continue to develop post-embryonically are particularly well suited for the study of developmental timing. Whereas embryonic development can be thought of as a continuous period from zygote to hatching, post-embryonic development is naturally punctuated by the molting cycle. Therefore, events that occur within a specific larval stage act as indicators of the developmental state of the animal. For example, in a wild-type animal, the vulva begins to develop during the L3 stage[11]. Therefore the initiation of vulval development is a proxy for the L3 stage. One specific set of hypodermal cell lineages, the seam cell lineages, has been used extensively for the study of developmental timing.

The seam cell lineage

The seam cells are a stem-cell-like population of hypodermal cells that undergo a synchronized pattern of cell division during each larval stage[11](Figure 2a). In a wild-type animal, the seam cells divide once at the beginning of the first, third and fourth larval stages to give rise to two daughter cells, one that fuses with the syncytial hypodermis and one (seam cell) that will divide again during the next larval stage. During the second larval stage, the seam cells undergo two rounds of division, the first being a symmetric division, giving rise to two daughter cells

both of which divide again asymmetrically in the pattern described for the other three larval stages. This symmetric division during the second larval stage results in the doubling of the number of seam cells and acts as an indicator of the L2 stage. During the final larval stage (L4) the seam cells undergo a process of terminal differentiation that indicates the end of development and the onset of adulthood.

Heterochronic mutants

Part of the fun of performing a genetic screen comes from the fact that you rarely know what you are going to find, and you usually find something you do not expect. This is especially true when you are working with an organism that is relatively unexplored. Early screens in *C. elegans* yielded several mutants with aberrant cell lineages. For example, in these mutants, specific cells would fail to divide or reiterate the division pattern of their parental or grandparental cells[12]. Upon closer inspection (and some keen insight) it was discovered that some of these mutants were reiterating the division pattern of a specific larval stage rather than of their parent or grandparent. These mutants were altered with respect to multiple aspects of developmental timing and were named "heterochronic" mutants.

The heterochronic mutants typically are categorized as "precocious mutants," which display late developmental traits too early, and "retarded mutants," which reiterate early developmental traits at later stages or retain juvenile characteristics. For example, precocious mutants might generate adult specific traits such as an adult cuticle (as evidenced by the presence of lateral alae) at the end of the L3 stage instead of the L4 stage. They might also show late-stage patterns of cell division and gene expression at earlier stages (Figure 2b). By contrast, retarded mutants might retain a juvenile cuticle as adults, undergo extra molts, or undergo extra rounds of

cell division associated with earlier stages (Figure 2c). Double mutant analyses with precocious and retarded mutants allowed the construction of a genetic pathway that has led to important insights into the regulation of developmental timing as well as to landmark discoveries concerning novel methods of post-transcriptional regulation with the discovery of microRNAs.

The early developmental timer

lin-4 controls the progression from L1 to L2

Early studies of developmental timing grew from the analysis of mutants with altered, and often reiterated, cell lineages[13]. In these mutants, abnormal lineages typically underwent one of three types of cell lineage change: a parental reiteration, a grandparental reiteration, or a proliferation. In a parental reiteration, one of the two daughter cells repeats the division pattern of its parent. In a grandparental reiteration, a cell repeats the division pattern of its grandparent. In a proliferative alteration, both daughter cells adopt the parental fate and reiterate the parental division. Interestingly, one mutant, *lin-4* (abnormal cell LINeage), caused not just the reiteration of cell divisions but also the reiteration of a general stage-specific developmental program. For example, while wild-type animals develop through four larval stages before becoming an adult, specific tissues within *lin-4* animals reiterate an L1 pattern of cell division and never progress to the L2 stage. Therefore, it was concluded that *lin-4* is required for the animal to progress from the L1 to the L2 stage. Because *lin-4* animals reiterate a developmental program, *lin-4* is considered a retarded mutant.

lin-14 is required for the execution of L1 developmental programs

A second mutant, *lin-14*, had the opposite phenotype to *lin-4*[14,15]. These animals appeared to skip L1-specific cell divisions and directly executed L2-specific developmental programs after hatching. Because *lin-14* animals skip a developmental stage and initiate a later developmental program prematurely, they are considered precocious. The absence of an L1 stage also resulted in the premature initiation of the larval-to-adult transition. It was concluded that *lin-14* is required for the animal to execute the L1 developmental program. A gain-of-function allele of *lin-14* was isolated that dominantly conferred lineage alterations that were identical to those conferred by the *lin-4* loss-of-function mutation discussed above. Double mutant analyses between *lin-4* and *lin-14* loss-of-function mutations suggested that *lin-4* acts upstream of *lin-14* to negatively regulate its activity[16]. It was proposed that the gain-of-function allele of *lin-14* uncoupled *lin-14* from *lin-4* regulation.

lin-28 is required for the execution of L2 developmental programs

Loss-of-function mutations in *lin-28* behave similarly to *lin-14* loss-of-function mutations, with one significant difference. While L1 stage developmental programs are skipped in *lin-14(*lf*)* mutants, L2 stage developmental programs are skipped in *lin-28(*lf*)* mutants[15]. For example, the L2 proliferative seam cell division does not occur, and therefore adult *lin-28* animals have roughly half the number of seam cells as wild-type animals. Furthermore, *lin-28* animals become adult one and sometimes two stages prematurely. *lin-28* was proposed to specify the L2 developmental program and to prevent the premature initiation of the L3 developmental program. Like *lin-14*, *lin-28* was shown to act downstream of *lin-4* genetically[16].

While *lin-4*, *lin-14*, and *lin-28* were found to control early developmental events, a fourth heterochronic gene, *lin-29*, was found to control late developmental events. In *lin-29* mutants, early development proceeds normally but animals fail to transition from larva to adult because they reiterate a late-stage developmental program[15]. Double mutant analyses demonstrated that *lin-29* was required for the precocious larval-to-adult transition that occurs in the absence of *lin-14* and *lin-28*, making *lin-29* the furthest downstream component of the heterochronic pathway[16].

The late developmental timer

When we discuss the larval-to-adult transition, we are referring to a set of events that occur during the L4 stage. Cells stop dividing and undergo a process of terminal differentiation. In the hypoderm, the larval-to-adult transition comprises four events. The stem cell-like population of seam cells stop dividing and fuse and form a syncytium. The hypoderm generates an adult cuticle and the organism exits from the molting cycle. *lin-29* mutants are defective in all four of these processes, and therefore LIN-29 is thought of as a master regulator of the larval-to-adult transition in the hypoderm[15].

While *lin-4*, *lin-14*, and *lin-28* control the timing of the larval-to-adult transition by regulating early developmental events, another set of heterochronic genes more directly regulates late developmental events. Animals with mutations in these late-acting genes undergo early development normally but either reiterate a late larval stage, as is the case with *let-7* (LEThal)[17], or skip a late larval stage, as is the case with *lin-41*[18] and *hbl-1* (HunchBack-Like)[19,20].

let-7 mutants undergo an extra larval stage

let-7 mutants were originally identified based on late-stage lethality as a result of bursting from the vulva[21]. A subsequent screen looking for suppressors of a synthetic sterility caused by the *lin-14* gain-of-function mutation and *egl-35* (EGg Laying defective) identified a second allele of *let-7* and suggested that it might be a heterochronic gene[17]. Upon closer examination, *let-7* mutants were found to undergo an extra larval stage. Instead of undergoing the larval-to-adult transition at the end of the L4 stage, *let-7* animals became adult after an inappropriate "L5" stage. It was observed that mutations that cause a precocious heterochronic defect could suppress the lethality associated with *let-7*, and this suppression of *let-7* lethality provided a powerful means for the identification of new precocious heterochronic mutants.

lin-41 mutants become adult prematurely

lin-41 mutants were isolated from a screen to identify mutations that suppress the lethality and the heterochronic defects conferred by *let-7*[18]. Like *let-7* mutants, early development in *lin-41* mutants proceeds normally. However, many *lin-41* hypodermal cells undergo the larval-to-adult transition one stage prematurely. This precocious differentiation suggested that *lin-41* acts to prevent premature LIN-29 activity. Because *lin-41* could suppress the heterochronic defects of *let-7*, *lin-41* was thought to act downstream of *let-7* to negatively regulate *lin-29*. The relationship between *let-7* and *lin-41* was similar to the relationship of *lin-4* and *lin-14* in that *let-7* and *lin-4* mutants both undergo extra larval stages and mutants that undergo too few larval stages can suppress these defects.

lin-4 and *let-7* encode small non-coding RNAs

lin-4 and *let-7* were cloned and found to encode small non-coding RNAs termed miRNAs (MIcroRNAs)[17,22]. Strikingly, the 3' UTRs of *lin-14* and *lin-28* contained several sites partially complementary to the *lin-4* miRNA (up to seven in *lin-14* and one in *lin-28*), and the *lin-41* 3' UTR contained six sites complementary to the *let-7* miRNA[17,22]. These observations provided the first hints into the mechanism of miRNA action. The genetic data suggested that *lin-4* acted to negatively regulate *lin-14* and *lin-28*, and the sequence complementarity suggested that *lin-4* might act by directly binding the 3' UTRs of the *lin-14* and *lin-28* mRNA to inhibit their translation. The finding that the gain-of-function allele of *lin-14* contained a deletion within the 3' UTR that removed several of the *lin-4* complementary sites corroborated this idea[23-25]. This seminal work with *lin-4* and *let-7* established miRNAs as a novel means of post-transcriptional regulation and as important regulators of developmental timing and differentiation.

The miRNA revolution

It is strange to think now that the cloning of *lin-4* was not the catalyst that started the miRNA revolution. Seven years passed between the cloning of *lin-4*[22] and the cloning of *let-7*[17,26]. Prior to the cloning of *let-7*, the *lin-4* miRNA existed as an oddity in the biological world. No obvious homologs were known to exist in other organisms, and it was the only example of a small RNA's acting via the 3' UTR of a target gene to control gene expression.

The cloning of *let-7* transformed miRNAs from a *C. elegans* oddity to a newly discovered evolutionarily conserved form of gene regulation. *let-7* homologs were identified

across a wide range of metazoans, including humans. While *lin-4* and *let-7* were identified from genetic screens, researchers quickly turned to biochemical approaches to identify candidate miRNAs in different organisms[27-29]. These approaches relied on the cloning of small endogenous RNAs, and they have proven incredibly fruitful for the identification of miRNAs in a high-throughput fashion. At the same time, computational biologists began creating algorithms to predict miRNAs and possible miRNA targets based on the known interactions involving *lin-4* and *let-7*[30-32].

To date, the characterization of miRNA/miRNA target interactions has had limited success. This limitation might be in part because of redundancy within miRNA families. In *C. elegans* for example, there are 23 families of miRNAs based on sequence homology within the seed region (nucleotides 2-6 of the mature miRNA). It is likely that these families share target genes, and the deletion of a single member of the family has no obvious effect on the phenotype of the animal[33]. Surprisingly, even the removal of entire miRNA families in many cases has no or little effect[34]. It is not clear at this point if the majority of miRNA families have very specific functions that when removed do not affect the gross development of the animal, if there is redundancy between miRNA families or with other non-coding or protein-coding genes, or if most of the miRNA families are simply not involved in development.

LIN-28 and LIN-41 likely regulate miRNA function

lin-28 likely has multiple functions

C. elegans lin-28 was found to encode a highly conserved cytosolic protein with two potential RNA-binding domains: a cold-shock domain and a pair of CCHC zinc fingers[35].

Humans have two LIN-28 homologs, LIN-28A and LIN-28B[36]. Recent work has demonstrated that mammalian LIN-28A is one of a combination of four factors that can promote reprogramming of differentiated human somatic cells into induced pluripotent stem cells[37]. Furthermore, LIN-28A has been shown to inhibit the activity of the *let-7* family of miRNAs by binding to the *let-7* pre-miRNA to prevent miRNA processing[38]. Evidence from studies of *C. elegans* suggests that LIN-28 might act to shuttle the primary *let-7* transcript to P-bodies for storage or degradation[39]. Taken together, these data suggest that LIN-28 homologs act in mammals to promote pluripotency by repressing the activity of the *let-7* family of miRNAs.

Interestingly, LIN-28A has also been suggested to increase the translation of cell cycle-related mRNAs by associating with their 3' UTRs[40]. It is possible then that LIN-28 might play multiple roles in the promotion of stemness, some that involve miRNA processing and others that involve the direct control of mRNA translation.

TRIM-NHL proteins can promote or inhibit miRNA activity

lin-41 mutants were originally identified based on their ability to suppress the lethality associated with mutations in *let-7*[18]. Strong *lin-41* loss-of-function mutations cause sterility and precocious hypodermal terminal differentiation. *lin-41* was cloned and found to encode a TRIM-NHL (TRIpartite Motif- Ncl-1, H2A, Lin-41) family member[18]. Recently, the mammalian TRIM-NHL protein TRIM32 has been shown to act as a ubiquitin ligase for the argonaute protein Ago1[41]. In this respect TRIM32 acts to inhibit miRNA activity by targeting the miRNA processing argonaute protein for degradation. Interestingly, the *C. elegans* TRIM-NHL protein NHL-2 was shown to promote miRNA activity by binding to the miRISC (miRNA Induced

Silencing Complex) and promoting translational repression[42]. It appears then that some TRIM-NHL proteins act to inhibit miRNA activity, while others act to promote it. It is not yet known if LIN-41 promotes miRNA activity, inhibits miRNA activity, or functions independently of miRNAs to control the timing of LIN-29 activity.

Developmental patterns are generated by protein gradients

lin-4 and *let-7* create temporal protein gradients

While miRNAs have been implicated in the control of several developmental processes in multiple organisms, their influence is best understood in the control of developmental timing in *C. elegans*. *lin-4* and *let-7* act on their target genes to create temporal protein gradients over the course of development[18,23,24]. *lin-4* is weakly expressed during the mid-L1 stage, increases expression to its highest point in the L2 stage and continues throughout development. LIN-14 protein levels are high early in the L1 stage when *lin-4* miRNA levels are low. As *lin-4* miRNA becomes more abundant, the level of LIN-14 decreases until it is undetectable by the L2 stage. Similarly, LIN-41 protein levels are high while *let-7* miRNA levels are low, until the L4 stage when *let-7* levels are elevated and LIN-41 levels dramatically decline. The generation of a temporal protein gradient is thought to act as a switch in the transition from one developmental stage to the next. The LIN-14 and LIN-28 gradients specifiy the transition from L1 to L2 and L3 respectively, and the LIN-41 gradient specifies the transition from L4 to adult.

Spatial protein gradients drive anterior-posterior patterning in *Drosophila*

The use of protein gradients to specify temporal developmental fates in *C. elegans* is an interesting parallel to the use of protein gradients to specify spatial developmental cell fates during *Drosophila* embryogenesis. *hunchback*, *bicoid*, *nanos*, and *caudal* mRNAs are maternally loaded into the oocyte during oogenesis. *bicoid* mRNA forms a gradient from anterior to posterior, *nanos* mRNA forms a gradient from posterior to anterior, and *hunchback* and *caudal* mRNA are evenly dispersed. Upon fertilization, the mRNAs are translated and Hunchback and Caudal protein gradients are formed. Bicoid in the anterior binds to the 5' cap of *caudal* mRNA and inhibits its translation[43]. Nanos in the posterior acts with another protein, Pumilio, to inhibit the translation of *hunchback* mRNA by binding to specific sequences, Nanos Response Elements, within the *hunchback* 3' UTR[44]. Therefore, Hunchback protein levels are high at the anterior end of the embryo and decline towards the embryo midline. Conversely, Caudal protein levels are high at the posterior end of the embryo and decline towards the embryo midline. Both the Hunchback and Caudal gradients are generated by post-transcriptional regulation. The Bicoid, Nanos, Caudal, and Hunchback gradients establish the anterior-posterior axis and lay the foundation for the spatially segmented body plan of the *Drosophila* embryo.

The anterior-to-posterior gradient of Hunchback promotes the expression of downstream Gap genes, including *hunchback* and *kruppel*[45,46], the expression of which roughly subdivides the anterior half of the embryo. High levels of Hunchback are found in the anterior of the embryo,[46] and a high level of Kruppel is found in a single band around the middle of the embryo[47]. The gap genes work together in a combinatorial fashion to promote and repress the expression of the downstream pair-rule genes, ultimately generating a pattern of seven characteristic stripes that

further subdivide the embryo. These stripes represent the rudimentary segmentation of the embryo into a series of distinct spatial domains.

Temporal patterning in *C. elegans* vs. Spatial patterning in *Drosophila*

The *hunchback* homolog *hbl-1* controls developmental timing in *C. elegans*

The parallel between *C. elegans* temporal and *Drosophila* spatial developmental patterning became more apparent when the *C. elegans* heterochronic gene *lin-57* was shown to encode a homolog of *Drosophila* Hunchback[19,20]. Loss-of-function mutations in *lin-57/hbl-1* (HunchBack-Like) cause a precocious heterochronic defect in which a small number of L2 proliferative seam cell divisions are skipped and the animal transitions from larva to adult prematurely. This premature larval-to-adult transition is the result of precocious activity of the Kruppel-type zinc finger protein LIN-29. While mutations in *hunchback* cause an anterior shift of Kruppel activity in *Drosophila*[48], mutations in *hbl-1* cause a precocious shift in the activity of LIN-29[19,20].

Like *lin-41*, *hbl-1* is also a target of the *let-7* family of microRNAs[19,20,49]. *hbl-1* expression is high during embryogenesis and the L1 stage, and declines over the course of development. By the L4 and adult stages the levels of *hbl-1* are undetectable[50]. HBL-1 protein forms a temporal gradient. This temporal gradient is generated by the combined activities of *mir-48*, *mir-84*, *mir-241*, and *let-7*, collectively known as the *let-7* family of miRNAs[49]. Loss of the functions of *mir-48*, *mir-84*, and *mir-241* causes a re-iteration of the L2 seam cell developmental program and increased levels of HBL-1 late in development. The loss of *hbl-1* function from the triply-mutant miRNA strain suppresses the L2 reiteration, suggesting that increased levels of *hbl-*

1 late in development can cause the reiteration of a previous developmental stage. The relief of *hbl-1* repression by the removal of *mir-48*, *mir-84* and *mir-241* is analogous to the relief of Hunchback repression by the removal of *nanos* in *Drosophila*. *nanos* mutant embryos develop normal anterior segments (acron and head) where Hunchback levels are normally high. However, the posterior abdominal segments of *nanos* embryos are replaced with an enlarged anterior thoracic region[51]. In *C. elegans*, an earlier developmental program replaces a later program, and in *Drosophila* an anterior program replaces a posterior program. Thus, miRNAs can generate a temporal gradient of a transcription factor like *hbl-1* within a cell lineage (seam cells) to control cell-fate specification.

rnt-1 promotes seam cell divisions

During *Drosophila* embryogenesis, the spatial gradient of Hunchback protein establishes the anterior-posterior segmentation plan of the early embryo. The gap genes, *giant*, *huckebein, tailless*[52], *hunchback*, *knirps* and *Kruppel,* act together to drive the expression of the pair-rule genes in a striped pattern of seven bands perpendicular to the anterior-posterior axis. Once the seven bands have formed, the syncytial blastoderm cellularizes and the cells within an individual stripe develop in a fashion unique to each stripe. One of the pair-rule genes expressed in this pattern of seven stripes is *runt*[53].

The RUNX (Runt X) family of proteins is a class of transcription factors that share a common evolutionarily conserved Runt domain. *C. elegans* has one RUNX family member, *rnt-1*[54], while more complex organisms typically have three or four. RUNX proteins can function as transcriptional activators or repressors depending on their biological context. While the RUNT

protein in *Drosophila* is crucial for the early patterning of the embryo, studies of *C. elegans* and vertebrate systems have shown important roles for RUNX proteins in a variety of developmental contexts, including the maintenance and proliferation of stem cells.

Runx genes are thought to act primarily at the junction of cell differentiation and proliferation. In some processes, such as hematopoiesis in *Drosophila*, Runx proteins act to promote differentiation[55]; in other contexts, such as during sea urchin embryogenesis, Runx proteins act to promote proliferation[56]. Recent work has demonstrated that Runx1 promotes the transition of hair follicle stem cells from a quiescent state (telogen) into a proliferative state (anagen). In *C. elegans*, *rnt-1* is required for the proliferation of the stem-cell-like seam cells during each larval stage. In the absence of *rnt-1*, seam cells fail to divide, and when *rnt-1* is overexpressed, the seam cells undergo ectopic divisions[57,58].

In *Drosophila*, the spatial gradient of Hunchback protein in combination with the other gap genes establishes the seven-stripe expression pattern of RUNT protein during embryogenesis. In *C. elegans*, the temporal gradient of HBL-1 controls the RNT-1-mediated division of the seam cells. A comparison of the gradients used to pattern anterior-posterior patterning in *Drosophila* with the gradients used to determine temporal identity in *C. elegans* suggests that each of these processes uses a conserved set of components and that the graded expression of *lin-4* and *let-7* family miRNAs acts to convert a spatial patterning program into a temporal patterning program.

Environmental cues drive developmental progression in *C. elegans*

DAF-12 integrates signals from the environment to control development

If the activities of *lin-4* and *let-7* family miRNAs act to create a temporal gradient of HBL-1, what then controls the activity of these miRNAs? Loss-of-function mutations in the gene *daf-12* (abnormal DAuer Formation) cause a reiteration of the L2 seam cell divisions, similar to what is observed in the *mir-48*, *mir-84*, *mir-241* triple mutant[59]. *daf-12* encodes a nuclear hormone receptor that is similar to the mammalian Vitamin D and Liver X receptors[60]. DAF-12 acts downstream of the TGF-β and insulin-like signaling pathway in response to environmental input[61]. In favorable environmental conditions, such as the presence of food, *C. elegans* achieves reproductive competence as rapidly as possible. DAF-12 binds its ligand (dafachronic acid)[62] and promotes the transcription of downstream target genes that promote continuous development. In unfavorable conditions, the amount of DAF-12 ligand is greatly reduced and DAF-12 is bound instead to its corepressor DIN-1[63]. Under these conditions DAF-12 acts as a transcriptional repressor and promotes entry into the dauer stage, an alternative third larval stage specialized for long-term survival. The organism-wide coordination of this transition from a developmental state to a quiescent dauer state suggests the involvement of an endocrine system.

DAF-12 promotes the expression of the *let-7* family of miRNAs in favorable environments

Recent work has demonstrated that DAF-12 promotes the expression of the *let-7* family of miRNAs under favorable conditions and inhibits their expression in unfavorable conditions[64]. In good conditions DAF-12 binds its ligand and promotes the expression of the *let-7* miRNAs that downregulate *hbl-1*, initiating the transition from the L2 to L3 stage. Under unfavorable conditions, DAF-12 inhibits miRNA expression and promotes either the L2 program or larval arrest. In *daf-12* null mutants, the *let-7* family of miRNAs is not upregulated and therefore *hbl-1* levels remain elevated during the L3 stage, leading to the reiteration of the L2 pattern of seam cell divisions. In this way, DAF-12 acts to integrate extrinsic signals from the environment to affect the intrinsic components that regulate developmental timing.

LIN-29 is the master regulator of the larval-to-adult transition

LIN-29 encodes a Kruppel type C2H2 zinc finger transcription factor that acts at several times during development but is most notably required for animals to take on adult characteristics at the end of development[15,65]. *lin-29* mutants were originally isolated from a screen for animals that were egg laying defective[66]. While these mutants had a very obvious protruding vulva defect, they also failed to generate an adult-specific cuticle and underwent extra molts. The seam cells, which normally exit the cell cycle during the L4 stage, fail to do so in a *lin-29* mutant. Based on these observations it was concluded that *lin-29* mutants were defective in the larval-to-adult transition. Double mutant analyses with all known precocious heterochronic

mutants placed *lin-29* as the furthest downstream component of the pathway. Subsequent work has demonstrated that the timing of LIN-29 activity is regulated by many different sets of genes.

Multiple pathways control the timing of LIN-29 activity

Circadian rhythm components control LIN-29 activity in *C. elegans*

Many organisms undergo physical or behavioral changes that occur in a 24-hour cycle. These traits or behaviors are said to act with a circadian rhythm. The first observations of circadian rhythms came from studies of the plant *Mimosa pudica* (Sensitive plant) in the 1700s[67]. The leaves of *Mimosa pudica* undergo changes in their orientation in coordination with the light-dark cycle. However, it was observed that these plants successfully changed their leaf orientation even in complete darkness, indicating that while the change in orientation coincided with the light/dark cycle, the light-dark cycle was not required.

lin-42 encodes the only *C. elegans* member of the PERIOD (PER) family of circadian rhythm proteins[68]. In several organisms, including flies and humans, PER proteins control circadian rhythms. Mutations that disrupt period homologs disrupt period length or cause arrhythmia with respect to circadian behaviors. For example, *Drosophila* has two peaks of increased locomotory behavior within a single circadian cycle; one peak occurs around dawn, and the second occurs around dusk. *per* mutants in which the circadian period is shortened still display two peaks of activity, but the second peak occurs earlier in the day[69].

While *C. elegans* has several of the core components that make up the circadian molecular clock, including *lin-42* (per), *tim-1* (timeless), and *kin-20* (doubletime), circadian behaviors have been elusive[68,70]. Recent reports suggest that *C. elegans* might have an adult-

specific circadian locomotory behavior[71], but it is not clear if the canonical circadian rhythm regulators control this behavior. Interestingly, in organisms that display circadian behaviors the mRNA levels of many of the molecular clock components, including *per*, rise and fall with the circadian cycle[72,73]. In *C. elegans*, where there do not appear to be obvious circadian behaviors, the levels of *lin-42*, *tim-1* and *kin-20* rise and fall with the molting cycle. It was recently proposed that the period just prior to molting, lethargus, represents a sleep-like state in *C. elegans* and that the oscillations observed in *lin-42*, *tim-1* and *kin-20* mRNA levels might be the correlate of the oscillations of mammalian clock mRNA levels during the light-dark cycle[74]. These observations suggest that in *C. elegans* the components of the molecular clock might play a central role in driving the timers that control the molting cycle and the progression from one developmental stage to the next.

In accordance with this hypothesis, *lin-42* mutants have developmental timing defects. In *lin-42* loss-of-function animals, seam cell development appears normal from the L1 to the L3 stage, but during the L3 molt, the seam cells precociously exit the cell cycle, fuse, and generate adult cuticle[75]. *lin-42*, like *lin-41* and *hbl-1*, acts to prevent the precocious activity of LIN-29.

A Cullin-RING complex prevents precocious LIN-29 activity

Studies of *daf-12* mutants, particularly with respect to gonad migration, suggested that there were likely factors that functioned in parallel to *daf-12* to control developmental timing. A screen to identify these parallel factors yielded *dre-1* (Daf-12 Redundant)[76]. *dre-1* encodes an F box protein that functions with *skr-1* (SKp1 Related ubiquitin ligase component) and *cul-1* (CULlin) to prevent precocious LIN-29 activity in the hypoderm. Cullin-RING complexes act as

ubiquitin ligases and target specific proteins for degradation. One hypothesis is that the DRE-1 complex targets LIN-29 for degradation, thus preventing LIN-29 activity prior to the L4 stage. However, an *in vitro* interaction between LIN-29 and DRE-1 has not been observed, suggesting that DRE-1 might act through another target protein. While *dre-1* acts as a negative regulator of *lin-29* with respect to the larval-to-adult transition, *dre-1* and *lin-29* mutants have a highly penetrant synthetic gonad migration defect. LIN-29 expression had been observed in the distal tip cells, but no significant gonad migration defect had been observed previously.

LIN-29 activity is controlled directly via HBL-1 and LIN-41, the circadian rhythm homologs LIN-42, TIM-1, and KIN-20, and an E3 ubiquitin ligase complex comprising DRE-1, SKR-1 and CUL-1 (Figure 3). It is not clear if these mechanisms represent one large connected pathway or if multiple pathways converge to ensure the proper timing of LIN-29 protein accumulation. Furthermore, it is not clear if these pathways regulate *lin-29* transcriptionally or post-transcriptionally.

LIN-29 likely has multiple target genes

LIN-29 is believed to promote the larval-to-adult transition by acting on many different target genes. In some cases LIN-29 might act to activate the expression of target genes that promote adulthood, and in other cases LIN-29 might act to repress genes required for larval development. The LIN-29-dependent activation and repression of collagen genes is a good example. During larval development, the *C. elegans* hypoderm expresses *col*-17[77]. When the animal transitions to adulthood, *col-17* is repressed and *col-19* is expressed. In *lin-29* mutants, *col-19* is never expressed and *col-17* continues to be expressed in all stages after L4[77]. When wild-type seam cells permanently exit the cell cycle during the L4 stage, they express an elevated level of the cell cycle inhibitor *cki-1* (Cyclin-dependent Kinase Inhibitor). This increase in *cki-1*

expression is abolished in *lin-29* mutants as the seam cells continue to undergo extra divisions[78]. Recently, it was shown that the seam cells fail to fuse during the larval-to-adult transition in animals that are lacking the fusogen *aff-1*[79]. Therefore, it seems likely that *aff-1* is a downstream target of LIN-29 with respect to seam cell fusion.

LIN-29 is expressed in multiple cell types throughout development

Expression analysis using an antibody that recognizes the LIN-29 C-terminus revealed that LIN-29 might also have developmental roles outside of the larval-to-adult transition[75]. For example, during the L1 stage, LIN-29 was detected primarily in the pharynx. During the L3 stage, LIN-29 accumulates in the vulval precursor cells, the descendants of the sex myoblasts, and the distal tip cells. It is not until the L4 stage that LIN-29 is seen in the seam cells and hypodermis. Interestingly, a *lacZ* transcriptional reporter driven by the *lin-29* promoter showed expression in the hypoderm as early as the L2 stage. This result might be an artifact of the promoter region used, or it might suggest that LIN-29 is regulated post-transcriptionally in the hypoderm.

LIN-29 controls male linker cell death

While *lin-29* has primarily been studied in the context of the terminal differentiation of the hypodermis, it is known to function in other tissues and at other times during development. It is not surprising that LIN-29 controls other processes given its expression pattern. Interestingly, *lin-29* has been shown to function in the gonad of both males and hermaphrodites. In males and hermaphrodites, the gonad starts as a small collection of somatic and germline cells. Over the course of development, the gonad elongates, driven by the migration of specific leading cells[80,81]. In males there is one leading cell, the linker cell, and in hermaphrodites there are two leading

cells, the distal tip cells. The linker cell undergoes a characteristic pattern of migration that begins at the animal's midbody and ends at the tail near the cloaca. Late in the L4 stage the linker cell undergoes a non-canonical programmed cell death and is engulfed by a neighboring cell. The removal of the linker cell fuses the lumen of the vas deferens to the cloacal tube and creates a passage for the transfer of sperm during mating[82,83]. LIN-29 appears to be involved in the timing of linker cell death, because in *lin-29* deficient animals the linker cell inappropriately survives well into adulthood[84].

The abnormal linker cell death shows that LIN-29 action during the larval-to-adult transition is not confined to the hypoderm and that different target genes are likely regulated in different tissues. It is not known if the same mechanisms that control the timing of LIN-29 activity in the hypoderm control the timing of LIN-29 activity in the linker cell.

LIN-29 acts redundantly with DRE-1 to control distal tip cell migration

In hermaphrodites, gonad elongation is controlled by the migration of the two distal tip cells[80,81]. These cells also migrate in a characteristic pattern, each making two specific turns at distinct points in development. During the L2 and L3 stages, the two arms of the gonad migrate along the ventral side of the animal from the midbody towards the anterior and posterior ends. During mid-L3 stage, the distal tip cells change their direction and migrate in a ventral-to-dorsal direction. Finally, near the beginning of the L4 stage, the distal tip cells change direction once more and migrate along the dorsal side of the animal back towards the midbody. This process generates two U-shaped gonad arms at the end of development.

While *lin-29* animals do not have obvious gonad migration defects on their own (although a mild defect has been reported), in combination with a *daf-12* null or a *dre-1* loss-of-function mutation, *lin-29* deficient gonads have a highly penetrant gonad migration defect. In

these doubly deficient animals, distal tip cell migration can be defective at either of the two gonad turns[76]. Therefore *lin-29* likely acts at multiple times within the distal tip cell to control gonad migration.

LIN-29 homologs in other organisms

LIN-29 is a Kruppel-type C2H2-zinc finger transcription factor. In *C. elegans* there are somewhere between 150 and 200 members of this class of protein. There are over 300 C2H2-zinc finger containing proteins in both *Drosophila* and humans, in the latter case accounting for 1-2% of the human genome[85]. Members of this group are defined by the presence of a C2H2 zinc finger motif that is highly conserved across metazoa. While the core sequence within the C2H2 domain is highly conserved, the number of C2H2 domains within an individual protein can vary dramatically.

In *Drosophila*, the closest homologs to LIN-29 are Rotund and Squeeze. Rotund is required for male and female fertility and for the normal development of many adult body structures[86]. Squeeze acts primarily in the central nervous system and controls the temporal specification of specific neuroblast lineages[87].

In mammals, the closest predicted homologs of LIN-29 based on conservation within the C2H2 zinc finger domains are CIZ (Castor-Interacting Zinc finger protein) and ZNF362 (ZiNc Finger protein 362)[65]. CIZ is thought to be a nucleocytoplasmic shuttling transcription factor involved in bone growth[88], and ZNF362 has not been extensively studied. Neither CIZ nor ZNF362 has been implicated in the control of developmental timing or terminal differentiation in mammals, and it is unclear if they share LIN-29-like function. There is little to no conservation between CIZ, ZNF362, and LIN-29 outside of the zinc finger domains.

The juvenile-to-adult transition in humans

Humans undergo a metamorphosis during puberty

In humans, the transition from adolescence to adulthood is known as puberty. More specifically, puberty refers to the process by which the body transforms from its juvenile form to its sexually mature adult form. The transformation includes the maturation of the gonads, the development of adult-specific secondary sex characteristics and changes in behavior. Quite a lot is known about the signaling and processes that occur during puberty, but much less is known about the mechanisms that control when puberty is initiated.

In females, puberty typically occurs around the age of 10[89], and in males around age 12[90], although there is a fair degree of variation for both sexes. While the onset of puberty is thought to be controlled by several contributing factors, the molecular initiation of puberty is thought to occur within a specific region of the hypothalamus known as the arcuate nucleus[91]. Neurons within the arcuate nucleus secrete pulses of Gonadotropin Releasing Hormone (GnRH), which signals to specific cells, gonadotropes, within the anterior pituitary gland to secrete Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH). LH and FSH are secreted into the blood, where LH activates either the Leydig cells in the testes or theca cells in the ovaries and FSH activates either the Sertoli cells and spermatogenic tissues in the testes or the follicle cells in the ovaries. These signaling events lead to increased production of testosterone and estradiol from the testes and ovaries, respectively. The increased levels of these hormones trigger the development of the adult-specific secondary sex characteristics throughout the body.

The pulsatile secretion of GnRH controls the onset of puberty

The pulses of GnRH are controlled by a mechanism known as the GnRH pulse generator[92]. The neurosecretory neurons within the arcuate region show a high degree of autorhythmicity, the nature of which is unknown. During fetal development and very early childhood, pulses of GnRH can be detected, but these pulses become repressed throughout the remainder of childhood. It is the derepression of the GnRH pulse generator that leads to increased pulses of GnRH and the initiation of puberty.

Heterochronic syndromes in humans?

Because mammalian development occurs in a more-or-less continuous fashion, it is difficult to imagine what a mammalian heterochronic mutant might look like. It has been postulated that mammalian heterochronic mutants might have defects in the timing of the onset of puberty[93]. Individuals with syndromes associated with deficits in hypothalamic function, like Kallmann Syndrome[94], fail to release GnRH and consequently fail to initiate puberty. In mice, mutations that affect pituitary function can also affect the onset of puberty by blocking the production of luteinizing hormone[95]. Interestingly, two whole-genome association studies looking at precocious puberty in humans identified LIN-28B, the homolog of *C. elegans* LIN-28, as a regulator of puberty onset[96]. In *C. elegans*, LIN-28 controls the timing of the larval-to-adult transition, and the loss of *lin-28* function causes the precocious onset of adulthood. This parallel suggests that despite the differences between the life cycles of *C. elegans* and humans, there may exist a shared ancestral mechanism that controls the timing of development.

Conclusion

Much is known about the mechanisms that control developmental timing in *C. elegans*. Many of the components of the *C. elegans* heterochronic pathway, which have been extensively studied in the context of the stem cell-like seam cell lineage, have mammalian homologs that have been implicated in the control of stem cell identity and stem cell proliferation. The seam cell lineage has proven to be an excellent system with which to study the progression of a stem cell from generation to terminal differentiation.

My work has focused primarily on the events that occur during the larval-to-adult transition. In this thesis I explore the function of the previously identified heterochronic mutant *mab-10* (Male ABnormal). *mab-10* mutants were originally isolated by Jonathan Hodgkin in the late 1970s in a screen for males that looked abnormal or that were unable to mate[97]. Chris Link later noticed that *mab-10* males undergo an extra molt. I originally pursued the project for two reasons: 1) extra molts are often associated with mutations in miRNAs, and 2) I was intrigued by the possibility that the mechanism controlling developmental timing in *C. elegans* might be sexually dimorphic. In Chapter 2, I demonstrate that *mab-10* is neither a miRNA nor a sexually dimorphic regulator of developmental timing. Thankfully, *mab-10* turned out to be interesting anyway. In Chapter 3, I expand on the activities of MAB-10 and LIN-29 showing that they are dynamically expressed and have activities outside of the hypodermis. In Chapter 4, I briefly discuss future projects based on my work that should further our understanding of developmental timing and terminal differentiation. I have also included two appendices describing screens I performed related to the control of programmed cell death.

Figure Legends

Figure 1

***C. elegans* is a soil nematode with a well-characterized life cycle. a,** Adult *C. elegans* are approximately 1 mm long and exist as either self-fertilizing hermaphrodites or males. **b,** The *C. elegans* life cycle consists of six stages beginning with the embryo, proceeding through four larval stages (L1-L4) and ending as an adult. Each of the larval stages is demarcated by a molting cycle in which the animal makes a new cuticle and sheds its old cuticle. (Images adapted from www.wormatlas.org)

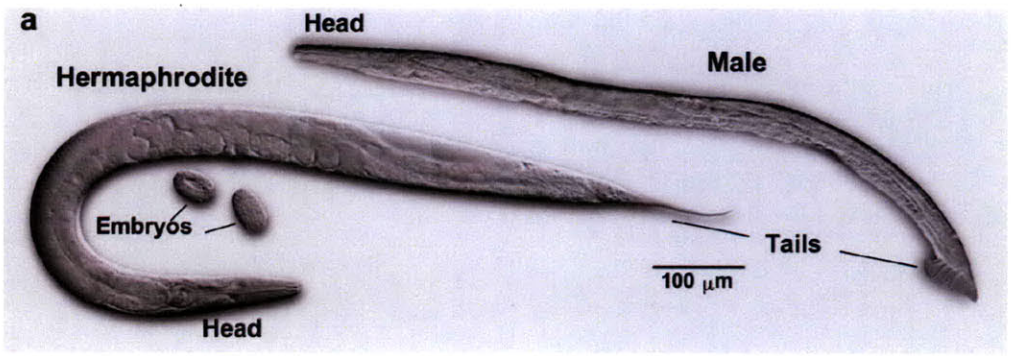
Figure 2

The seam cell lineage is an excellent indicator of developmental stage and heterochrony. a, Wild-type seam cells divide in a stem cell-like pattern during each of the larval stages[11]. The anterior daughter of the seam cell fuses with the surrounding hypodermis. The vertical axis represents absolute time and the horizontal lines on the axis represent molts. The colored boxes represent the developmental program that is being executed at a specific stage. **b,** In precocious heterochronic mutants, such as *lin-28* and *lin-41,* the seam cells skip certain developmental programs[15,18]. **c,** In retarded heterochronic mutants, such as *lin-4* and *lin-29*, the seam cells reiterate certain developmental programs[15].

Figure 3

Multiple mechanisms converge at LIN-29 to control the timing of the larval-to-adult transition. This diagram is a simplified schematic of the mechanisms that control LIN-29. The removal of any of these mechanisms leads to precocious LIN-29 activity and the premature onset of adulthood.

Figure 1


a
Head
Male
Hermaphrodite
Embryos
Tails
100 μm
Head

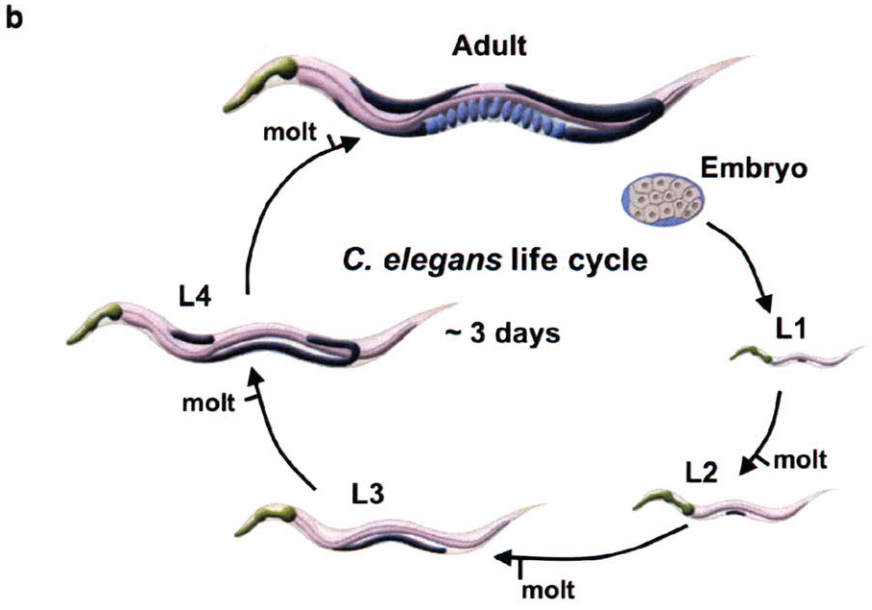

b
Adult
molt
Embryo
C. elegans life cycle
L4
~ 3 days
L1
molt
molt
L2
L3
molt

Figure 2

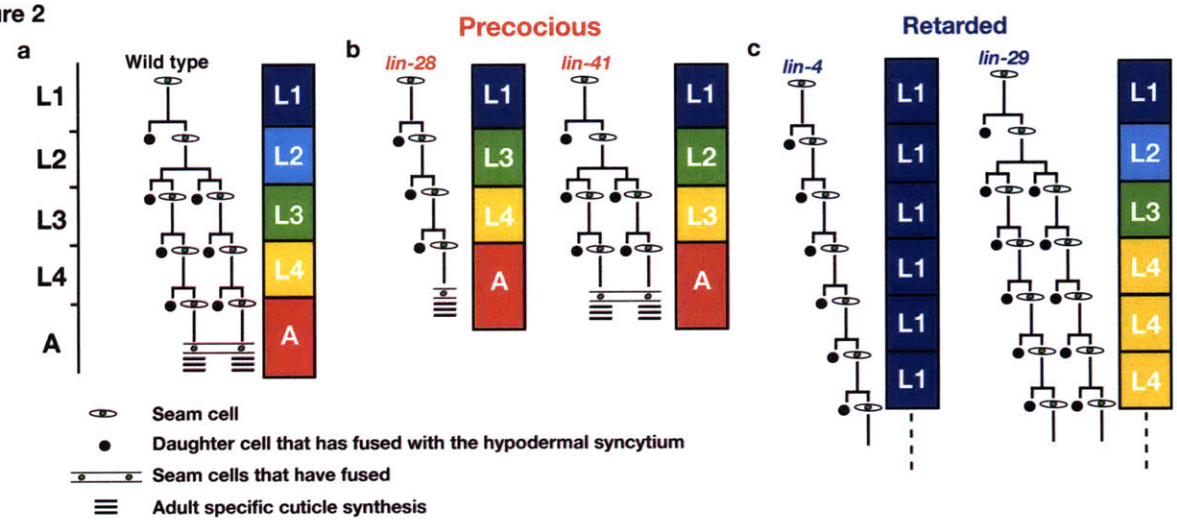
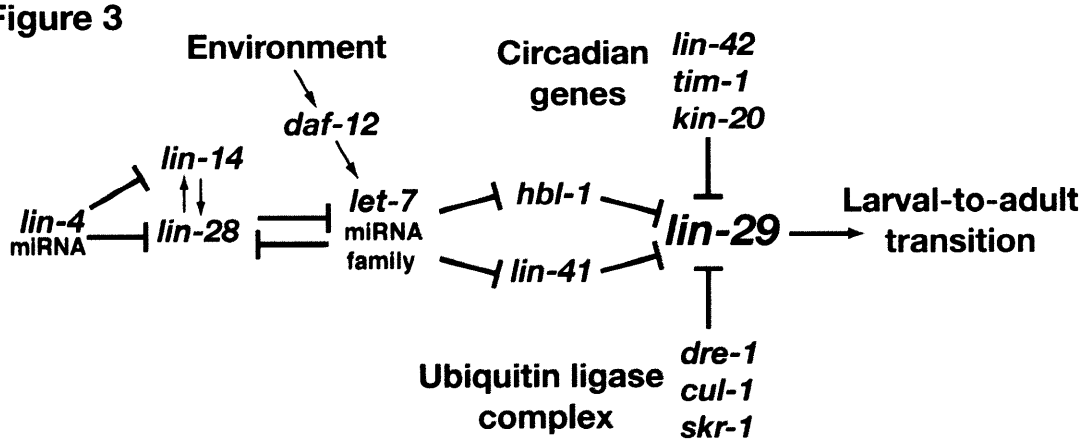

a
Wild type
L1
L2
L3
L4
A
b
Precocious
lin-28
lin-41
c
Retarded
lin-4
lin-29
Seam cell
Daughter cell that has fused with the hypodermal syncytium
Seam cells that have fused
Adult specific cuticle synthesis

Figure 3


Environment
daf-12
Circadian genes
lin-42
tim-1
kin-20
lin-14
lin-4
miRNA
lin-28
let-7
miRNA
family
hbl-1
lin-41
lin-29
Larval-to-adult transition
Ubiquitin ligase complex
dre-1
cul-1
skr-1

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Chapter Two

The *C. elegans* heterochronic pathway controls NAB/EGR-mediated terminal differentiation

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Summary

To identify genes involved in the control of the larval-to-adult transition in *C. elegans*, we performed a genetic screen to isolate mutations that cause adult males to undergo an extra molt. We isolated two new alleles of *mab-10*, a gene that was previously isolated based on abnormal tail morphology and an inability to mate. We show that *mab-10* encodes the *C. elegans* member of the NAB family of transcriptional cofactors. We demonstrate that *mab-10* mutants are defective in specific aspects of the larval-to-adult transition and that MAB-10 acts with LIN-29 to promote terminal differentiation. Our analysis reveals evolutionary conservation between LIN-29 and mammalian EGR proteins and suggests that the mechanism controlling the onset of adulthood is conserved between *C. elegans* and mammals.

Introduction

The mechanisms governing the progression of a stem cell from an undifferentiated state to a differentiated state are basic to stem cell biology and to metazoan development in general. In the nematode *Caenorhabditis elegans*, the differentiation of the seam cells, a population of hypodermal stem cells (Figure 1a), is regulated by heterochronic genes that control the timing of specific developmental events (Figure 1b). The conserved *lin-4*[1] microRNA (miRNA) and the conserved *let-7*[2,3] family of miRNAs act on their target genes *lin-28*[4], *hbl-1*[5,6] and *lin-41*[7] to regulate the activity of the Kruppel-family zinc finger transcription factor LIN-29[8], the central regulator of hypodermal cell terminal differentiation. Recent work has shown that homologs of core components of the *C. elegans* heterochronic pathway act in mammals to control stem cell identity[9,10] and cell differentiation[11]. Here we identify a new heterochronic gene, *mab-10*, and show that *mab-10* encodes a NAB (NGFI-A Binding protein) transcriptional cofactor that acts with LIN-29 to regulate a subset of hypodermal cell differentiation events during the transition from larva to adult. MAB-10 and LIN-29 are co-expressed temporally and spatially, MAB-10 interacts with LIN-29 *in vitro* via a conserved domain in the LIN-29 C-terminus, and the LIN-29 C-terminus is required *in vivo* for complete terminal differentiation. The NAB-interaction domain of LIN-29 is conserved in the Kruppel-family early growth response proteins EGR-1/NGFI-A, EGR-2/Krox20, and EGR-3, suggesting that LIN-29 and EGR proteins share a common NAB-interacting ancestor. In mammals, EGR proteins control stem cell proliferation[12] and, like LIN-29 in *C. elegans*, interact with NAB proteins, control terminal differentiation and promote the onset of adulthood[13-15]. Our work suggests that homologs of the *C. elegans* heterochronic genes act in mammals in a conserved pathway to control NAB/EGR-mediated differentiation in multiple cell lineages, many of which are important in human disease.

Results

The *C. elegans* seam cells are hypodermal cells that undergo a stem-cell-like pattern of asymmetric cell division during each larval stage to generate an anterior daughter cell that fuses with the surrounding syncytial hypoderm and a posterior daughter cell that retains the seam cell identity and divides again during the next larval stage[16] (Figure 1a). Prior to the asymmetric division during the second (L2) larval stage, the seam cells undergo a symmetric proliferative division that doubles the total number of seam cells. At the end of each larval stage the hypoderm generates a new cuticle, and the animal molts. After the final (L4) larval stage, the hypoderm undergoes a process of terminal differentiation comprising four events: i) synthesis of the adult-specific cuticle, ii) exit from the molting cycle, iii) seam cell fusion, and iv) seam cell exit from the cell cycle. Terminal differentiation is initiated via the downregulation of the Hunchback/Ikaros homolog *hbl-1* and the TRIM-NHL gene *lin-41* by the *let-7* family of miRNAs. This downregulation triggers the activity of the Kruppel family zinc finger protein LIN-29, which promotes all four aspects of terminal differentiation (Figure 1b)[2,5,6].

A screen to identify males that undergo an extra molt

To identify new genes required for the terminal differentiation of the hypoderm, we performed a screen of ~4500 genomes for mutants that failed to exit the molting cycle and inappropriately initiated an adult molt. Because extra molts are difficult to observe in adult hermaphrodites, we focused on the identification of mutant males. We identified two isolates, *n5117* and *n5118*, both of which failed to complement the previously isolated mutant *mab-10(e1248)*. *mab-10(e1248)* was originally identified in a screen for morphologically

abnormal males (male abnormal)[17], and *mab-10* males were later shown to undergo an extra molt (C. Link, personal communication).

mab-10 mutants make a normal adult cuticle, but undergo an extra molt

We observed that both *mab-10* males and hermaphrodites underwent an extra molt (Figure 1c-f). *mab-10* mutants synthesize apparently normal larval cuticles and synthesize an adult cuticle appropriately at the end of the fourth larval stage (data not shown); however, *mab-10* mutants fail to exit the molting cycle and generate an extra cuticle with adult-specific features known as alae about 18 hours after the larval-to-adult transition. Adult *mab-10* hermaphrodites that had begun to synthesize the second adult cuticle were typically consumed by internally hatched progeny prior to completion of the extra molt. In short, *mab-10* is not required for adult-specific cuticle synthesis but is required for both males and hermaphrodites to exit the molting cycle.

Seam cells of *mab-10* mutants fuse normally, but fail to exit the cell cycle

To determine if the seam cells of *mab-10* mutants fuse appropriately during the larval-to-adult transition, we used the adherens junctions marker AJM-1::GFP *(wIs78)*[18] to label seam cell membranes. In *mab-10* mutants the seam cells fused at the appropriate time and were indistinguishable from those of the wild type (Figure 1g-j). We conclude that *mab-10* is not required for seam cell fusion.

To detect alterations of the seam cell lineage caused by mutations in *mab-10*, we observed the seam cell divisions on one side each of approximately 20 *mab-10* animals during each of the four larval stages; we saw no abnormalities (data not shown). We then observed adult

hermaphrodites using the seam cell reporter *scm::gfp* (*wIs78*)[18] and found that seam cell nuclei within the newly formed seam syncytium inappropriately divided about 15 hours after the first adult cuticle was generated. In *mab-10(n5117)* mutants approximately five of the 11 V lineage-derived seam cells underwent an extra division by 24 hours after the larval-to-adult transition (Figure 1k and l) (Supplementary Table 1). This observation indicates that *mab-10* is required to promote seam cell exit from the cell cycle.

Thus, *mab-10*, like *lin-29*, has a retarded heterochronic defect and is required for exit from the molting cycle and seam cell exit from the cell cycle; however, *mab-10*, unlike *lin-29*, is not required for adult-specific cuticle synthesis or seam cell fusion (Figure 1m and n).

mab-10 acts downstream of or parallel to *lin-28* and *lin-41*

To determine the position of *mab-10* in the heterochronic pathway, we performed epistasis tests with the precocious mutants *lin-28* and *lin-41*. Seam cells of *lin-28* and *lin-41* animals exit the cell cycle prematurely at the end of the L3 stage instead of the L4 stage in a *lin-29*-dependent fashion. Seam cells of *lin-28; mab-10* and *lin-41; mab-10* double mutants continued to divide after the larval-to-adult transition, indicating that *mab-10,* like *lin-29*, is required for the early terminal differentiation of seam cells in *lin-28* and *lin-41* mutants (Supplementary Table 1). *mab-10* therefore likely functions downstream of or parallel to both *lin-28* and *lin-41*.

mab-10 encodes the only *C. elegans* NAB transcriptional cofactor

Using a combination of SNP mapping, genomic rescue, and DNA sequence determination (Supplementary Figure 1), we determined that *mab-10* is the gene *R166.1* (Figure 2a) and encodes a NAB transcriptional cofactor (Figure 2b). The NCD1 (Nab Conserved Domain)

domain is thought to be required for physical interactions with the R1 (Repressor 1) domains of the mammalian immediate early genes EGR-1 -2 and -3[19], and the NCD2 domain has been shown to function both as a transcriptional activator[20] and as a repressor[21]. *mab-10(n5117)* and *mab-10(n5118)* are both early nonsense mutations located within the NCD1 domain, and *mab-10(e1248)* causes a serine-to-phenylalanine amino acid substitution within the NCD2 domain (Figure 2c).

Mammalian NAB proteins are thought to act as cofactors for EGR proteins to regulate the terminal differentiation and function of several cell types: keratinocytes, chondrocytes, Schwann cells, macrophages and gonadotropes[13-15,20]. While R166.1 can bind mammalian EGR-1 in a yeast-two-hybrid assay[22], an EGR-like NAB-interacting protein has not yet been identified in *C. elegans*.

Based on the observations that *mab-10* is required for a subset of the *lin-29*-dependent terminal differentiation events and likely acts downstream of *lin-41* and that *lin-29* encodes a Kruppel family zinc finger protein that shares homology with mammalian EGR proteins, we hypothesized that MAB-10 acts as a cofactor for LIN-29 to promote specific aspects of terminal differentiation during the larval-to-adult transition.

mab-10 and *lin-29* are co-expressed in multiple tissues during development

To determine if MAB-10 and LIN-29 are co-expressed, we generated rescuing MAB-10::GFP and LIN-29::mCHERRY fusion proteins. Expression of the LIN-29::mCHERRY fusion protein largely resembled the previously published pattern of LIN-29 expression[23]. Both MAB-10 and LIN-29 localized to specific nuclei in the pharynx throughout development (Figure

3a-c), and both were expressed in vulval precursor cells during the third larval stage (Figure 3d-f). Both were present in the seam cells throughout the L4 stage and later in the hypodermal nuclei derived from the seam cell lineage (Figure 3g-i). These observations are consistent with our hypothesis that MAB-10 is a nuclear factor that acts with LIN-29 in hypodermal cells during the late L3 and/or L4 stage to promote terminal differentiation.

MAB-10 interacts with an evolutionarily conserved domain within LIN-29

To test for a direct physical interaction between LIN-29 and MAB-10 we performed a GST pull-down assay. We found that *in vitro*-translated LIN-29 interacted with MAB-10::GST but did not interact significantly with GST alone (Figure 3j). A similar *in vitro* interaction between *Drosophila* NAB and the two *Drosophila* LIN-29 homologs RN and SQZ was reported recently[24]. We were unable to identify an obvious R1 domain within LIN-29, RN, or SQZ using protein alignment algorithms. To determine the region of LIN-29 required for the MAB-10/LIN-29 interaction, we tested a series of large LIN-29 deletions (Supplementary Figure 2) for interactions with MAB-10. We found that amino acids C-terminal to the zinc finger domains were required for efficient MAB-10 binding. We showed that deletion of a 17 amino acid sequence (390-406) (Figure 3k) within the LIN-29 C-terminus decreased MAB-10 binding by about 80% as compared to the binding by wild-type LIN-29 (Figure 3l lane 3). To determine if this LIN-29 region is evolutionarily conserved we compared *C. elegans* LIN-29 to the closest homologs of the distantly related nematodes *Brugia malayi* and *Pristionchus pacificus*. Alignment of the LIN-29 homologs revealed significant identity within only the zinc finger domains and this MAB-10 interacting domain (Supplementary Figure 3). Strikingly, alignment of this 17 amino acid sequence with the R1 domains of mammalian EGR-1, -2 and -3 revealed a conserved motif (Figure 3m). Furthermore and unexpectedly, we identified the same conserved

region within the domains of *Drosophila* RN and SQZ shown previously to bind to *Drosophila* NAB, but not previously known to be similar to EGR proteins. Mutation of the completely conserved isoleucine (268) to asparagine in EGR2 causes a recessive form of the hypomyelinating neuropathy Charcot-Marie-Tooth Disease in humans[25] and eliminates NAB/EGR binding *in vitro*[26]. Based on these observations we conclude that LIN-29, RN, SQZ and the mammalian EGR proteins all share a common NAB-interacting domain and likely descend from a common NAB-interacting ancestor.

The LIN-29 C-terminus is required for terminal differentiation

To determine if the LIN-29 C-terminus is required for terminal differentiation *in vivo*, we used *lin-29(n546)*, an allele that carries a nonsense mutation immediately following the last zinc finger domain (Figure 4a). In an otherwise wild-type background, *lin-29(n546)* animals are completely defective in all four aspects of hypodermal differentiation. However, some *lin-29* activity can be restored to *lin-29(n546)* mutants by disrupting nonsense-mediated mRNA decay using a mutation in a *smg* (suppressor with morphological effect on genitalia) gene[27]. The lack of nonsense-mediated decay presumably allows for the translation of a LIN-29 product that is missing the C-terminus. We characterized the extent of terminal differentiation in *smg-1(e1228); lin-29(n546)* double mutant animals and found them to be normal with respect to seam cell fusion, adult cuticle synthesis and exit from the cell cycle. However, about 93% of *smg-1(e1228); lin-29(n546)* males failed to exit the molting cycle and generated an extra adult cuticle (Figure 4b and c) (Supplementary Table 1). This defect strongly resembles the *mab-10* mutant phenotype. We therefore conclude that MAB-10 likely binds directly to a conserved R1 domain in the LIN-29 C-terminus to regulate at least exit from the molting cycle and possibly also seam cell exit from the cell cycle (Figure 4d).

Discussion

By showing that MAB-10 acts with LIN-29 through an evolutionarily conserved R1 domain, we implicate LIN-29 and the *Drosophila* LIN-29 homologs RN and SQZ as EGR-like molecules. We propose that NAB proteins and EGR proteins act together in temporal developmental programs to control terminal differentiation. In *Drosophila*, the LIN-29 homolog SQZ acts with *Drosophila* NAB to control neuroblast dfferentiation[28]. In *C. elegans*, we show that LIN-29 and MAB-10 act together to control the differentiation of a hypodermal stem cell lineage during the transition from larva to adult. Homologs of the *C. elegans* heterochronic pathway act in mammals to control stem cell identity, and our work suggests that NAB/EGR proteins could mediate this control. Indeed, EGR-1 has recently been shown to be required to prevent the overproliferation of at least one stem cell population[12]. We note that mice lacking EGR-1, like *lin-29* mutants of *C. elegans*, fail to enter adulthood. EGR-1 is required within the gonadotrope lineage of the pituitary gland for the production of Luteinizing Hormone and the onset of puberty[15]. Furthermore, the *C. elegans* homologs of other factors known to control the differentiation of gonadotropes, GATA-2 (ELT-1/3/5/6 in *C. elegans*) and SF1 (NHR-25 in *C. elegans*), are required for specifying seam cell identity and hypodermal differentiation[18,29,30]. These observations suggest an evolutionary conservation of ectodermal cell-fate determinants and a mechanistic link between the control of developmental timing in *C. elegans* and the onset of puberty in mammals.

Materials and Methods

Strains and genetics

C. elegans was grown as described[31] and maintained at 20° C unless otherwise noted. N2 was the wild-type strain. The mutations used in this study were: LGI: *smg-1(e1228)*[27], *lin-28(n719)*[32], *lin-41(n2914)*[7]; LGII: *dpy-10(e128)*[31], *mab-10(e1248)*[17], *mab-10(n5117)*, *mab-10(n5118)*, *mab-10(tm2497)*, *lin-29(n546)*[32], *lin-29(n836)*[33], *rol-1(e91)*[31]; LGIV: *him-8(e1489)*[34]; LGV: *him-5(e1467)*[34].

Information about *tm2497* (kindly provided by S. Mitani, Tokyo Women's Medical University, Japan) can be found at www.wormbase.org. The following balancer chromosomes were used: *hT2 [qIs48]* LGI; LGIII, *mIn1 [mIs14]* LGII and *mnC1* LGII.

Screen for males with an extra cuticle

The F2 progeny of mutagenized *him-8(e1489)* hermaphrodites were screened clonally using a dissecting microscope for adult males with an extra cuticle. We screened about 4500 genomes and isolated two mutants *(n5117)* and *(n5118)*, both of which failed to complement *mab-10(e1248)*.

Phenotypic analyses

Cuticles were observed using a Zeiss Axioskop 2 with Nomarski optics. Seam cell fusion was assayed in early L4 and young adult animals using the *ajm-1::gfp (wIs78)*[18] reporter. Adult molting was assayed by picking L4 males to plates and scoring 24 hours later for the presence of an extra cuticle. Synchronized wild-type and *mab-10(e1248)* males and hermaphrodites were grown until the *mab-10* mutants had synthesized an extra cuticle. These animals were then fixed

and electron microscopic analysis was performed as described previously. Seam cell divisions were followed using either Nomarski microscopy or fluorescence microscopy to observe *scm::gfp (wIs78)*[18] or *col-19::gfp (maIs105)*, as noted. To assay adult seam cells, we picked late L4 animals to fresh plates and scored 24 hours later.

Plasmids

pDH04 contains about 9 kb of the *mab-10* genomic locus on LG II from 10525019 to 10534380 with *gfp* inserted in place of the stop codon. pDH36 contains about 18 kb of the *lin-29* genomic locus on LG II from 11917276 to 11936077 with *mCherry* inserted in place of the stop codon. *mab-10* cDNA was isolated by RT-PCR from a mixed-stage RNA preparation and cloned into the PCR8GW Gateway entry vector creating pDH08. pDH23 contains the *mab-10* cDNA fused downstream of *gst* and was created by the Gateway LR reaction between pDH08 and pDestGST2TK. Full-length *lin-29* cDNA was amplified from the plasmid AAC37255 (Open Biosystems) and cloned into the PCR8GW entry vector, creating pDH06. pDH24 contains the *lin-29* cDNA fused downstream of 6XHis tag and was created by the Gateway LR reaction between pDH06 and pDest17. All plasmids containing *lin-29* deletion variants were generated by site-directed mutagenesis of pDH24.

Oligos

pDH06 5'-atggatcaaactgttctagattcggc-3',
5'-ttaataggaatgatttttcatattat-3'
pDH08 5'-atgtcatcatcgtcgtcgtcgtcgtta-3',
5'-tcaagattccgggagctcacccttcatttttcgattatcgccgcccat-3'
pDH25 (LIN-29 Del 1-144)
5'-aaagcaggctccgaattcgcccttatgcagatgcgggaagcaaaaccttacaagt-3',
5'-acttgtaaggttttgcttcccgcatctgcataagggcgaattcggagcctgcttt-3'
pDH26 (LIN-29 Del 147-300)
5'-caaaattgctggccttcgatcgatccgctgcttcccgcatctgcatctgttgctg-3',
5'-cagcaacagatgcagatgcgggaagcacaaactgataagccattcaaatgtaac-3'

pDH27 (LIN-29 Del 301-459)
5'-catgacaaagcacgcggatcgatcgtaaaagggcgaattcgacccagctttc-3',
5'- gaaagctgggtcgaattcgccctttttacgatcgatccgcgtgctttgtcatg-3'
pDH29 (LIN-29 Del 371-389)
5'-cagtggggttatcatgttgaacccaggaatattaaagagctgactgaacgcact-3',
5'-agtgcgttcagtcagctctttaatattcctgggttcaacatgataaccccactg-3'
pDH30 (LIN-29 Del 390-406)
5'-acgagtacgaagaatggagaacgagcttcatcttcctcggccacggctgtcgta-3',
5'-tacgacagccgtggccgaggaagatgaagctcgttctccattcttcgtactcgt-3'
pDH31 (LIN-29 DEL 404-440)
5'-ctggagaacatccaacgctacaacgggcagggaggcgtgttcaacccacaatca-3',
5'-tgattgtgggttgaacacgcctccctgcccgttgtagcgttggatgttctccag-3'
pDH32 (LIN-29 441-459)
5'-tcctcgtcagcaggttcgtcctcaagttacccagctttcttgtacaaagtggtt-3',
5'-aaccactttgtacaagaaagctgggtaacttgaggacgaacctgctgacgagga-3'
pDH37 (LIN-29 Del 147-232)
5'-cagcaacagatgcagatgcgggaagcacaaactgataagccattcaaatgtaac-3',
5'-gttacatttgaatggcttatcagtttgtgcttcccgcatctgcatctgttgctg-3'
pDH38 (LIN-29 Del 233-300)
5'-aatctccaatctcactctcgatgccatgcggatcgatcgaaggccagcaat-3',
5'-attgctggccttcgatcgatccgcatggcatcgagagtgagattggagatt-3'

Transgenics

The integrated arrays *wIs78*[18], which contains *scm::gfp* and *ajm-1::gfp,* and *maIs105*[35], which contains *col-19::gfp*, were used to assay seam cell fusion and seam cell number. The *mab-10::gfp* array (*nEx1655*) was formed by the injection of pDH04 at 35 ng/ul and pSN359 (*pgp-12::gfp*[36] kindly provided by S. Nakano) at 40 ng/ul. The *lin-29::mCherry* array (*nEx1681*) was formed by the injection of PCR product (~50 ng/ul) containing LG II sequence from 11917298 to 11927996 from template pDH36 with *ttx-3::gfp*[37] at 40 ng/ul. *nEx1681* was integrated into the genome using gamma irradiation to form *nIs408.*

GST pull-down Assay

GST pull-downs were performed as previously described[38] and quantified using a Typhoon phosphorimager (GE Healthcare). Binding efficiency is expressed as the percent of wild-type binding after correcting for binding to GST-alone.

Figure Legends

Figure 1

***mab-10* promotes hypodermal terminal differentiation. a**, Diagram of a wild-type seam cell lineage (V1-V4,V6)[16]. Terminal differentiation is indicated by the fusion of the seam cells (eye-shaped cells with green nucleus) and the formation of adult-specific lateral alae (three horizontal bars). To the right is a diagram of the H-, V- (green) and T-derived seam cells of L1, L4 and adult animals. Anterior daughters of a division (black) fuse with the hypodermal syncytium, while posterior daughters (green) retain stem cell characteristics. The anterior progeny from only the V1 lineage are shown (dorsal and ventral to the newly V1-derived seam cells). **b**, Diagram of the heterochronic gene pathway that controls the four aspects of terminal hypodermal differentiation. Mammalian homologs are listed below the *C. elegans* genes. **c-f**, Electron micrographs of cross-sections of wild-type and *mab-10(e1248)* 18-hr adult males and hermaphrodites. Arrows indicate adult-specific lateral alae. Scale bar, 2 μm **g-j**, Wild-type and *mab-10(n5117)* seam cells visualized using the adherens junction marker AJM-1::GFP (*wIs78*)[18]. **k** and **l**, Seam cell nuclei of 24-hr adult wild-type and *mab-10(n5117)* hermaphrodites visualized using the seam cell reporter SCM::GFP (*wIs78*)[18]. Scale bar, 10 μm. Anterior is left and dorsal is up. **m**, Seam cell lineage diagrams of the wild type, *mab-10* and *lin-29* animals. **n**, Comparison of the *mab-10* and *lin-29* mutant phenotypes. A (+) symbol indicates a wild-type phenotype, and a (-) symbol indicates a mutant phenotype.

Figure 2

***mab-10* encodes the only *C. elegans* member of the NAB family of transcriptional co-factors. a**, Diagram of the *mab-10* (*R166.1*) open reading frame showing the locations of introns and mutations. **b**, MAB-10 contains the conserved NCD1 and NCD2 domains characteristic of NAB proteins. Shown are NAB1 and NAB2 from *Mus musculus*. **c**, Within the NCD1 domain, MAB-10 shows 54% identity with NAB1 and 56% identity with NAB2. Within the NCD2 domain, MAB-10 shows 58% identity with NAB1 and 45% identity with NAB2. Black boxes represent regions of 100% similarity and grey boxes represent regions of 66% similarity.

Figure 3

***mab-10* and *lin-29* are co-expressed *in vivo* and physically interact *in vitro*. a-i**, MAB-10::GFP and LIN-29::mCherry proteins localize (**a-c**) to pharyngeal nuclei throughout development (L1 shown), (**d-f**) to the nuclei of vulval precursor cells (L4 shown), and (**g-i**) to seam cell nuclei (arrows) and to the majority of the seam-cell-derived hypodermal nuclei during the L4 stage. Scale bar 10 μm. **j**, GST pull-downs using *in vitro* translated, S^{35}-labeled LIN-29, MAB-10::GST, and GST alone. An asterisk indicates full-length LIN-29 product. **k**, Diagram of LIN-29 protein and deletion variants. **l**, GST pulldowns performed with LIN-29 deletion variants and MAB-10::GST. **m**, Comparison of the MAB-10 interaction domain of LIN-29 with a region of amino acids within the R1 domains of EGR1, EGR2 and EGR3 (*H. sapiens*) and the C-terminus of RN and SQZ (*Drosophila*)[24]. Black boxes represent regions of 100 percent similarity, and gray boxes represent regions of 66 percent similarity. Isoleucine 268 (asterisk) of EGR2 is mutated to asparagine in the recessive form of the familial hypomyelinating neuropathy Charcot-Marie-Tooth disease[25].

Figure 4

Animals lacking the LIN-29 C-terminus generate a normal adult cuticle but undergo an extra molt. a, The generation of C-terminally truncated LIN-29. *lin-29(n546)* is a C-to-T transition mutation that introduces a premature opal stop codon at amino acid 294, immediately downstream of the zinc finger coding region. *lin-29(n546)* mRNA is degraded by nonsense-mediated mRNA decay. Prevention of nonsense-mediated mRNA decay by inactivation of the kinase SMG-1[27] should result in truncated LIN-29 protein. **b and c**, Electron micrographs of cross-sections of adult male *lin-29(n546)* and *smg-1(e1228); lin-29(n546)* animals undergoing an extra molt. Arrows indicate individual cuticles, while arrowheads indicate adult-specific lateral alae. Scale bar, 2 μm **d**, Model of the pathway for the regulation of hypodermal terminal differentiation in *C. elegans* showing that *mab-10* acts with *lin-29* to promote exit of the animal from the molting cycle and seam cell exit from the cell cycle.

Supplementary Table 1

***mab-10* is required for animals to exit the molting cycle and for seam cell exit from the cell cycle**. Animals were scored for the presence of complete adult alae and indicated as a percentage of sides scored. Adult males were assayed for extra molts and are indicated as a percentage of animals assayed. Animals were scored for complete seam cell fusion and are indicated as a percentage of sides scored. The V-lineage-derived seam cells of hermaphrodites were scored 24 hrs after the initiation of the L4 lethargus and are indicated as total number ± the standard

deviation. *a* Strains contained *wIs78*[18] and *him-5(e1467ts). b* Strains contained *him-8(e1489)* and *maIs105*[35]. *c* P-value <0.005 by Student's T-test.

Supplementary Figure 1

Cloning of *mab-10.* a. Based on preliminary mapping results by C. Link (personal communication), we tested deficiencies on LGII for their ability to complement *mab-10(e1248).* Males carrying deficiencies balanced over *mIn1 [mIs14]* LGII were crossed with homozygous *mab-10(e1248)* hermaphrodites, and male non-GFP-positive non-balancer cross progeny were scored for an extra molt. Red solid lines represent regions genetically determined to be missing in each deficiency. Black dotted horizontal lines represent regions that might or might not be missing. Blue dotted vertical lines delineate the region determined to contain *mab-10.* **b.** We performed SNP mapping using the polymorphic Hawaiian strain CB4856 and a Bristol strain carrying a triply marked *dpy-10(e128) mab-10(e1248) rol-1(e91)* chromosome. Rol non-Dpy progeny were picked, and recombinant chromosomes were made homozygous. Strains were assayed for the Egl/vulval bursting defect found in *mab-10* adult hermaphrodites at 25 degrees. x/y represents the number of *mab-10* mutant strains / the total strains with a recombination breakpoint within that interval. SNP mapping placed *mab-10* within an interval defined by the SNPs uCE2-1912 and pkP2129. **c.** The interval between uCE2-1912 and pkP2129 contains eight open reading frames. A PCR product carrying the indicated genomic fragment rescued the *mab-10* mutant phenotype, and point mutations were identified in the open reading frame of *R166.1* in *mab-10(e1248)*, *mab-10(n5117)*, and *mab-10(n5118).*

Supplementary Figure 2

Removal of the LIN-29 C-terminus disrupts MAB-10 binding. a. Diagram of LIN-29 protein and deletion variants. **b.** GST pull-downs with LIN-29 deletion variants and MAB-10::GST. *In vitro* translated, S^{35}-labeled LIN-29 variants were incubated with recombinant MAB-10::GST protein bound to glutathione sepharose beads and separated by SDS-PAGE. Removal of the LIN-29 C-terminus (Lanes 3 and 9) dramatically reduced MAB-10 binding.

Supplementary Figure 3

The MAB-10 interaction domain is conserved among distantly related nematodes. An alignment of *C. elegans* LIN-29 to *Brugia malayi* RoE and the predicted protein encoded by the *Pristionchus pacificus* predicted gene SNAP300000076224_CE28257. Black boxes indicate amino acids similar in all three proteins. Grey boxes indicate similar amino acids or identical amino acids shared by only two proteins. The blue bar indicates the C2H2 zinc finger domain, and the red bar indicates the MAB-10 interaction domain (R1).

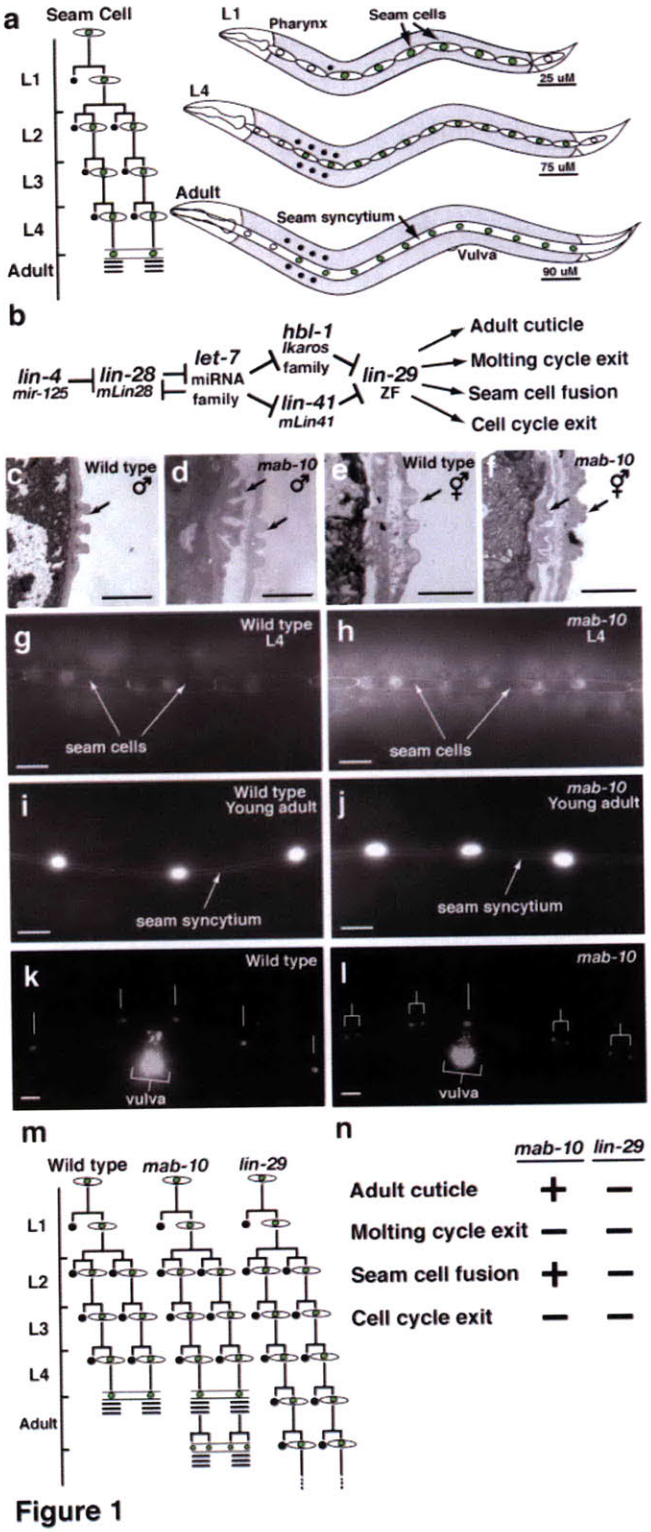
a
Seam Cell
L1
L2
L3
L4
Adult
L1
Pharynx
Seam cells
25 uM
L4
75 uM
Adult
Seam syncytium
Vulva
90 uM
b
lin-4
mir-125
lin-28
mLin28
let-7
miRNA
family
hbl-1
Ikaros
family
lin-41
mLin41
lin-29
ZF
Adult cuticle
Molting cycle exit
Seam cell fusion
Cell cycle exit
c
Wild type
d
mab-10
e
Wild type
f
mab-10
g
Wild type
L4
seam cells
h
mab-10
L4
seam cells
i
Wild type
Young adult
seam syncytium
j
mab-10
Young adult
seam syncytium
k
Wild type
vulva
l
mab-10
vulva
m
Wild type
mab-10
lin-29
L1
L2
L3
L4
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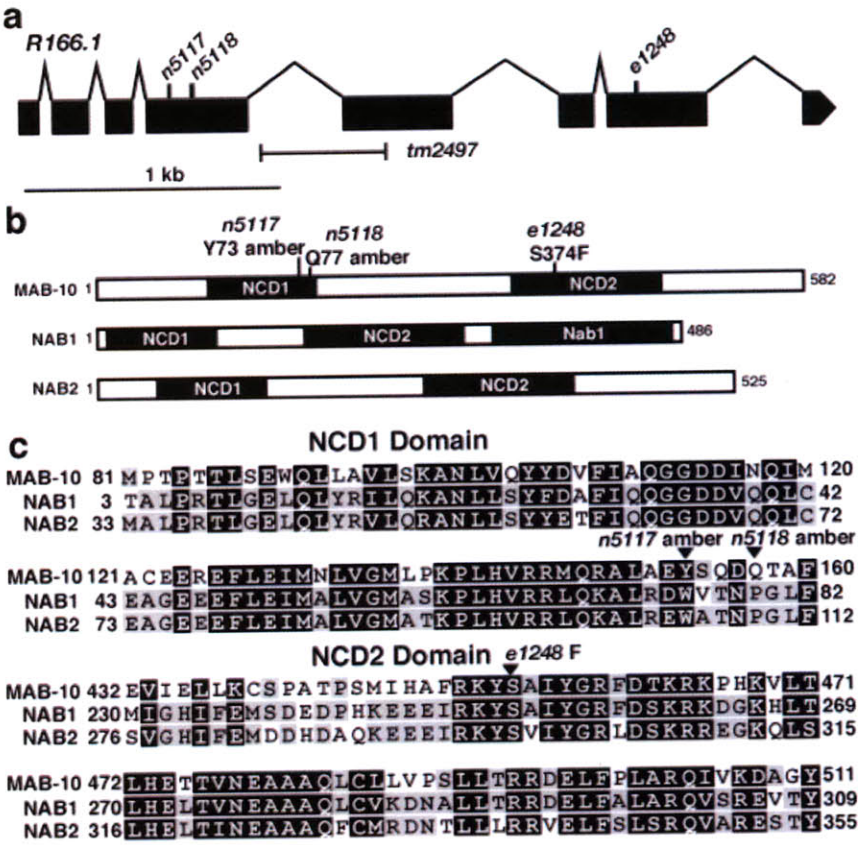
Figure 1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S374F
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NCD1
NCD2
582
NAB1 1
NCD1
NCD2
Nab1
486
NAB2 1
NCD1
NCD2
525
c
NCD1 Domain
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NAB1 3 TALPRTLGELQLYRILQKANLLSYFDAFIQQGGDDVQQLC 42
NAB2 33 MALPRTLGELQLYRVLQRANLLSYYETFIQQGGDDVQQLC 72
n5117 amber n5118 amber
MAB-10 121 ACEERFFLEIMNLVGMLPKPLHVRRMQRALAEYSQDQTAF 160
NAB1 43 EAGEEEFLEIMALVGMASKPLHVRRLQKALRDWVTNPGLF 82
NAB2 73 EAGEEEFLEIMALVGMATKPLHVRRLQKALREWATNPGLF 112
NCD2 Domain e1248 F
MAB-10 432 EVIELLKCSPATPSMIHAFRKYSAIYGRFDTKRKPHKVLT 471
NAB1 230 MIGHIFEMSDEDPHKEEEIRKYSAIYGRFDSKRKDGKHLT 269
NAB2 276 SVGHIFEMDDHDAQKEEEIRKYSVIYGRLDSKRREGKQLS 315
MAB-10 472 LHETTVNEAAAQLCLLVPSLLTRRDELFPLARQIVKDAGY 511
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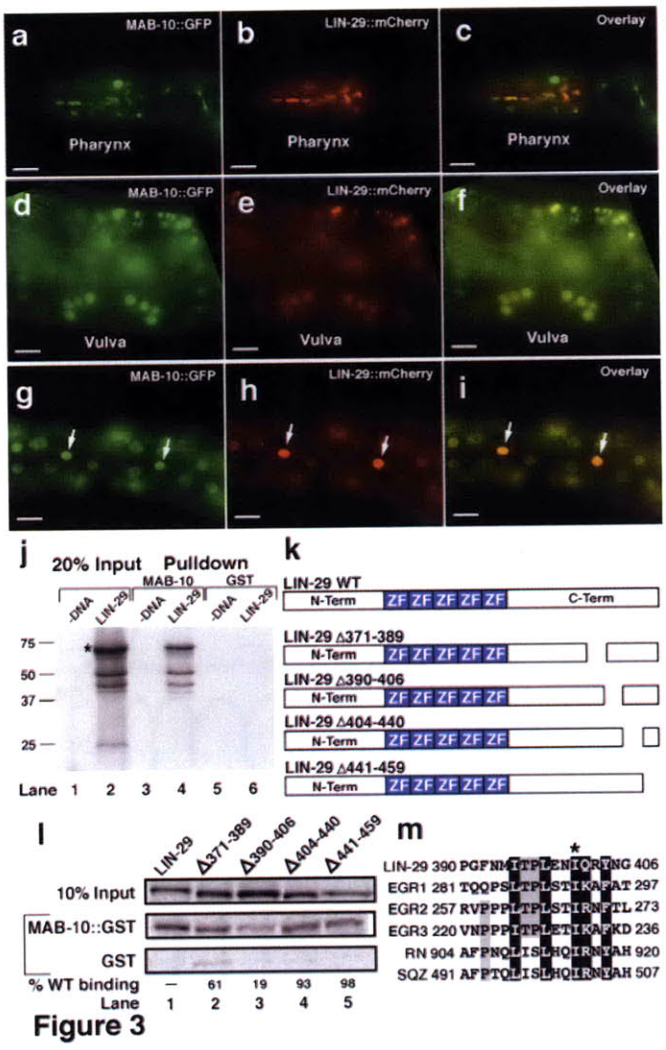
Figure 2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Pharynx
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Pharynx
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Vulva
f
Overlay
Vulva
g
MAB-10::GFP
h
LIN-29::mCherry
i
Overlay
j
20% Input
Pulldown
MAB-10
GST
-DNA
LIN-29
75
50
37
25
Lane 1 2 3 4 5 6
k
LIN-29 WT
N-Term
ZF ZF ZF ZF ZF
C-Term
LIN-29 Δ371-389
LIN-29 Δ390-406
LIN-29 Δ404-440
LIN-29 Δ441-459
l
LIN-29
Δ371-389
Δ390-406
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10% Input
MAB-10::GST
GST
% WT binding — 61 19 93 98
Lane 1 2 3 4 5
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LIN-29 390 PGFNMLTPLENQRNG 406
EGR1 281 TQQPSLTPLSTLKAPAT 297
EGR2 257 RVPPPLTPLSTLRNPTL 273
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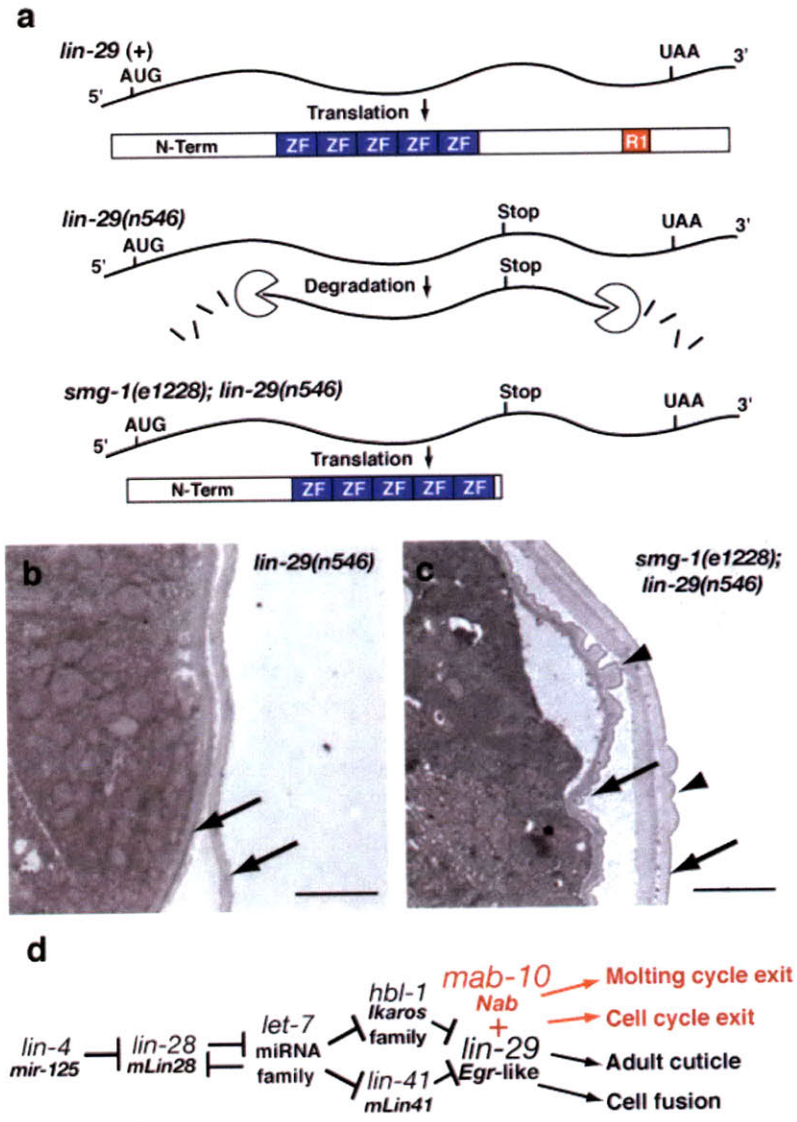
SQZ 491 AFPTQLISLHQLRNYAH 507

Figure 3


a
lin-29 (+)
AUG
5'
UAA
3'
Translation
N-Term
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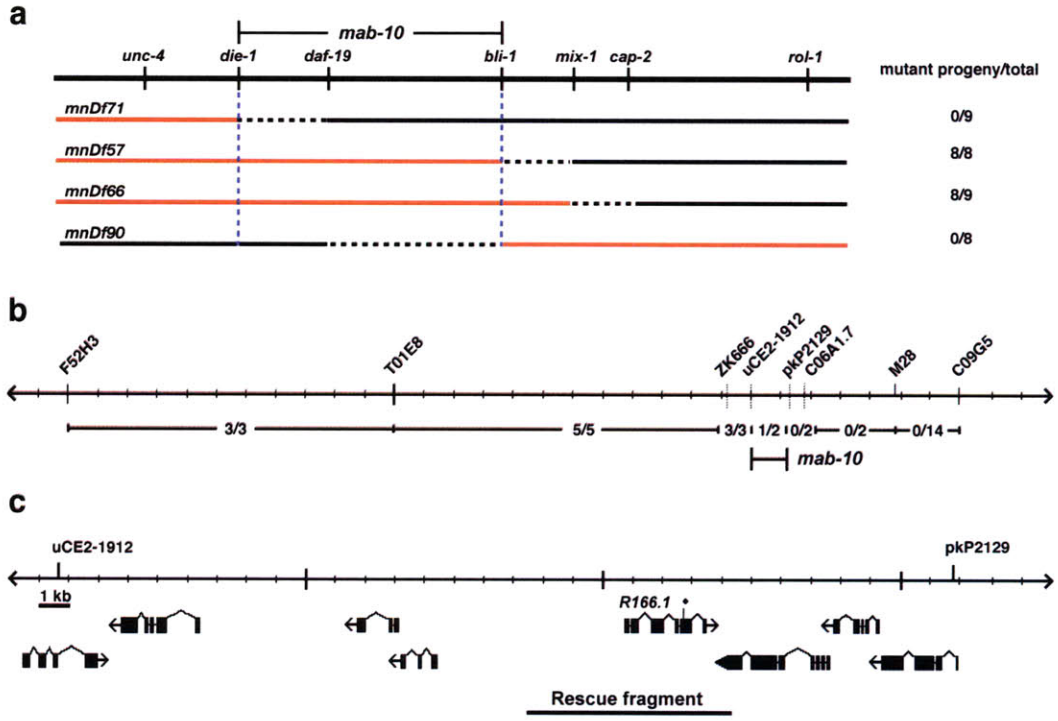
R1
lin-29(n546)
AUG
5'
Stop
UAA
3'
Degradation
Stop
smg-1(e1228); lin-29(n546)
AUG
5'
Stop
UAA
3'
Translation
N-Term
ZF ZF ZF ZF ZF
b
lin-29(n546)
c
smg-1(e1228);
lin-29(n546)
d
lin-4
mir-125
lin-28
mLin28
let-7
miRNA
family
hbl-1
Ikaros
family
lin-41
mLin41
mab-10
Nab
+
lin-29
Egr-like
Molting cycle exit
Cell cycle exit
Adult cuticle
Cell fusion

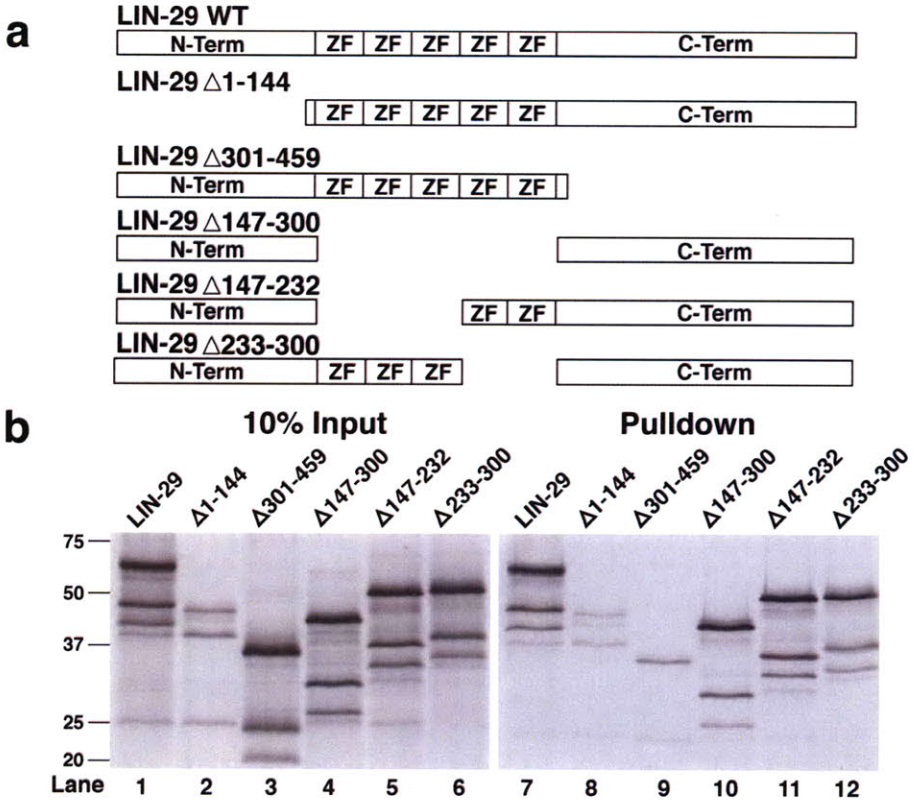
Figure 4

Supplementary Table 1 |

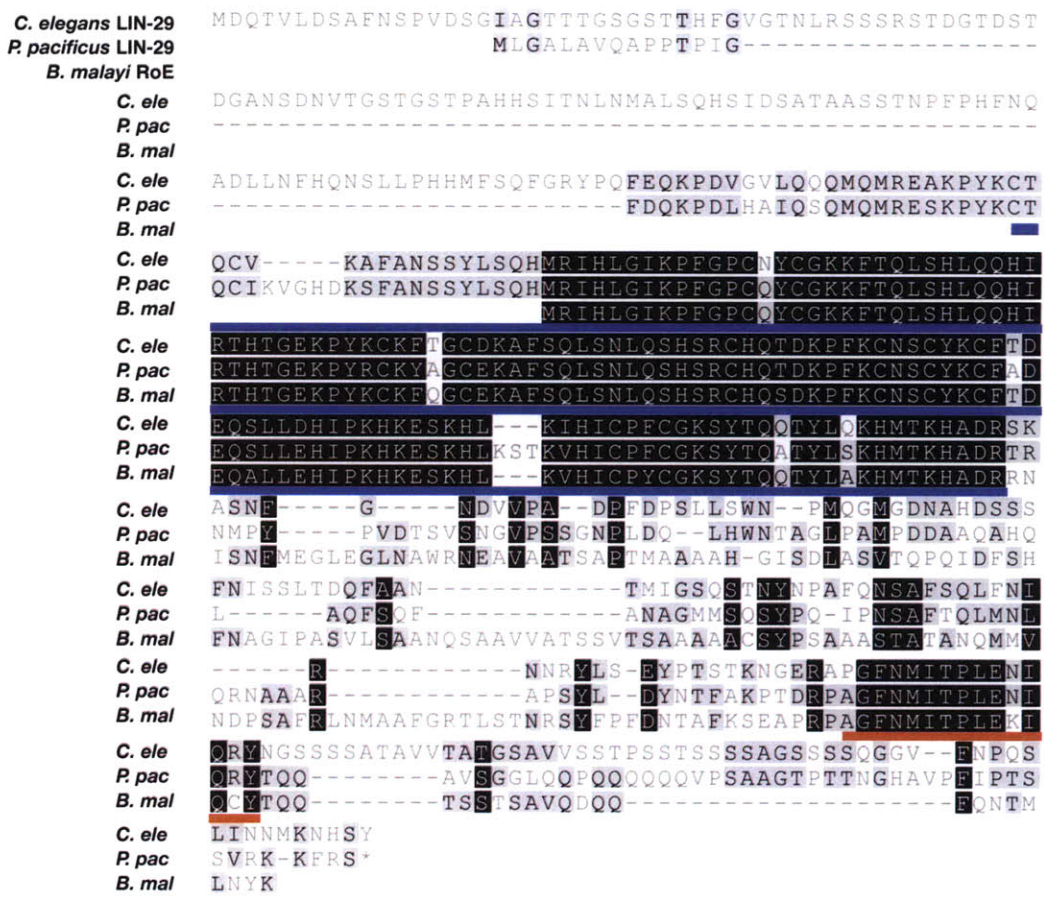
	% Adult alae (n)	% Extra molt (n)	% Seam cell fusion (n)	Seam cell number (n)
Wild-type [a]	100 (34)	0 (50)	100 (34)	11.1 ± 0.4 (34)
mab-10(e1248) [a]	100 (99)	100 (50)	100 (30)	13.1 ± 2.0 (99) [c]
mab-10(tm2497) [a]	100 (99)	100 (50)	100 (30)	15.1 ± 2.4 (99) [c]
mab-10(n5118) [a]	100 (93)	100 (50)	100 (30)	15.3 ± 2.4 (93) [c]
mab-10(n5117) [a]	100 (40)	100 (50)	100 (40)	15.8 ± 2.6 (40) [c]
lin-28(n719) [b]	100 (15)	ND	ND	7.0 ± 0.9 (15)
lin-28(n719); mab-10(n5117) [b]	100 (20)	ND	ND	13.0 ± 2.3 (20) [c]
lin-41(n2914) [b]	100 (30)	ND	ND	10.8 ± 1.0 (30)
lin-41(n2914); mab-10(n5117) [b]	100 (34)	ND	ND	15.5 ± 3.5 (34) [c]
lin-29(n546) [b]	0 (50)	100 (50)	0 (50)	18.8 ± 3.8 (22)
smg-1(e1228); lin-29(n546) [b]	100 (20)	93 (15)	100 (20)	11.1 ± 0.5 (20) [c]



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

Acknowledgments

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Chapter Three

mab-10 and *lin-29* are expressed dynamically throughout development and control multiple terminal differentiation events

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Daniel Denning performed linker cell death assays and Christoph Engert assisted with the single molecule FISH.

Summary

The larval-to-adult transition in *C. elegans* is controlled by the activities of the EGR-like protein LIN-29 and its NAB cofactor MAB-10. The heterochronic pathway controls the timing of LIN-29 protein accumulation and therefore controls the timing of the larval-to-adult transition. Here we show that the heterochronic pathway controls the timing of MAB-10::GFP accumulation and that MAB-10::GFP localization to seam cell nuclei requires *lin-29*. We use single molecule Fluorescence *in situ* hybridization to establish the wild-type expression patterns of *mab-10* and *lin-29* and demonstrate that *mab-10* expression does not require *lin-29* activity. We show that *mab-10* and *lin-29* are expressed in multiple tissues and that *mab-10* promotes the terminal differentiation of a non-hypodermal cell. Finally, we show that *mab-10* promotes seam cell exit from the cell cycle by antagonizing the activities of RNT-1, the *C. elegans* homolog of mammalian RUNX proteins, and BRO-1, the homolog of CBF-Beta (Core Binding Factor-Beta).

Introduction

Cell-fate specification requires the combined activities of multiple transcriptional regulators. In *C. elegans,* the heterochronic pathway controls the terminal differentiation of a stem cell lineage and the timing of the larval-to-adult transition by regulating the activities of the EGR-like transcription factor LIN-29 and its NAB-family cofactor, MAB-10 (Chapter 2). In mammals, NAB proteins act with EGR proteins to control the terminal differentiation of multiple cell lineages and the onset of puberty[1,2].

Previous studies using transcriptional reporters containing portions of the *lin-29* promoter suggested that *lin-29* might be transcribed in the hypoderm as early as the L2 stage and that the timing of LIN-29 activity is controlled by a post-transcriptional mechanism. LIN-29 and MAB-10 proteins first accumulate in the lateral hypoderm during the L4 stage (Chapter 2). In *lin-28*, *hbl-1*, and *lin-41* mutants, LIN-29 accumulates during the L3 stage, initiating the larval-to-adult transition precociously[3-6]. While each of these precocious mutants initiate the larval-to-adult transition precociously, their degree of seam cell terminal differentiation varies. For example, the seam cells of *lin-28* and *lin-41* mutants undergo a relatively complete precocious terminal differentiation while the seam cells of *hbl-1* mutants only partially terminally differentiate[5-7]. These differences might reflect changes in the regulation of either *lin-29* or *mab-10*.

In mammals, NAB proteins are thought to be transcriptional targets of their EGR binding partners. *Nab* transcription is often induced following the activation of *Egr* transcription, and it has been suggested that NAB proteins act in a negative feedback loop to repress EGR target

genes[8]. NAB proteins can also activate the transcription of EGR target genes. For example, the pulsatile release of GnRH (Gonadotropin Releasing Hormone) from the hypothalamus triggers the periodic transcription of *Egr1* in the gonadotrope lineage of the anterior pituitary gland[9,10]. EGR1 then acts with NAB1 and NAB2 to promote the expression of luteinizing hormone, the primary regulator of the onset of puberty[11].

Like mammalian NAB and EGR proteins, MAB-10 and LIN-29 reporters are co-expressed in multiple cell types throughout development, suggesting that they might act together in several different processes (Chapter 2). For example, in the male gonad, *lin-29* is required for the programmed cell death of the linker cell[12]. In Chapter 2, we demonstrated that LIN-29 acts with MAB-10 to control terminal differentiation in the hypoderm but the terminal differentiation of other cell types was not explored (Chapter 2).

Here we show that the heterochronic pathway controls the timing of MAB-10::GFP accumulation and that *lin-29* is required for MAB-10::GFP accumulation in the seam cells. We use single molecule FISH (Fluorescence *In Situ* Hybridization) to determine the wild-type expression patterns of *lin-29* and *mab-10* during development and to show that *lin-29* is not required for *mab-10* transcription. We find that *mab-10* controls the terminal differentiation of the male linker cell by promoting its programmed cell death and that MAB-10 prevents overproliferation of the seam cells by antagonizing the activities of RNT-1 and BRO-1, the *C. elegans* AML1/RUNX (Acute Myeloid Leukemia/RUNtX) and CBF-B (Core Binding Factor-Beta) homologs.

Our work suggests that the heterochronic pathway controls the timing of MAB-10 expression independent of LIN-29 to coordinate exit from the molting cycle and seam cell exit from the cycle with seam cell fusion and adult cuticle synthesis. Our work also suggests that

MAB-10 and LIN-29 act together to control the terminal differentiation of multiple lineages during the larval-to-adult transition.

Results

The timing of MAB-10::GFP accumulation is controlled by *hbl-1* and *lin-41*

Since *mab-10* acts with *lin-29* to control terminal differentiation and the expression patterns of *mab-10* and *lin-29* were highly correlated (Chapter 2), we asked if *mab-10* expression, like *lin-29* expression, is controlled by components of the heterochronic pathway. The *C. elegans* Hunchback homolog HBL-1 and the TRIM-NHL protein LIN-41 act to prevent the premature accumulation of LIN-29 and the precocious onset of adulthood[4-6]. We crossed a *mab-10::gfp* translational reporter into *hbl-1(ve18)* and *lin-41(n2914)* mutant backgrounds to determine if *hbl-1* and *lin-41* prevent the precocious accumulation of MAB-10. We observed precocious accumulation of MAB-10::GFP in the seam cell nuclei as well as the syncytial hypodermal nuclei of *hbl-1(ve18)* animals (Figure 1d-f). In *lin-41(n2914)* mutants we observed precocious accumulation in several cell types including the non-seam hypodermal nuclei (Figure 1g-i). However, we did not detect precocious MAB-10::GFP accumulation in the seam cell nuclei.

MAB-10::GFP nuclear localization in the seam cells requires *lin-29*

One possible explanation for the co-expression of MAB-10 and LIN-29 and the precocious accumulation of MAB-10::GFP in *hbl-1* and *lin-41* backgrounds is that LIN-29 promotes *mab-10* expression. To test this hypothesis, we observed MAB-10::GFP in a

lin-29(n836) mutant background. We found that the presence of MAB-10::GFP was unaltered in the pharynx, vulva, and non-seam hypodermal nuclei. However, MAB-10::GFP was absent in the seam cells during the L4 stage (Figure 1g-i), suggesting that LIN-29 promotes *mab-10* expression in the seam cells, and that other factors likely control *mab-10* expression in other tissues.

To determine if LIN-29::mCherry localization requires *mab-10*, we crossed the *lin-29::mcherry* reporter into a *mab-10(n5117)* mutant background. We saw no change in expression or localization of LIN-29::mCherry in the absence of *mab-10*, suggesting that MAB-10 is not required for proper LIN-29 expression or localization (Figure 1s-u).

mab-10 transcription is not dependent on *lin-29*

To determine if LIN-29 controls the expression of *mab-10* in the seam cells, we performed Single Molecule FISH[13] to detect the endogenous *mab-10* transcripts in wild-type and *lin-29(n836)* mutant backgrounds.

During the L1 stage (Figure 2a), we observed *mab-10* transcripts in the anterior and posterior bulbs of the pharynx as well as a small number of transcripts throughout the hypoderm. L2-stage animals (Figure 2b) showed increased expression of *mab-10* mRNA in hyp7 and the rectal epithelium in addition to the nerve ring and the ventral nerve cord, where several cells contained one or two transcripts.

During the early L3 stage (Figure 2c), *mab-10* was weakly expressed in hyp7 but showed high expression in the vulval and uterine precursor cells. *mab-10* mRNA was also present within the distal tip cells and a pair of bilaterally symmetric cells that we believe to be the CAN neurons

based on their position and neuronal nuclear morphology. The CAN neurons are associated with the excretory canal and are thought to regulate excretory function.

By the late L3 stage (Figure 2d), *mab-10* expression was almost absent from hyp7 but was highly expressed in the seam cells, distal tip cells and developing vulva. *mab-10* transcript was also detected in the gonadal sheath cells.

During the L4 stage (Figure 2e), *mab-10* transcripts increased in abundance throughout the pharynx, hypoderm, and somatic gonad, including the distal tip cells.
We found no change in the level or pattern of *mab-10* transcription in a *lin-29(n836)* mutant background compared to the wild type (Figure 2f), suggesting that *mab-10* is not a transcriptional target of LIN-29 and that the absence of MAB-10::GFP localization to the nuclei of L4 seam cells reflects a post-transcriptional regulation of MAB-10 by LIN-29.

lin-29 expression oscillates in the hypoderm

LIN-29 protein localization has been assayed previously using an anti-LIN-29 antibody[3] and a LIN-29::mCherry fusion reporter (Chapter 2). LIN-29 protein begins to accumulate in the hypoderm during the L4 stage of wild-type animals. Previous work using a lacZ reporter driven by variants of the *lin-29* promoter suggested that *lin-29* might be expressed as early as the L2 stage in the hypoderm[3]. Furthermore, we observed significant overlap between the expression patterns of MAB-10::GFP and LIN-29::mCherry reporters (Chapter 2), and we wanted to determine if this overlap was the result of co-regulated transcription. To address these issues we determined the expression pattern of endogenous *lin-29* transcripts using Single Molecule FISH.

During the L1 stage (Figure 3a), a small number of *lin-29* transcripts were visible in the anterior and posterior bulbs of the pharynx with low levels of transcript throughout the

hypoderm. *lin-29* mRNA was much more abundant in one ventral hypodermal cell, located just anterior to the rectum, that we believe to be P12.p.

During the L2 stage (Figure 3b), *lin-29* transcripts became much more abundant in both the pharynx and the hypoderm. Additionally, *lin-29* was highly expressed in a cell located immediately dorsal to P6.p, consistent with being the anchor cell.

During the early L3 stage (Figure 3c), following the first division of the Pn.p cells, *lin-29* transcript was highly abundant in the pharynx, hyp7, and the rectal epithelium. We saw expression in the migrating distal tip cells, the anchor cell, and all of the daughters of P3.p-P8.p. *lin-29* mRNA was also present in several cells of the ventral nerve cord. There was very low expression of *lin-29* in the body wall muscles at this time.

After the divisions of the daughters of the Pn.p cells, the *lin-29* expression pattern underwent significant changes (Figure 3d). The number of transcripts in the syncytial hypoderm decreased substantially while the number of transcripts in the seam cells remained high. Expression in the body wall muscles increased and *lin-29* transcript remained present in the distal tip cells as well as the anchor cell. *lin-29* transcript levels became elevated in the vulval precursor cells that adopted a secondary fate, while the number of *lin-29* transcripts in the cells that adopted the primary cell fate remained unchanged. Additionally, *lin-29* transcript could now be observed in the daughter cells of the sex myoblasts.

By the middle of the L4 stage (Figure 3e), *lin-29* transcripts again became abundant throughout the hypodermal syncytium and were present within the invaginating vulva and within a small number of uterine cells. The distal tip cells no longer showed *lin-29* expression.

By late L4 (Figure 3f), *lin-29* transcripts became greatly reduced in the hypodermal syncytium and were highly abundant throughout the seam. At the time of vulval eversion, *lin-29* was expressed in all vulval cells as well as in most of the uterine cells.

MAB-10 promotes male linker cell death

The male linker cell is a somatic gonad cell that is generated during the second larval stage[14]. Like the distal tip cells in hermaphrodites, the linker cell acts as the leading cell of the gonad and undergoes a characteristic pattern of migration throughout development. The linker cell is born near the middle of the animal and migrates to its final location in the tail near the cloaca. At the end of the L4 stage, the linker cell undergoes a *ced-3*-independent programmed cell death[12,14]. The engulfment of the linker cell corpse by the rectal epithelium results in the connection of the lumen of the vas deferens to the cloaca, forming a passage for the transfer of sperm[14].

It was recently reported that this programmed cell death occurs in the absence of the canonical cell-death execution pathway and is instead controlled by LIN-29[12]. In the absence of LIN-29, the linker cell survives well into adulthood. We postulated that *mab-10* might also play a role in promoting the linker cell death since *mab-10* males were originally identified based on a minor tail abnormality and their inability to successfully mate[15]. We found that MAB-10::GFP and LIN-29::mCherry were co-expressed throughout the L4 stage in the migrating linker cell (Figure 4a-d). Late L4 animals showed increased expression of both *mab-10* and *lin-29* just prior to linker cell death (Figure 4e-h). We used a *lag-2::gfp* reporter, which is expressed in the linker cell, to assay linker cell death in *mab-10* and *lin-29* mutants. We found that the linker cell inappropriately survived in 66% of *lin-29(n836)* males, consistent with the previous report [12],

and the linker cell survived in 42% of *mab-10(n5117)* males (Figure 4i). Therefore, *mab-10* likely acts with LIN-29 to promote the linker cell death.

Loss of *rnt-1* or *bro-1* suppresses the *mab-10* seam cell defect

Although the seam cells of *mab-10* and *lin-29* mutants undergo extra divisions, their lineages are not identical (Figure 5a). *lin-29* seam cells inappropriately divide during the L4 stage, whereas *mab-10* seam cells do not divide during the L4 stage, but inappropriately divide as young adults. This difference in lineage suggests that LIN-29 functions independently of MAB-10 to establish the initial seam cell exit from the cell cycle. It was previously observed that there is a *lin-29*-dependent upregulation of the p27/KIP homolog CKI-1 (Cyclin-dependent Kinase Inhibitor) in the seam cells during the L4 stage[16]. We found that *mab-10* was not required for *cki-1* expression during the L4 stage, as CKI-1 levels were normal in *mab-10* mutants (Figure 5b-d).

However, we noticed that CKI-1 levels decreased in the seam cells prior to the extra divisions (data not shown), suggesting that there might be factors inappropriately repressing *cki-1* activity in *mab-10* adults. RNT-1 (RuNT), the *C. elegans* homolog of the mammalian RUNX proteins, and its binding partner BRO-1 (BROther homolog), the Core-binding factor Beta homolog, act together to promote seam cell divisions by downregulating *cki-1* expression during development[17,18]. If either *rnt-1* or *bro-1* is inactivated, the seam cells undergo fewer divisions. If either *rnt-1* or *bro-1* is overexpressed, the seam cells overproliferate[17,18].

To determine if the extra seam cell divisions in *mab-10* mutants required the activity of *rnt-1* or *bro-1*, we performed epistasis tests between *mab-10* and either *rnt-1* or *bro-1*. In both *rnt-1; mab-10* and *bro-1; mab-10* double mutants, the extra seam cell divisions were completely

suppressed (Figure 5e), suggesting that both *rnt-1* and *bro-1* are required for the extra seam cell divisions seen in *mab-10* animals.

Discussion

MAB-10::GFP accumulates precociously in *hbl-1* and *lin-41* mutants

The heterochronic genes *hbl-1* and *lin-41* act in parallel to prevent the precocious initiation of the larval-to-adult transition[4-6]. In *hbl-1* and *lin-41* mutant backgrounds, LIN-29 protein accumulates in the hypoderm during the L3 stage instead of the L4 stage. LIN-29 is thought to act by promoting the expression of adult-specific genes and repressing the expression of larval-specific genes[7,19,20]. To determine if *mab-10* expression is controlled by the heterochronic pathway, we crossed *mab-10::gfp* into *hbl-1(ve18)*, *lin-41(n2914),* and *lin-29(n836)* mutant backgrounds.

We found that, like LIN-29, MAB-10::GFP accumulated in the hypoderm precociously in both *hbl-1* and *lin-41* mutant backgrounds. In *hbl-1(ve18)* animals, MAB-10::GFP accumulated during the L3 stage in the nuclei of hyp7 as well as in the seam cell nuclei. MAB-10::GFP accumulated in the hypoderm during the L3 stage in *lin-41(n2914)* animals but it was restricted to the nuclei of hyp7 and did not accumulate precociously in seam cell nuclei. These results suggest that *hbl-1* and *lin-41* act to prevent the precocious accumulation of MAB-10. The difference in the extent of precocious terminal differentiation observed in *hbl-1* and *lin-41* mutants might reflect differences in the precocious accumulation of both MAB-10 and LIN-29.

Seam cell localization of MAB-10::GFP requires LIN-29

Because *hbl-1* and *lin-41* mutants have precocious LIN-29 activity, and LIN-29 promotes the expression of genes required for adulthood, we wanted to determine if *mab-10* expression was dependent on *lin-29* function. We found that the pattern of MAB-10::GFP expression in *lin-29(n836)* animals was similar to the wild-type expression pattern with the notable exception of the seam cells. During the L4 stage, MAB-10::GFP was not visible within the seam cells suggesting that either *mab-10* transcription is controlled by LIN-29 in the seam cells or that LIN-29 promotes MAB-10::GFP localization to the seam cell nuclei via a post-transcriptional mechanism. We demonstrated previously that MAB-10 and LIN-29 physically interact (Chapter 2). One possibility is that LIN-29 acts to stabilize MAB-10 in the seam cells. In the absence of LIN-29, MAB-10 is either degraded or excluded from the nucleus.

MAB-10::GFP nuclear localization in the *lin-29(n836)* mutant background was not affected in other cell types, including the nuclei of hyp7, suggesting that *mab-10* expression in the majority of cells is not controlled by *lin-29*. Furthermore, the fact that we see precocious MAB-10::GFP accumulation in the nuclei of hyp7 in both *hbl-1* and *lin-41* mutants suggests that *hbl-1* and *lin-41* regulate *mab-10* independently of *lin-29*. This also suggests that *hbl-1* and *lin-41* act to prevent the premature initiation of the larval-to-adult transition by regulating multiple factors and not LIN-29 alone.

LIN-29::mCherry localization does not require MAB-10

Since MAB-10::GFP localization to seam cell nuclei requires *lin-29*, we wanted to know if *mab-10* was required for the proper localization of LIN-29::mCherry. We found that

LIN-29::mCherry localization was unaltered in *mab-10(n5117)* animals, suggesting that neither LIN-29 expression nor localization requires *mab-10*.

mab-10 transcription does not require *lin-29*

While *mab-10* mutants have only been shown to be defective in processes occurring during the larval-to-adult transition, *mab-10* is expressed in multiple cell types throughout development. Interestingly, our previous analysis using MAB-10::GFP suggested that MAB-10 protein was not present in the hypoderm and seam cells until the L4 stage. Using single molecule FISH, we detected *mab-10* transcript in the hypoderm as early as L2, with strong expression in the seam cells during late L3. This suggests that *mab-10* might undergo some form of post-transcriptional regulation.

In mammals, EGR proteins regulate the expression of their NAB cofactors in certain contexts[8]. The EGR protein promotes the expression of NAB1 or NAB2, which then acts with the EGR protein to regulate a specific subset of target genes.

During the L4 stage, *mab-10* expression in the hypoderm increased substantially, but this expression was not dependent on *lin-29*. This *lin-29* independence suggests that *mab-10* is not a downstream target of LIN-29, and that other factors, possibly *hbl-1* and *lin-41*, act upstream of *mab-10* to coordinate exit from the molting cycle and exit from the cell cycle with seam cell fusion and adult cuticle synthesis.

lin-29 is expressed dynamically throughout development

Previous studies of *lin-29* expression focused primarily on the accumulation and localization of LIN-29 protein[3]. Transcriptional reporters driving lacZ expression from portions of the *lin-29* promoter showed some hypodermal expression as early as the L2 stage, but it is not clear if this accurately reflects the expression of endogenous *lin-29*[3].

Using single molecule FISH, we have shown that *lin-29* is expressed dynamically throughout development. During the L2 stage, the number of *lin-29* transcripts detected in the hypoderm is quite low. During the early L3 and L4 stages, *lin-29* transcript is detected in high numbers throughout the hypoderm and absent from the body wall muscles. By the end of the L3 and L4 stages, *lin-29* transcript is detected in the seam cells and body wall muscle but greatly reduced in hyp7. These oscillations in hypodermal expression are undetectable using LIN-29 antibody or translational reporters, as the protein does not accumulate until the L4 stage.

While it was demonstrated that single molecule FISH in human cells can likely detect individual mRNAs, it is not yet clear if this is also the case in *C. elegans*. One possible approach to address this concern would be to compare the intensities of fluorescence for several of the detected "dots" in a given area of the animal. If each "dot" represents a single molecule, we would expect to see a unimodal distribution of intensities with a single peak and a tail extending towards zero fluorescence.

These observations confirm that *lin-29* transcript is present in the hypoderm prior to the L4 stage and that *lin-29* is likely regulated post-transcriptionally. Furthermore, the oscillation of *lin-29* expression in the hypoderm might suggest the transcriptional regulation of *lin-29* by the *C. elegans Period* homolog *lin-42*, the expression of which also oscillates in the hypoderm and when mutated causes precocious LIN-29 activity[21,22].

MAB-10 promotes a *ced-3*-independent programmed cell death

The male linker cell death occurs during the larval-to-adult transition and is controlled by components of the heterochronic pathway. *mab-10* had previously been implicated in promoting specific aspects of the larval-to-adult transition within the hypoderm (Chapter 2). Here, we have shown that *mab-10,* like *lin-29,* is required for the gonadal linker cell death.

Therefore, *mab-10* activity is not limited to promoting exit from the cell cycle and the cessation of the molting cycle, but also includes promoting a *ced-3*-independent cell death. This also suggests that MAB-10 likely regulates the expression of different genes in different cell types. Interestingly, *mab-10* mutants were originally identified based on a very subtle mail tail defect and an inability to mate[15]. The failure of the linker cell to die in *mab-10* mutants might contribute to their infertility.

MAB-10 prevents RNT-1/BRO-1 mediated overproliferation

NAB proteins are required to prevent the overproliferation of multiple cell lineages during mammalian development. Mice lacking both NAB1 and NAB2 have overproliferation defects within the chondrocyte lineage of the bone, the Schwann cell lineage of the peripheral nervous system, and the keratinocytes of the epidermis[2]. In *C. elegans*, we have shown that MAB-10 is important to prevent the overproliferation of the seam cells, a hypodermal stem cell lineage.

RUNX proteins control the decision to proliferate or differentiate in multiple tissues across a wide range of organisms. In mammals, RUNX proteins promote differentiation during

hematopoiesis and chondrogenesis[23,24]. In sea urchins, RUNX proteins promote cell proliferation during embryogenesis[25]. In *C. elegans*, RNT-1 and its binding partner, BRO-1, act together during development to promote seam cell proliferation by repressing the expression of the cyclin-dependent kinase inhibitor CKI-1 to allow entry into the S phase of the cell cycle[17,18,26].

We have shown that the seam cell overproliferation defect observed in *mab-10* animals requires the activities of *rnt-1* and *bro-1*. Our results suggest that RNT-1 and BRO-1, which normally act to promote seam cell divisions during development, are inappropriately active during adulthood in *mab-10* mutants. One possibility is that MAB-10 acts to repress *rnt-1* and *bro-1* during the larval-to-adult transition. In the absence of MAB-10, the initial increase of CKI-1 mediated by LIN-29, prevents seam cell divisions during the L4 stage. However, inappropriate expression of *rnt-1* and *bro-1* results in the repression of *cki-1* and re-entry into the cell cycle.

EGR and NAB proteins have conserved roles in the terminal differentiation of cell lineages and the onset of adulthood

The larval-to-adult transition in *C. elegans* is dependent on the activities of the EGR-like molecule LIN-29 and its NAB cofactor MAB-10 (Chapter 2). At the end of development, several cells undergo unique terminal differentiation events[14,27]. In the hypoderm, LIN-29 and MAB-10 control seam cell exit from the cell cycle, seam cell fusion, adult-specific cuticle synthesis and exit from the molting cycle (Chapter 2). In the male gonad, LIN-29 and MAB-10 promote the programmed cell death of the linker cell.

In mammals, NAB1 and NAB2 act with EGR proteins to promote the terminal differentiation of multiple cell lineages during development. In the Schwann cell lineage, NAB

proteins act with EGR2 to prevent the overproliferation of Schwann cell precursors and to promote their differentiation into a myelin-secreting state. In the chondrocyte and keratinocyte lineages, NAB proteins also act to prevent overproliferation[2]. We have shown that MAB-10 and LIN-29 prevent the overproliferation of the seam cells by antagonizing the proliferative activity of RNT-1 and BRO-1, the *C. elegans* homologs of mammalian RUNX proteins and CBF-B(Core Binding Factor-Beta) respectively[17,28].

The initiation of puberty in mammals is dependent on the activities of EGR1, NAB1, and NAB2[1,11,29]. Prior to the initiation of puberty, the GnRH (Gonadotropin Releasing Hormone) pulse generator becomes de-repressed and oscillating expression of EGR1 drives the pulsatile release of luteinizing hormone (LH) into the blood stream[30]. This expression of EGR1 is under circadian control as the amplitude of the LH pulses increase at specific times during the night[31]. Here we show that, like EGR1, *lin-29* expression oscillates prior to the onset of the larval-to-adult transition. Furthermore, it was already shown that the *C. elegans* homologs of the circadian rhythm genes, *period* (*lin-42*), *timeless* (*tim-1*), and *doubletime* (*kin-20*), regulate the timing of LIN-29 activity[22,32]. Recently, a SNP closely linked to LIN28B, a human homolog of *C. elegans* LIN-28 was shown to be associated with the premature onset of puberty[33]. In *C. elegans*, LIN-28 prevents precocious LIN-29 activity and the premature onset of the larval-to-adult transition[7]. While the results of the human association studies are tantalizing, the cohort size was small, as was the specific SNPs contribution to the phenotype. However, based on our findings as well as those in humans, we propose that the mechanisms that control the onset of adulthood are evolutionarily conserved from *C. elegans* to humans and we hypothesize that LIN-28B prevents precocious puberty by preventing precocious EGR1 activity.

Materials and Methods

Strains and genetics

C. elegans was grown as previously described[34] and maintained at 20° C unless otherwise noted. N2 was the wild-type strain. The mutations used in this study were: LGI: *lin-28(n719)*[7], *lin-41(n2914)*[4], *rnt-1(ok351)*, *bro-1(tm1183)*; LGII: *mab-10(n5117)*, *lin-29(n836)*[35]; LGIV: *him-8(e1489)*[36]; LGV: *him-5(e1467)*[36]; LGX *hbl-1(ve18)*.

The following balancer chromosomes were used: *hT2 [qIs48]* LGI; LGIII, *mIn1 [mIs14]* LGII and *mnC1* LGII.

Single Molecule FISH

Single molecule FISH (Fluorescence *In Situ* Hybridization) was performed based on protocols previously described[13] and at www.singlemoleculefish.com. Briefly, synchronized populations of N2 animals were grown on NGM agar plates seeded with OP50. Animals were harvested by the addition of 2-3 mLs of M9 solution and subsequent collection in 15 mL conical tubes. Animals were washed twice with PBS and transferred to a 1.5 mL microfuge tube. This yielded approximately 35 uL of packed animals per large NGM plate. PBS was removed and replaced with 1 mL of fixation solution (4% Formaldehyde in PBS). Animals were fixed for 45 minutes and washed twice in PBS. After the second wash, the PBS was removed and replaced with 70% ethanol. Animals were kept in 70% ethanol for at least one night.

Hybridization

Hybridizations were performed essentially as previously described[13] and at www.singlemoleculefish.com. For *mab-10* probes conjugated to Cy5, a probe dilution of 1/10000 from the original stock solution was used. For *lin-29* probes conjugated to either Alexa594 or Cy5, a probe dilution of 1/5000 from the original stock solution was used.

Probes

mab-10 and *lin-29* probes were designed following the guidelines specified at www.singlemoleculefish.com and purchased from Biosearch Technologies Inc. (Novato, CA). A total of 48 probes were used for *mab-10* and 45 probes were used for *lin-29*.

mab-10 probes

The top strand is the *mab-10* transcript read 5'->3'. The bottom strand is the probe sequence read 3'->5'.

```
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gaccatcagcatcacatcacttggagtccatacttagctcgtcttcatcttctccatcaattttatcatc

c  gtagtcgtagtgtagtgaac  caggtatgaatcgagcagaa                    gtag


tcttacaactcaatccacttcatcaatacccacctcttcatcatcatcatcacaacgtcaatcaaccagc

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lin-29 probes

The top strand is the *lin-29* transcript read 5'->3'. The start codon of the *lin-29* short isoform is indicated by an asterisk.

*

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gcagtcgtccaa aggagttcagtccctccgca agttgggtgttagtgactat attatacttttt

tcattcctattaa
agtaagga

Phenotypic analyses

Linker cell death was assayed using a Zeiss Axioskop 2 with Nomarski optics. Animals were synchronized by hatching overnight in M9 solution. Linker cell death was assayed using the integrated array *qIs56* which carries a *lag-2::gfp* reporter. Young adult males (4-8 hrs) were assayed for the presence or absence of *lag-2::gfp* expression. Seam cell divisions were followed using either Nomarski microscopy or fluorescence microscopy to observe *col-19::gfp (maIs105)*. To assay adult seam cells, we picked late L4 animals to fresh plates and counted seam cell number 24 hours later.

Figure Legends

Figure 1

MAB-10::GFP expression is controlled by the heterochronic pathway. a-i, DIC and epifluorescence images showing MAB-10::GFP localization in L3 stage animals of (a-c) wild-type, (d-f) *hbl-1(ve18)*, and (g-i) *lin-41(n2914)* mutant backgrounds. **j-o,** DIC and epifluorescence images showing MAB-10::GFP localization in L4 animals of (j-l) the wild type and (m-o) *lin-29(n836)* mutant background. **p-u,** DIC and epifluorescence images showing LIN-29::mCherry localization in L4 animals of (p-r) the wild type and (s-u) *mab-10(n5117)* mutant background.

Figure 2

***mab-10* expression pattern in wild-type and *lin-29(n836)* mutant backgrounds.** Each red dot corresponds to one *mab-10* transcript. The scale bars equal 10 μm. **a,** L1 animal showing expression in the anterior and posterior bulbs (Ab and Pb) of the pharynx, and a very low level of expression in the hypoderm. **b,** L2 animal showing expression in the pharynx, the nerve ring (Nr), the ventral nerve cord (VNC), the rectal epithelium, and the hypoderm (hyp7). **c,** Early L3 animal (Pn.p cells have divided once) showing expression in the pharynx, the nerve ring, the distal tip cells (Dtc), the Canal-associated neuron (CAN) and all of the cells of the vulval and uterine precursor cells. There is also a low level of expression throughout hyp7. **d,** Late L3 animal (Pn.p daughters have divided once) showing reduced expression in the pharynx, increased expression in the seam cells, developing vulva, uterus, and distal tip cells. **e,** Mid-L4 animal showing high levels of *mab-10* expression throughout the pharynx, hyp7 and gonad. **f,** Mid-L4

lin-29(n836) animal showing levels of *mab-10* expression that are indistinguishable from the wild type.

Figure 3

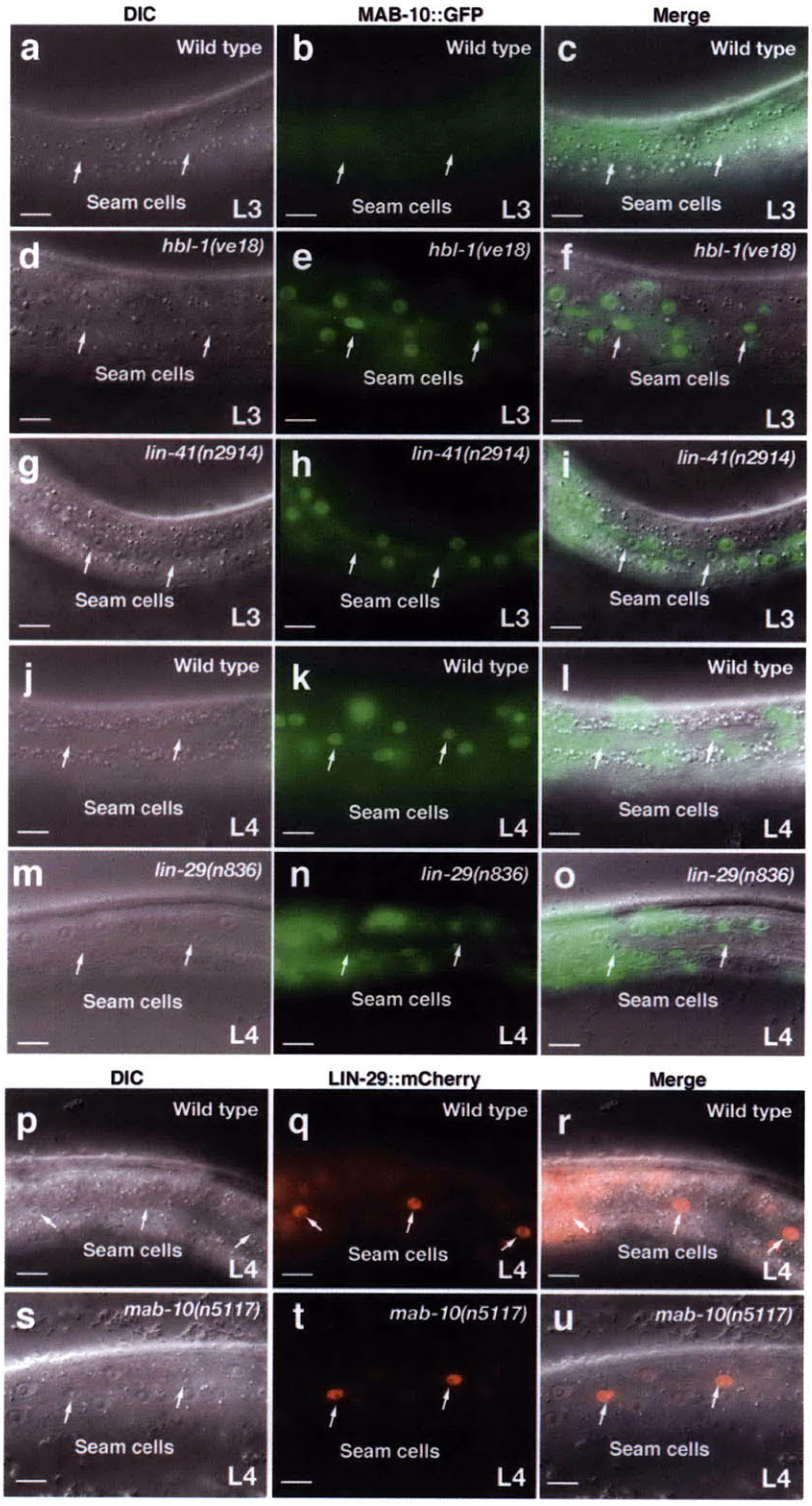
***lin-29* expression oscillates in the hypoderm during development.** Each green dot corresponds to one *lin-29* transcript. The scale bars are 10 μm. **a,** L1 animal showing expression in the anterior and posterior bulbs of the pharynx and a low level of expression in the hypoderm. **b,** L2 animal showing increased expression in the pharynx, ventral nerve cord, hypoderm, and the anchor cell (Ac). **c,** Early L3 animal (Pn.p cells have divided once) showing high levels of expression throughout hyp7, the distal tip cells, the anchor cell, and all daughters of P3.p-P8.p. **d,** Late L3 animal (Pn.p daughters have divided once) showing very little expression within hyp7, high levels of expression within the row of seam cells, the daughters of the sex myoblasts (Sm), the distal tip cells, the anchor cell, and the vulval precursor cells. Vulval precursor cells that will/have adopted the secondary vulval cell fate express at a much higher level than those that will/have adopted the primary vulval cell fate.

Figure 4

***mab-10* promotes male linker cell death. a,** DIC image of a male gonad during the mid-L4 stage. The linker cell is indicated. **b-d,** Epifluorescence images showing MAB-10::GFP and LIN-29::mCherry co-expressed in the migrating linker cell. **e,** DIC image of a late L4 male tail, shortly before the linker cell death. **f-h,** Epifluorescence images showing MAB-10::GFP and LIN-29::mCherry with increased expression in the linker cell prior to its death. **i,** Table showing that *mab-10* mutants are defective in the execution of the linker cell death.

Figure 5

***mab-10* prevents *rnt-1/bro-1*-mediated seam cell overproliferation. a,** Seam cell lineages (V1-V4, V6)[27] of wild-type, *mab-10*, and *lin-29* mutant strains. *lin-29* mutant seam cells **b-g,** DIC and epifluorescence images showing (b-c) CKI-1::GFP expression in the seam cells during the larval-to-adult transition of a wild-type animal, (d-e) the absence of CKI-1::GFP expression in the seam cells of *lin-29(n836)* animals during the late L4 stage, and (f-g) the wild-type expression of CKI-1::GFP in *mab-10(n5117)* mutants. **h,** Table showing that the extra cell divisions of *mab-10* mutants are suppressed by *rnt-1* and *bro-1*.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MAB-10::GFP
Merge
a
Wild type
Seam cells
L3
b
Wild type
Seam cells
L3
c
Wild type
Seam cells
L3
d
hbl-1(ve18)
Seam cells
L3
e
hbl-1(ve18)
Seam cells
L3
f
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L3
g
lin-41(n2914)
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L3
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lin-41(n2914)
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L3
i
lin-41(n2914)
Seam cells
L3
j
Wild type
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L4
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m
lin-29(n836)
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lin-29(n836)
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Seam cells
L4
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Merge
p
Wild type
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r
Wild type
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mab-10(n5117)
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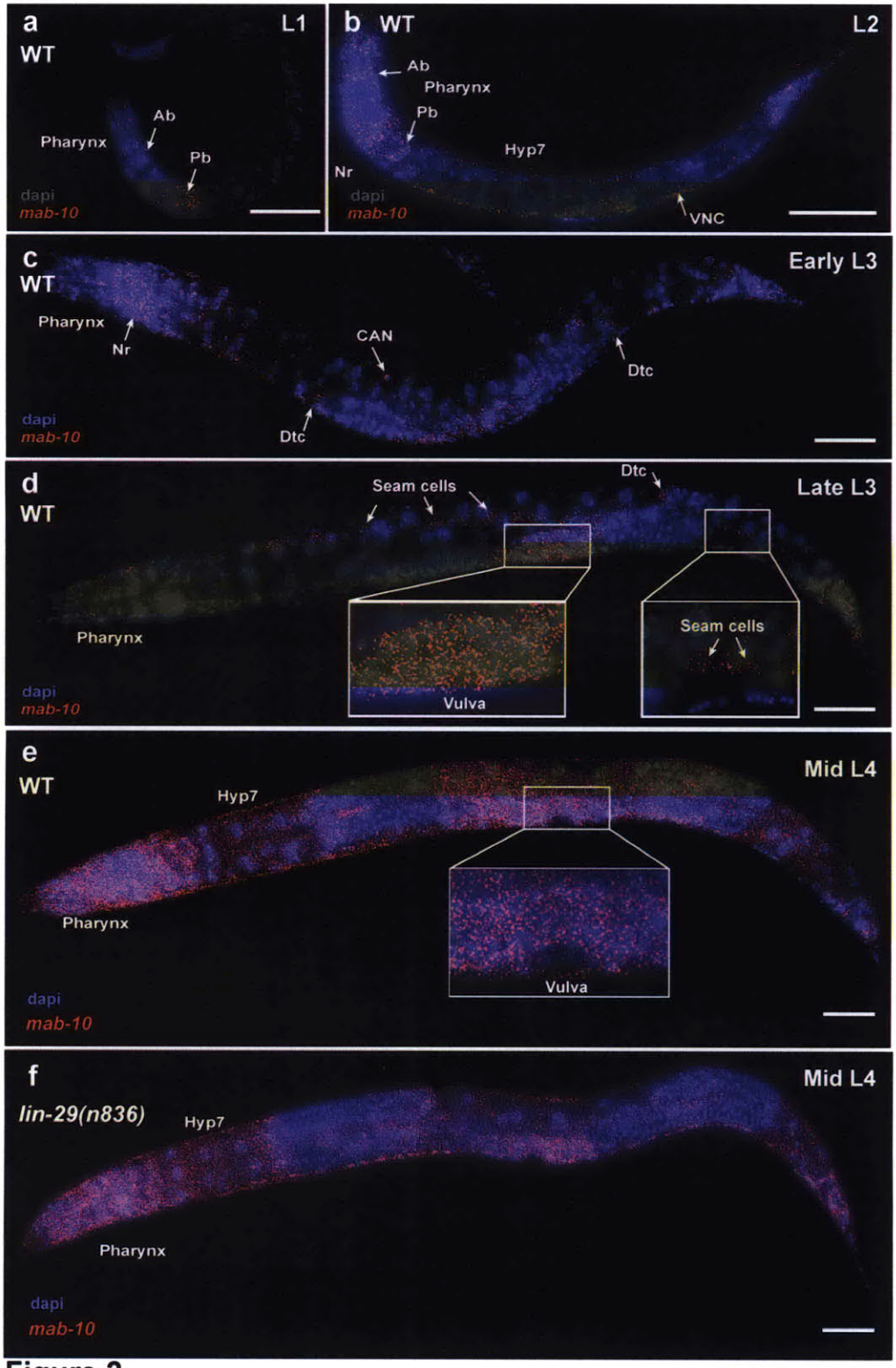
Seam cells
L4

Figure 1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WT
L1
Ab
Pharynx
Pb
dapi
mab-10
b WT
L2
Ab
Pharynx
Pb
Hyp7
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VNC
c
WT
Early L3
Pharynx
Nr
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Dtc
Dtc
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Seam cells
Dtc
Pharynx
Seam cells
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e
WT
Mid L4
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lin-29(n836)
Mid L4
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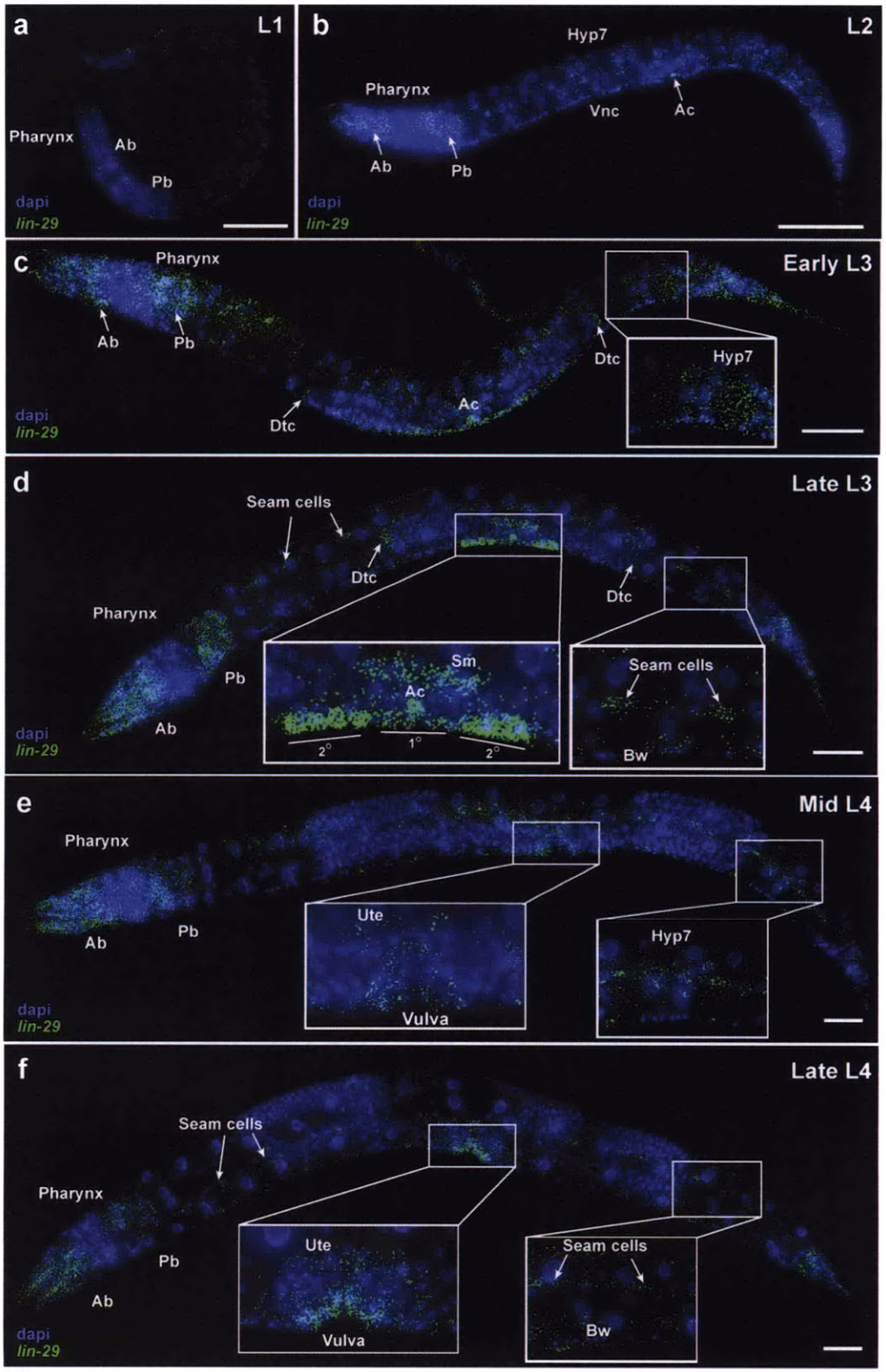
mab-10

Figure 2


a
L1
Pharynx
Ab
Pb
dapi
lin-29
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Hyp7
L2
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Vnc
Ac
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c
Pharynx
Early L3
Ab
Pb
Dtc
Hyp7
Ac
Dtc
dapi
lin-29
d
Late L3
Seam cells
Dtc
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Pharynx
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Pb
Ac
Seam cells
Ab
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2°
Bw
dapi
lin-29
e
Mid L4
Pharynx
Ute
Pb
Ab
Hyp7
Vulva
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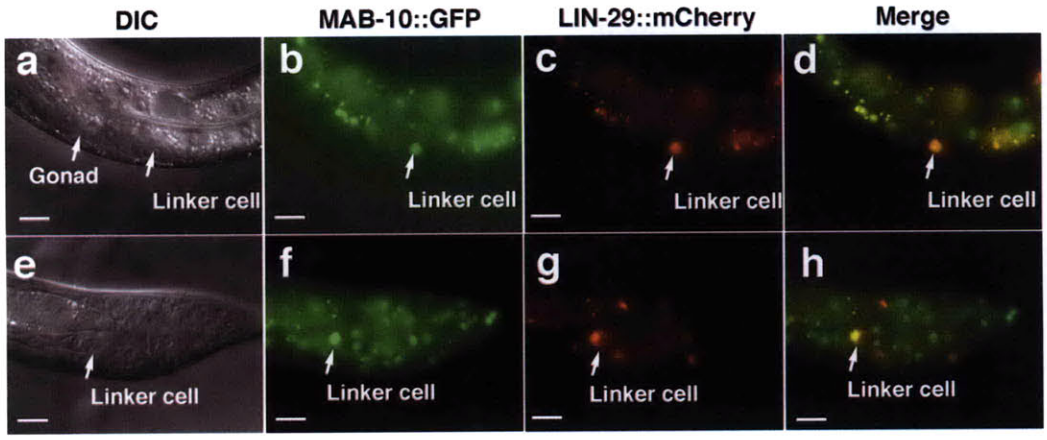
Figure 3

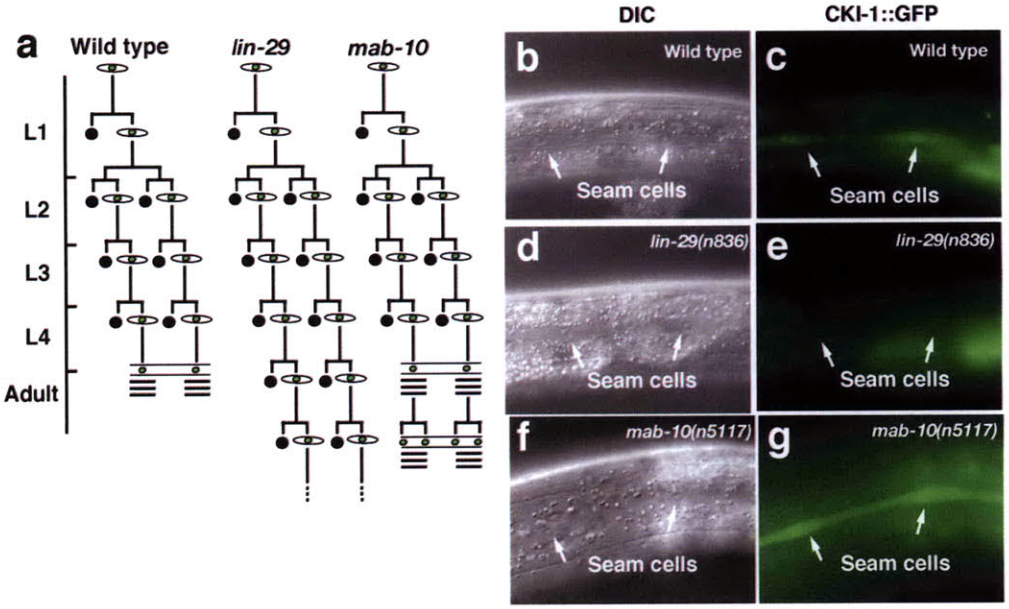


i

Genotype *	% males w/surviving linker cell	n
+	0	27
lin-29(n836)	66	32
mab-10(n5117)	42	31

* all strains contain: *him-8(e1489)*; *qIs56* [P_{lag-2}::*gfp*]

Figure 4



h

Genotype	Seam cell #	n
Wild type	11.1 ± 0.4	34
mab-10(n5117)	15.8 ± 2.6	40
rnt-1(ok351)	8.9 ± 1.6	23
bro-1(tm1183)	9.1 ± 1.3	22
rnt-1(ok351); mab-10(n5117)	9.2 ± 2.1	29
bro-1(tm1183); mab-10(n5117)	9.9 ± 0.8	23

all strains contain *mals105 [col-19::gfp]*

Figure 5

Acknowledgments

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Chapter Four

Future Directions

David Harris

Introduction

The identification of MAB-10 as a cofactor for LIN-29 opens up several interesting avenues of research with respect to hypodermal terminal differentiation in *C. elegans* and to NAB/EGR regulation in general. Our work along with studies of *Drosophila* and mammalian systems has demonstrated that NAB and EGR-like proteins act together as a conserved module to 1) control the terminal differentiation of cell lineages and 2) to promote the onset of adulthood[1-4]. Future work to uncover the specific mechanisms by which NAB proteins exert their function on EGR-like protein target genes is required for a better understanding of these processes. The study of *C. elegans* seam cell terminal differentiation provides an excellent opportunity to address these mechanisms and perhaps also discover new pathways that work in parallel.

A combination of genetic and biochemical approaches would allow for the identification not only of factors that work with MAB-10 and LIN-29 but also of those factors that work in parallel to control the same processes. New insights into the terminal differentiation process will contribute to our understanding of *C. elegans* biology and will also likely contribute to our understanding of NAB/EGR function.

Identifying regulators of MAB-10 and LIN-29 expression

In Chapter 3 we showed that *mab-10* and *lin-29* are expressed dynamially in several tissues throughout development. We also showed that the presence of a MAB-10::GFP fusion protein in the seam cells during the L4 stage is dependent on *lin-29*; however, endogenous *mab-10* mRNA was not diminished in a *lin-29* mutant background. Therefore *mab-10* is unlikely to be

a downstream target of LIN-29, and other factors must control the expression of both *mab-10* and *lin-29*.

Screens to identify mutants with altered MAB-10::GFP or LIN-29::mCherry expression

F2 non-clonal screens for mutants that disrupt the expression patterns of MAB-10::GFP and LIN-29::mCherry might allow for the identification of factors that control their expression either temporally or spatially. This approach might identify new regulators of developmental timing and also new factors that integrate timing with tissue specific cell-fate specification. Furthermore, because both the MAB-10::GFP and LIN-29::mCherry reporters are translational fusions, the screen could also identify factors required for proper MAB-10 and LIN-29 localization.

Does the heterochronic pathway control expression of *mab-10* and *lin-29*?

While MAB-10 and LIN-29 first appear in seam cells and surrounding hypoderm during the L4 stage, a transcriptional *lin-29*::*lacZ* reporter was expressed in the hypoderm as early as the L2 stage[5]. We showed in Chapter 3 that *mab-10* and *lin-29* are both expressed in the hypoderm as early as the L2 stage. These observations suggest that both *mab-10* and *lin-29* might be subjected to some form of post-transcriptional regulation.

Single molecule FISH (Fluorescence *In Situ* Hybridization) could be used to determine if *mab-10* and *lin-29* are regulated at the mRNA level by any of the previously characterized heterochronic mutants. This approach allows for a quantitative analysis of *mab-10* and *lin-29* mRNA levels in a cell- and tissue-specific fashion. For example, since *hbl-1* is a transcription factor[6-8], we might predict increased or precocious accumulation of *lin-29* mRNA during the L2

or L3 stage in an *hbl-1* mutant background. This approach could be followed for all known heterochronic mutants.

Identifying MAB-10 interactors

Screens to identify factors that control seam cell exit from the cell cycle

A screen for mutants in which the seam cells undergo extra divisions during adulthood might identify factors that regulate seam cell exit from the cell cycle. Previous screens have been performed to identify mutants that generate an abnormal number of seam cells during development[9]. However, the seam cells of *mab-10* animals inappropriately divide when the animals are adults, and therefore *mab-10* mutants have not been isolated from any seam cell based screens.

Focusing on the identification of mutants that undergo abnormal adult seam cell divisions might allow for the identification of animals with a *mab-10*-like seam cell defect. To facilitate the identification of these mutants, two different seam cell reporters could be used. The integrated array *wIs78,* which expresses *scm::gfp* (Seam Cell Marker) and *ajm-1::gfp* (Adherens Junction Marker), allows for the easy visualization of seam cell nuclei and seam cell boundaries[10]. The presence of *ajm-1::gfp* would allow for the identification of mutants that are also defective in seam cell fusion, another aspect of seam cell terminal differentiation.

An alternative approach would be to use the integrated transgene *maIs105*, which expresses *col-19::gfp*[11]. COL-19 is an adult-specific collagen that is expressed in the hypoderm beginning in the late L4 stage and continuing into the adult stage. COL-19::GFP labels seam cell nuclei as well as all of the hypodermal nuclei derived from the seam cell lineage. The total

number of non-seam hypodermal nuclei present can be used to assess abnormalities that occur within the seam cell lineage early in development. Additionally, *col-19::gfp* itself is an indicator of adulthood, as its expression is adult-specific. *col-19::gfp* expression is almost completely absent in *lin-29* mutants and is reduced in *mab-10* mutants. Therefore, mutants that are defective in *col-19::gfp* expression might lead to the identification of more factors that act with *lin-29* and *mab-10*.

A candidate gene approach

NAB family proteins have been shown to act as either transcriptional co-activators or co-repressors in different biological contexts. The mammalian protein NAB2 has been shown to repress transcription by recruiting the CHD4 subunit of the NuRD (Nucleosome Remodeling and Deacetylase) complex[12]. It is possible that MAB-10 acts by recruiting LET-418 or CHD-3, the *C. elegans* CHD4 homologs. This interaction could be tested directly using *in vitro* pulldown techniques. However, the region within NAB2 that is thought to be responsible for CHD4 binding is not conserved in MAB-10[12]. A broader approach would be to knock down by RNAi, all known chromatin remodeling factors and look for defects in aspects of the larval-to-adult transition.

Biochemical purification of MAB-10 and LIN-29 interactors

Biochemical approaches could be used as an alternative to genetic screening to identify MAB-10 and LIN-29 interactors. LIN-29::mCherry or MAB-10::GFP could be isolated from whole worm extracts using either an anti-dsRed antibody or an anti-GFP antibody. Proteins that are bound to LIN-29::mCherry and MAB-10::GFP could be identified using mass spectrometry. We might expect that these two immunoprecipitations would pull down an overlapping set of

proteins. Co-IPs with LIN-29::mCherry could be performed in wild-type and *mab-10* mutant backgrounds. Proteins bound to LIN-29::mCherry in the wild-type but not in the *mab-10* mutant background might bind LIN-29 via an interaction with MAB-10. These factors might represent a MAB-10 complex that is recruited to LIN-29 target genes to control transcription.

The identification of MAB-10 and LIN-29 target genes

Screens for enhancement or suppression of *mab-10(e1248)* mutant phenotypes

mab-10 enhancer and suppressor screens might be a powerful way to identify *mab-10* interactors. *mab-10(e1248)* is an intermediate strength allele of *mab-10* with approximately three seam cells undergoing an extra cell division. An enhancer screen for mutants that increase the number of extra seam cell divisions in a *mab-10(e1248)* mutant background might identify factors that would not have an extra seam cell division defect in a non-sensitized background.

Alternatively, a screen for mutants that suppress the extra seam cell division defect of *mab-10* might identify factors that act downstream of or parallel to *mab-10*. Based on our results from Chapter 3 we would expect to recover loss-of-function alleles of both *rnt-1*[13,14] and *bro-1*[9].

A screen for suppressors of the *mab-10* molting defect might identify factors that act downstream of *mab-10* to control exit from the molting cycle. *mab-10* males have a completely penetrant extra molt phenotype that is easy to observe using a dissecting microscope. This screen could be performed in a *mab-10*; *him-8* (High Incidence of Males) mutant background to maximize the generation of male animals. Previous work has shown that loss-of-function mutations in *nhr-23* or *nhr-25* or overexpression of *mir-84* can suppress the extra molt defect of

lin-29 animals[15]. Therefore we might expect to recover alleles of *nhr-23* and *nhr-25* from this screen.

Chromatin immunoprecipitation to identify target genes

LIN-29 and MAB-10 likely regulate several downstream target genes during the larval-to-adult transition. Whole-genome approaches to identify relevant candidate genes have not been explored and might reveal targets that are otherwise unattainable. Recent work in *C. elegans* has established an efficient pipeline for the use of Chromatin Immunoprecipitation followed by deep sequencing (CHIP-Seq) to identify transcription factor binding sites across the genome[16]. This approach involves cross-linking a GFP-tagged variant of the candidate transcription factor to chromatin and performing an immunoprecipitation with anti-GFP antibody. The isolated DNA is then subjected to deep sequencing. The sequencing results are then compared to sequencing results from non-immunoprecipitated control DNA and sites of enrichment are treated as candidate binding sites.

To identify candidate LIN-29 binding sites, a chromatin immunoprecipitation can be performed with an anti-dsRED antibody on a strain containing a LIN-29::mCherry fusion protein. To identify target genes regulated by MAB-10 and LIN-29 together, chromatin immunoprecipitations can be performed with an anti-GFP antibody on wild-type and *lin-29* mutant backgrounds carrying a MAB-10::GFP fusion protein. Binding sites identified from ChIP-Seq from the wild-type background represent the total pool of possible MAB-10 target genes, and these binding sites can be compared to sites obtained from the LIN-29::mCherry ChIP-seq. Performing the MAB-10::GFP chromatin immunoprecipitation in a *lin-29* mutant background would theoretically eliminate MAB-10 binding to those target genes that require LIN-29 activity. This approach might be very effective at identifying binding sites both specific

to LIN-29 and shared by LIN-29 and MAB-10. The identification of binding sites would give us a list of candidate target genes as well as a possible consensus binding site that could be used to predict more targets throughout the genome. These candidates could be tested for enhancement or suppression of *lin-29* and *mab-10* mutant phenotypes using available mutations or RNAi.

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Appendix I

A screen to identify negative regulators of programmed cell death in *C. elegans*

David T. Harris and H. Robert Horvitz

Summary

Mutations that prevent programmed cell death in *C. elegans* lead to the inappropriate survival of cells normally fated to die. Screens designed to identify these mutations have identified numerous pro-apoptotic genes required both for the specification of programmed cell death and for its execution. Far fewer negative regulators of programmed cell death have been identified. *ced-9*, the best studied negative regulator of programmed cell death, was isolated as a rare gain-of-function mutation that prevented programmed cell death. Loss-of-function mutations in *ced-9* cause embryonic lethality resulting from the ectopic programmed cell death of cells not normally fated to die. The lethality of *ced-9* mutants suggests that there might be more anti-apoptotic factors that have not been isolated from previous screens because of a similar lethality. We designed and performed a pilot screen that allows for the identification of mutants with ectopic programmed cell death while preventing the associated lethality. We isolated three candidates potentially defective in the regulation of programmed cell death and we provide thoughts on possible ways in which they might be further studied.

Introduction

Apoptosis or programmed cell death (PCD) evolved in metazoans to shape developing tissues and organs, maintain tissue homeostasis, and remove damaged or potentially damaging cells. This process is crucial for the normal development of most metazoans, and a failure to properly regulate programmed cell death has been implicated in a multitude of disorders including Alzheimer's disease[1], heart attacks, cancer[2] and autoimmunity[3].

Programmed cell death in *C. elegans*

During development of the *C. elegans* hermaphrodite 131 of the 1090 somatic cells generated undergo programmed cell death[4,5]. Genetic studies have identified over 20 genes that regulate four different phases of this process: 1) specification, the decision of a cell to die; 2) execution, the process by which a cell dies; 3) engulfment, the enveloping of a dead cell by a neighboring cell; and 4) degradation, the process by which the dead cell is broken down and disposed of. The analysis of programmed cell death in *C. elegans* lead to the discovery of four genes that regulate the execution of PCD in all somatic cells[6-8]. Strong loss-of-function mutations in *egl-1*, *ced-4* or *ced-3* prevent nearly all somatic programmed cell deaths from occurring but have virtually no effect on the health of the animal, while loss-of-function mutations in *ced-9* result in embryonic lethality as a result of ectopic PCD. This embryonic lethality is completely suppressed by loss-of-function mutations in either *ced-3* or *ced-4* but not *egl-1*. These observations suggested that *egl-1* acted upstream of *ced-9* and that *ced-3* and *ced-4* acted downstream of *ced-9*. Interestingly, weak *ced-3* or *ced-4* loss-of-function mutations, which on their own have virtually no cell death defect, can completely suppress the lethality associated

with *ced-9*. This suppression suggests that even cells that are not fated to die, possess just enough killing activity to induce apoptosis in the absence of an anti-apoptotic factor.

Programmed cell death is evolutionarily conserved

Early work using mammalian systems led to the discovery of the first molecularly identified gene to play a role in the regulation of apoptosis. The *Bcl-2* gene was originally identified by the mapping of the breakpoints of a translocation commonly associated with B-cell lymphoma. The fusion of the *Bcl-2* gene downstream of the immunoglobulin heavy chain locus results in the inappropriate expression of *Bcl-2* and an increase in B cell survival[2,10,11]. Like *Bcl-2*, the gene *ced-9* was originally identified by a rare gain-of-function mutation that caused an increase in cell survival[8,12]. The cloning of *ced-9* and the discovery that CED-9 protein is homologous to BCL-2 revealed that the execution phase of the PCD pathway is conserved between *C. elegans* and vertebrates[9]. The cloning of the genes *ced-4* and *ced-3* provided the first implication that their vertebrate homologues may play a role in regulating apoptosis. The cloning of *ced-4* was followed five years later by the biochemical purification of the mammalian counterpart Apaf-1 (Apoptosis Protease Activating Factor-1)[13,14]. *ced-3* was found to encode a cysteine protease known as a caspase (Cysteine Aspartate Specific Protease), providing the first evidence for a mechanism of PCD execution[15]. *egl-1* encodes a protein containing a single BH3 (Bcl-2 Homology domain 3) domain that is found in BCL-2, joining a family of proteins known as the BH3-domain only proteins[6], which, like EGL-1, are also pro-apoptotic.

In the current model of PCD in *C. elegans*, CED-9 (BCL-2) binds to CED-4 (APAF-1) sequestering it to the mitochondrial membrane and preventing the activation of the caspase CED-3. In cells fated to die, the BH3-only protein EGL-1 is expressed and interacts with CED-9,

resulting in the re-localization of CED-4 (APAF-1) to the nuclear membrane[16] and the subsequent activation of CED-3 (Caspase).

Negative regulators of PCD

While *ced-9* is the best characterized negative regulator of PCD in *C. elegans*, recent works have suggested that two caspases might also have anti-apoptotic functions. One lab has reported that CSP-2 (CaSPase) and CSP-3 inhibit programmed cell death by binding to CED-3 to prevent its autoactivation[17,18]. Loss-of-function mutations in either CSP-2 or CSP-3 are reported to cause a small increase in the number of cell deaths observed in the germline in the case of CSP-2 and in the anterior pharynx in the case of CSP-3. However, attempts by another have failed to reproduce these findings (D. Denning, personal communication).

In other organisms, IAP (Inhibitors of APoptosis) proteins inhibit apoptosis by binding to and inactivating caspases[19]. While *C. elegans* encodes two proteins related to IAP proteins, BIR-1 and BIR-2, neither of them seem to play a role in regulating apoptosis[20].

Anti-apoptotic proteins are common targets for cancer therapeutics and the identification of new anti-apoptotic proteins opens the door to new therapies. Previous studies of *C. elegans* have contributed greatly to our understanding of both pro- and anti-apoptotic mechanisms. However, genetic screens for anti-apoptotic factors are complicated by the fact that the disruption of an inhibitor of apoptosis, like *ced-9*, is likely to cause embryonic lethality as the result of too much programmed cell death. This embryonic lethality has made identifying genes that function similarly to *ced-9* difficult. Therefore, we designed a screen that allows for the detection of increased PCD in a small number of cells while the rest of the animal remains cell-death deficient.

Results

A screen to identify negative regulators of programmed cell death

We designed a screen that would allow the rapid isolation of mutations that enhance the ability of ectopically expressed *ced-3* to kill. The strategy for the screen involved the creation of transgenic *ced-3(n717)* animals that express *gfp* and *ced-3* under the control of two different promoters in an overlapping subset of cells. The *mec-3* promoter drives the expression of *gfp* in the six touch neurons (ALML/R, AVM, PLML/R, and PVM) as well as four non-touch neurons (FLPL/R, PVDL/R)[21]. The *mec-7* promoter drives the expression of the *ced-3* killer gene in the six touch neurons only (ALML/R, AVM, PLML/R, and PVM) (Figure 1)[22]. *ced-3(n717)* animals carrying this transgene would be lacking functional CED-3 in all cells except for the touch neurons. Therefore, only the touch neurons are competent to undergo apoptosis. In this strain, mutations that inactivate anti-apoptotic factors would cause the death of the touch neurons, but would not affect viability, as the rest of the animal is cell-death deficient. For this strategy to be successful it is important to create transgenic animals that express *ced-3* at a low enough level as to not induce apoptosis on their own, but at a high enough level to induce apoptosis in the absence of an anti-apoptotic factor.

We selected transgenic lines in which all ten *gfp*-expressing cells were visible. Animals consistently missing one or more of the touch neurons were likely expressing *ced-3* at a level sufficient to kill the cell and were not suitable for screening. A candidate extrachromosomal array was selected and integrated into the genome using gamma irradiation to generate *nIs174*.

Loss of *ced-9* function causes the death of touch neurons carrying *nIs174*

To verify that animals carrying *nIs174* were expressing *ced-3* in the touch neurons and that the touch neurons were competent to undergo programmed cell death, we crossed *nIs174* into a *ced-9(n2812); ced-3(n717)* mutant background. In the absence of *ced-9* function, there was a substantial decrease in the number of GFP-positive touch neurons (Table 1). *ced-9(n2812)* does not alter the number of GFP-positive FLPs and PVDs. We verified that the absence of touch neurons is caused by programmed cell death using Nomarski optics to observe GFP-positive cell corpses at the expected positions in the embryos of this strain (Figure 3). These results demonstrate that the removal of *ced-9* from a *ced-3(n717) nIs174* mutant background causes the observable death of the touch neurons while maintaining the viability of the animal.

Pilot Screening

ced-3(n717) nIs174 animals were subjected to EMS mutagenesis[23], and their F_2 progeny were screened for the absence of the CED-3-expressing touch neurons. We screened approximately 10,000 haploid genomes and picked a total of 35 animals that were missing some or all six touch neurons. Of the 35 isolates, 18 gave rise to only wild-type progeny, 10 were missing all six touch neurons but failed to develop to adults, three were missing all 10 GFP-positive neurons, and four were missing the touch neurons specifically (Table 2).

We proceeded to quantify the absence of the ALMs and PLMs in those mutants that were lacking the touch neurons specifically (Table 3). We found that the ALMs and PLMs of isolates *n4772*, *n4773*, and *n4774* died, and these animals exhibited normal *mec-3::gfp* expression. The touch neurons of *n4679* animals were absent, but the remaining *mec-3::gfp* expressing cells

showed an obvious increase in GFP fluorescence. The increased fluorescence suggests that *n4679* affects transgene expression and not endogenous PCD regulation.

Discussion and Future Directions

The preliminary results from my pilot screen suggest that I might be able to identify factors that are necessary for the proper regulation of programmed cell death. However, further experiments demonstrated that the expression of the *mec-3::gfp* transgene was quite variable in different mutant backgrounds, suggesting that the expression level of the *mec-7::ced-3* transgene might also be variable. Because increased expression of the *mec-7::ced-3* transgene can lead to ectopic touch neuron death, it is not clear if the touch neuron death that I observed in other mutant backgrounds was the result of the mutation in question or of increased *mec-7::ced-3* expression in that specific background. Furthermore, *nIs174* was sufficient to kill the touch neurons in the polymorphic CB4856 mapping strain, significantly reducing the feasibility of mapping screen isolates using single nucleotide polymorphisms.

One possible way to avoid the expression variability of *nIs174* is to generate and test several more transgenic lines. It is possible that the variability observed with *nIs174* is specific to this integrated transgene and other transgenes might be more stable.

Recent advances in whole-genome sequencing technology might allow for the direct sequencing of screen isolates and avoid the complications that arise by introducing the *nIs174* transgene into different mutant backgrounds. Using this approach, a large number of isolates could be generated, complementation tests could be performed between the isolates, and two isolates from each complementation group could be submitted for whole-genome sequencing.

The identification of mutations in the same gene from isolates within the same complementation group would likely be sufficient to identify causative mutations.

Once causative mutations are identified, they could be crossed out of the *ced-3(n717) nIs174* background into either a wild-type or a *ced-3(n2427)* mutant background. Isolates that are inviable or abnormal in the presence of wild-type copies of *ced-3* should have the highest priority with respect to further investigation, as they would likely have the most robust PCD defect. Isolates that have a PCD defect in a *ced-3(n2427)* background likely have a more subtle cell-death defect.

Materials and Methods

Strains and Genetics

Strains were cultured as described[23] and grown at 20° C on NGM agar using *E. coli* OP50 as a food source. The Bristol strain N2 was the wild-type strain. The mutations used in the study are as follows and are previously described[24] unless otherwise indicated:

LGIII, *ced-9(n1950 n2077), ced-9(n2812).*

LGIV, *ced-3(n717), nIs174*[*mec-3::gfp*; *mec-7::ced-3*; *unc-76(+)*] (this study).

LGV, *unc-76(e911)*

Plasmid construction

Plasmid pDH10 (*mec-7::ced-3*) was generated by digesting pS126 with SpeI and SmaI. The resulting fragment was ligated into pPD52.102 digested with NheI and EcoRV.

Transgenics

mec-3::gfp (pPD118.17), *mec-7::ced-3* (pDH10), *unc-76(+)*, and 1 kb plus DNA ladder were injected into *ced-3(n717); unc-76(e911)* at 20 ng/ul, 1 ng/ul, 50 ng/ul, and 50 ng/ul respectively. Stable extrachromosomal arrays were selected based on the presence of ten *mec-3::gfp* expressing neurons. Arrays were integrated into the genome using gamma irradiation.

Quantitation of touch neuron death

L4 animals expressing *mec-3::gfp* in the touch neurons were visualized using a dissecting microscope equipped with fluorescence optics (M2BIO, Kramer Scientific, Valley Cottage, NY). Touch neurons were scored as absent if no *mec-3::gfp* expression was observed.

Screen for mutants without touch neurons

We mutagenized *ced-3(n717) nIs174; unc-76(e911)* hermaphrodites with EMS as previously described[23]. Mutagenized hermaphrodites were picked to large plates in groups of five. Five to six days later, gravid adults were washed from each plate and bleached to release embryos. Embryos were allowed to hatch overnight in M9 to synchronize the population and arrested L1 animals were spotted the next day. Touch neuron death was assayed two days later.

Figure Legends

Figure 1

A diagram showing the expression of GFP and *ced-3* driven by the *mec-3* and *mec-7* promoters, respectively. The top panel shows *Pmec-3::gfp* expression (green) in the six touch

neurons and the four non-touch neurons. The bottom panel shows *Pmec-7::ced-3* expression (orange) in only the touch neurons.

Figure 2

Diagram for the F_2 non-clonal screen to identify hermaphrodites that are missing the touch neurons. Hermaphrodites containing the transgene expressing GFP and CED-3 were mutagenized with EMS. Their F_2 progeny were screened non-clonally for animals that had lost GFP in the six touch neurons but not the four non-touch neurons.

Table 1

The transgene *nIs174* kills touch neurons in *ced-9(lf)* animals but not in *ced-9(+)* animals. Animals were scored for the presence or absence of the ALM neurons in either a *ced-3(n717)*, a *ced-9(n2812); ced-3(n717)* background. The percent of surviving ALM neurons is shown.

Figure 3

The dying ALM and PLM neurons become refractile corpses. To determine if the absence of the ALM and PLM neurons induced by the *nIs174* transgene in a *ced-9(n2812)*; *ced-3(n717)* background was the result of programmed cell death, we directly observed corpse formation in late stage embryos. Arrows indicate refractile corpses.

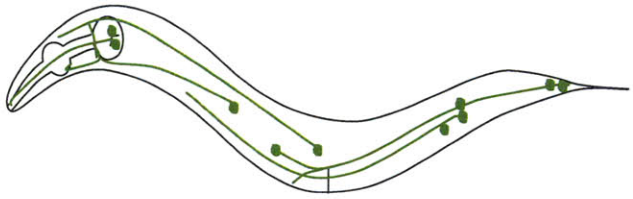
Table 2

Results of a pilot screen for mutants defective in the control of programmed cell death. Approximately 10,000 haploid genomes were screened for the absence of the touch neurons in *ced-3(n717) nIs174* animals and isolates were grouped into four categories.

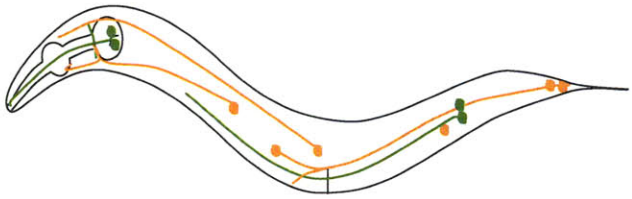
Table 3

Three isolates have increased touch neuron death without increased GFP fluorescence. Isolates *n4772*, *n4773*, and *n4774* showed increased cell death in the ALMs and PLMs indicating that those strains might be deficient for an anti-apoptotic factor. Isolate *n4769* had increased cell death but also had increased GFP fluorescence, suggesting that this strain might have increased transgene expression. Loss-of-function mutations in the anti-apoptotic gene *ced-9* also cause increased touch neuron death mediated by *nIs174*.

Figure 1

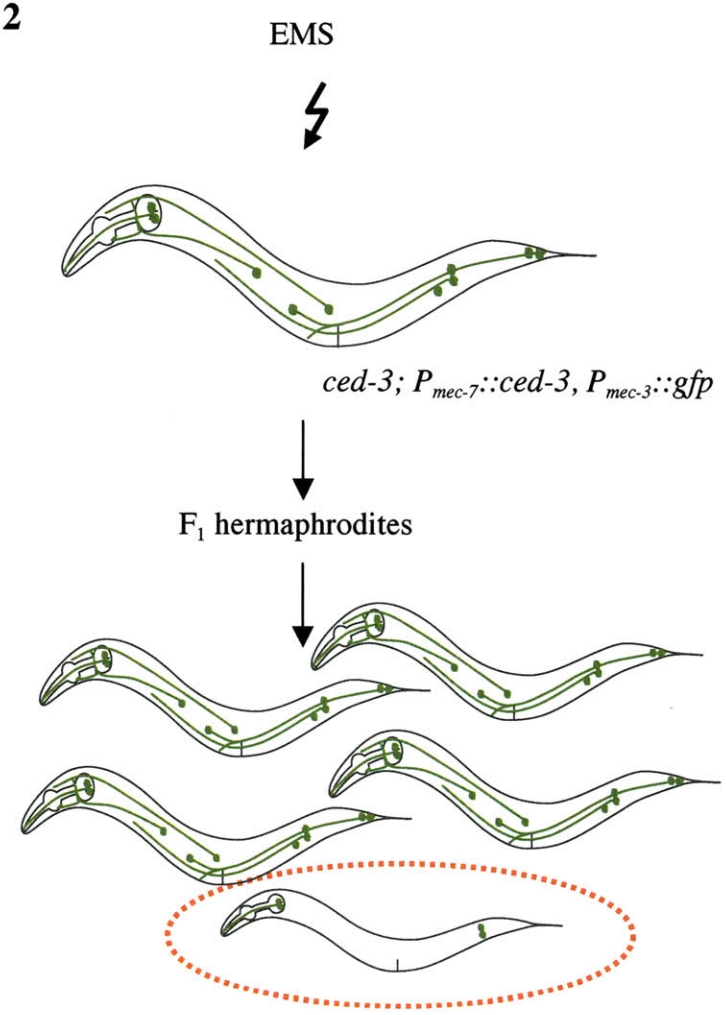


$P_{mec\text{-}3}$*::gfp* is expressed in 10 neurons



$P_{mec\text{-}7}$*::ced-3*, $P_{mec\text{-}3}$*::gfp*
Orange cells represent the *ced-3*-expressing touch neurons

Figure 2



Screen for F_2 hermaphrodites without GFP expression in the touch neurons.

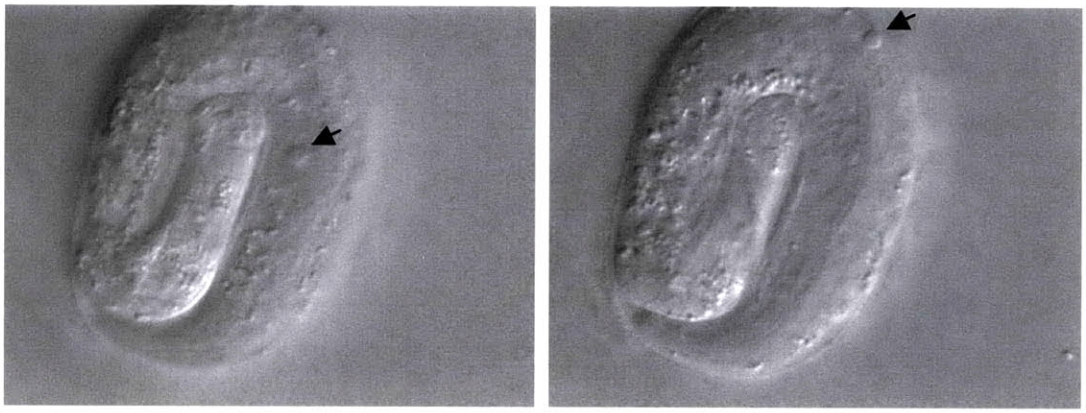
Table 1

	Percent surviving ALMs (no. ALMs/no. sides scored)	
	Genetic background	
	ced-9(n2812); ced-3(n717)	*ced-3(n717)*
$P_{mec\text{-}7}$*::ced-3 nIs174*	40 (16/40)	100 (40/40)
$P_{mec\text{-}3}$*::gfp*	100 (48/48)	100 (50/50)

Figure 3 **Cell corpses corresponding to touch neurons**

ced-9(n2812); ced-3(n717) nIs174

ALML and ALMR



PLML and PLMR

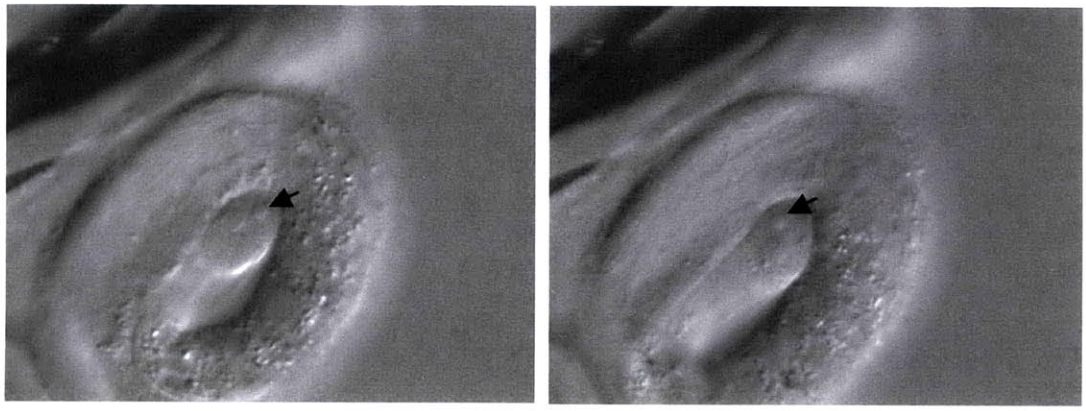


Table 2

Results of pilot F2 screen (~10,000 genomes)

4	**Positively re-tested for the absence of the touch neurons**
3	Positively re-tested for the absence of all GFP-positive neurons
10	Missing all six touch neurons but produced no progeny
18	Failed to re-test for the absence of GFP-positive neurons
35	Total candidates

Table 3

Characterization of Isolates

Percent surviving neurons (no. neurons/no. sides scored)

	L1 larvae		Adult
Genotype	**PLMs**	**ALMs**	**ALMs**
+	**55 (22/40)**	**100 (40/40)**	**95 (19/20)**
n4774	**4.7 (2/42)**	**26 (11/42)**	**17 (7/42)**
n4773	**15 (5/34)**	**79 (27/34)**	**43 (20/46)**
n4772	**21 (9/42)**	**29 (12/42)**	**10 (4/40)**
n4769*	**0 (0/40)**	**20 (8/40)**	**ND**
ced-9(n1950 n2077)	**13 (5/40)**	**63 (25/40)**	**40 (16/40)**
ced-9(n2812)	**9 (3/32)**	**59 (19/32)**	**ND**

All strains contained *ced-3(n717)* and *nIs174*

*** GFP intensity was noticeably increased in surviving GFP-positive cells.**

Acknowledgments

We thank Daniel Denning for comments on this appendix.

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Appendix II

A screen to identify tissue-specific alleles of *egl-1*

Brendan D. Galvin, David T. Harris, and H. Robert Horvitz

I performed the non-complementation screen and characterized *egl-1(n4629)* during my rotation in the Horvitz lab. Brendan Galvin designed the screen and constructed the necessary strains.

Introduction, Result, and Discussion

The BH3-only killer gene *egl-1* is the most upstream gene that functions in the execution phase of programmed cell death in *C. elegans* and is thought to be expressed primarily in cells that die[1]. The mechanisms by which *egl-1* gene expression is regulated are known for only a small number of cells that die, including the HSNs[2], the NSM sister cells[3-5], and P11.aaap[6] (a neuron located in the posterior ventral nerve cord). In the *C. elegans* ventral nerve cord of the hermaphrodite, 12 cells are generated in the lineal position Pn.aap (the posterior daughter of the anterior daughter of the anterior daughter of one of 12 P blast cells); six of those 12 (the two anterior-most, P1.aap and P2.aap, and the four posterior-most, P9-P12.aap) die by programmed cell death[7] (Figure 1). The six Pn.aap cells that survive differentiate into class VC motor neurons, some of which innervate the vulval muscles of the adult hermaphrodite. The *lin-11::gfp* reporter *nIs106* is expressed in Pn.aap cells, including Pn.aap cells that are normally fated to die but the deaths of which have been prevented by mutations blocking programmed cell death[8] (Figure 1). This reporter is useful in studying programmed cell death, because it provides a measure of programmed cell death that is both quantifiable at the single-animal level and easily scored using a dissecting microscope.

Previously, a screen for programmed cell death mutants using *lin-11::gfp* as a reporter identified a mutation in *egl-1*, *egl-1(n4045)*, that resides in an evolutionarily conserved DNA element four kb downstream of the coding sequence. This mutation specifically prevents the death of cells within the ventral nerve cord and likely defines a ventral nerve cord-specific *egl-1* regulatory element. However, because of the distance from the *egl-1* coding sequence and the non-coding nature of the mutation, it was not certain if the point mutation identified in *egl-1(n4045)* was causative.

To identify additional alleles of *egl-1* that might define new regulatory elements and to better define this previously identified regulatory element, we performed an *egl-1* non-complementation screen using the *lin-11::gfp* reporter. We mated mutagenized males with *egl-1(n1084 n3082)* hermaphrodites and screened for animals with undead VC-like neurons. In a screen of approximately 13,000 haploid genomes, we isolated the single *egl-1* allele *n4629*. *n4629* contains the identical base change identified in *egl-1(n4045)*. The identification of a second mutation in the exact base that is mutated in *egl-1(n4045)* is strong evidence that both *n4045* and *n4629* cause the programmed cell death defect. Based on this result we have named the conserved region surrounding *n4045* and *n4629* the VCK (VC-like Killing) element (Figure 2).

Materials and Methods

Strains and Genetics

Strains were cultured as previously described[9] and grown at 20° C on NGM agar using *E. coli* OP50 as a food source. The Bristol strain N2 was the wild-type strain. The mutations and balancer chromosomes used in the study are as follows and have been previously described[10] unless otherwise indicated:

LGI, *nIs133*[*pkd-2::gfp*; *lin-15AB(+)*][11,12]

LGV, *rol-4(sc8)*, *unc-76(e911)*, *egl-1(n1084 n3082)*, *egl-1(n4045 and n4629)* (this study).

LGX, *nIs106*[*lin-11::gfp*; *lin-15AB(+)*][8]

egl-1 non-complementation screen

We mutagenized L4 *nIs133*; *nIs106* males with EMS as previously described[9] and mated them with *nIs133*; *rol-4(sc8) egl-1(n1084 n3082) unc-76(e911)*; *nIs106* hermaphrodites. After 3-4 days, F_1 non-Rol non-Unc cross progeny were screened using a fluorescence microscope to identify animals with surviving GFP-positive VC-like neurons. Animals carrying putative *egl-1* mutations were picked to single plates and allowed to self. Twelve non-Rol non-Unc progeny from each candidate were picked individually to plates. Progeny from plates containing only non-Rol non-Unc animals were scored for VC-like neuron survival.

nIs133 was included in the background because it expresses GFP in the male-specific CEM (Cephalic Male) sensory neurons. In hermaphrodites, the CEM neurons die during embryogenesis in an *egl-1* dependent fashion; therefore, mutations that prevent the death of the CEM neurons might also be detected. However, during screening we focused on the cells within the ventral cord and therefore mutations affecting the death of the CEM neurons were likely missed.

Determination of mutant allele sequences

We used PCR-amplified regions of genomic DNA to determine mutant sequences. For *egl-1(n4629),* we determined the sequence of the coding region and the interval containing the *egl-1(n4045)* mutation.

Figure Legends

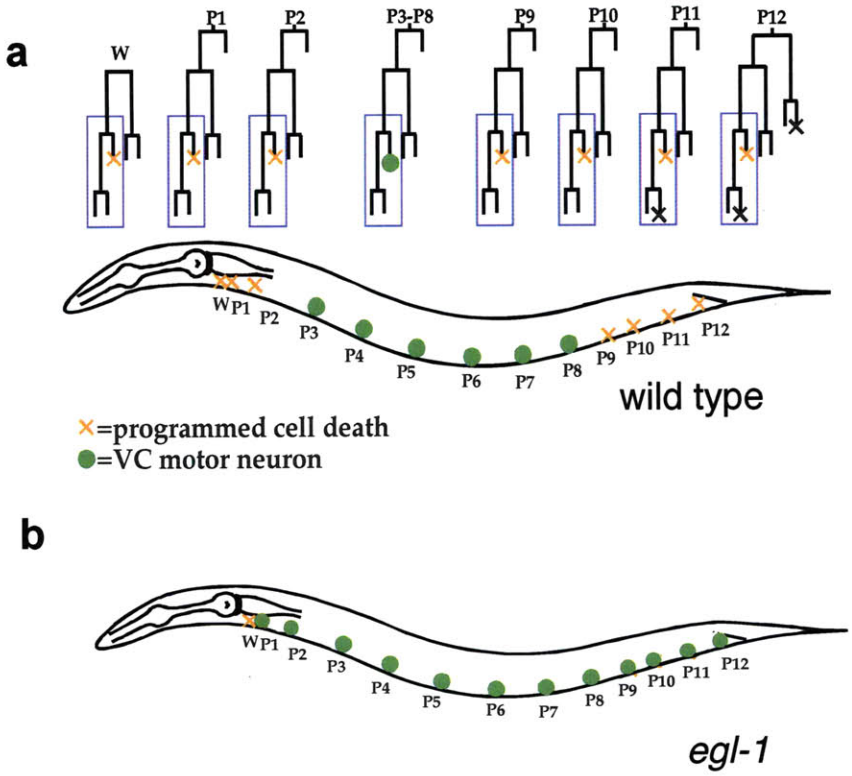
Figure 1

The *lin-11::gfp* reporter is an excellent marker for programmed cell death in the ventral nerve cord. a, In the ventral nerve cord of wild-type animals, six of the 12 Pn.aap cells that are born die by programmed cell death. The surviving six Pn.aap cells express the *lin-11::gfp* reporter. **b,** In a cell death defective mutant, several surviving Pn.aap cells express *lin-11::gfp*.

Figure 2

The *egl-1(n4629)* mutation is identical to the *egl-1(n4045)* mutation. *egl-1(n4629)* and *egl-1(n4045)* carry the identical mutation in an evolutionarily conserved block of sequence approximately 4 kb downstream of the *egl-1* coding region.

Figure 1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wild type
×=programmed cell death
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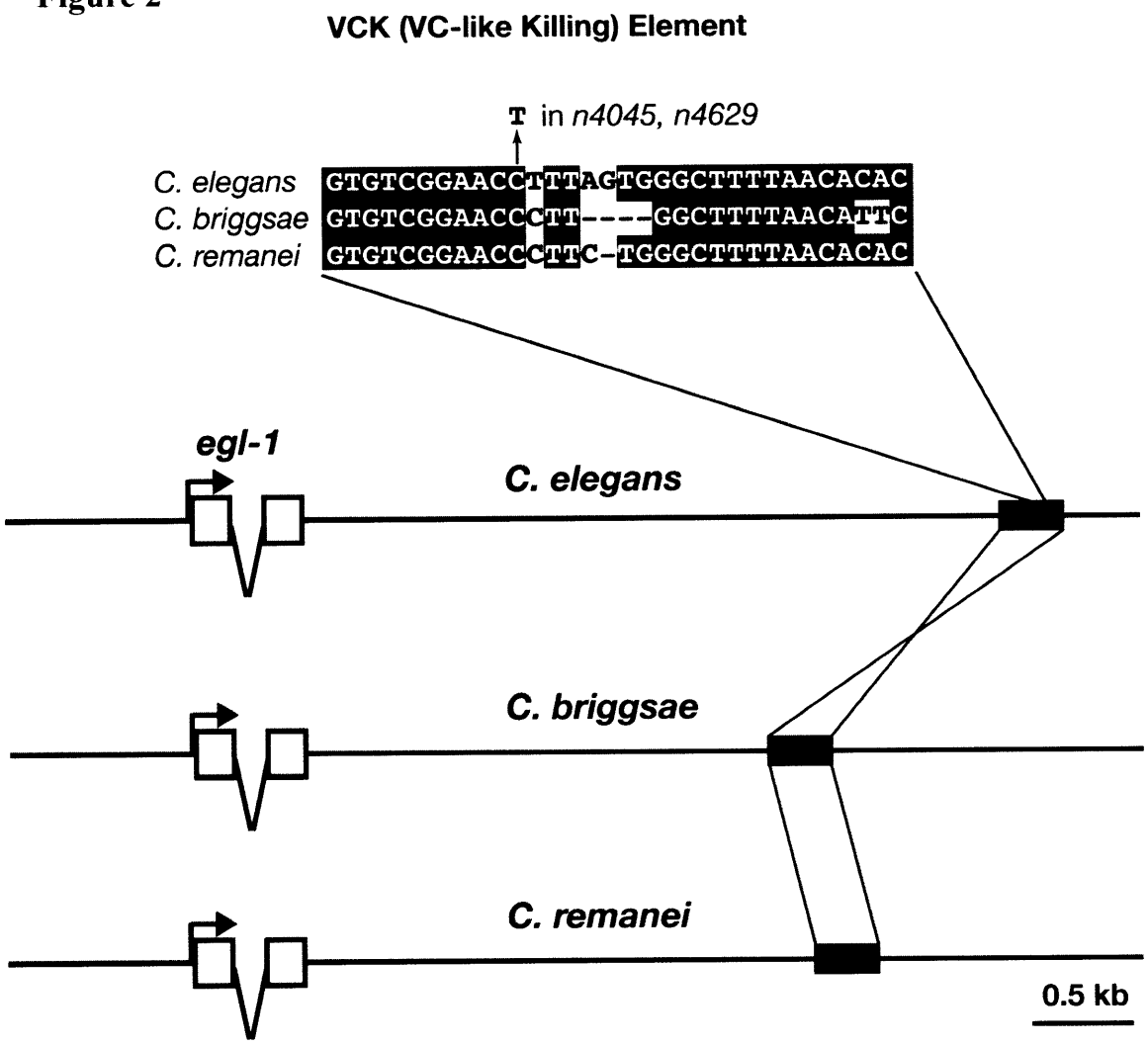
egl-1

Figure 2


VCK (VC-like Killing) Element
T in n4045, n4629
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C. briggsae GTGTCGGAACCCTT----GGCTTTTAACATTC
C. remanei GTGTCGGAACCCTTC-TGGGCTTTTAACACAC
egl-1
C. elegans
C. briggsae
C. remanei
0.5 kb

Acknowledgments

We thank Na An and Beth Castor for technical assistance and Daniel Denning for comments on this appendix.

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