Control of developmental timing in *Caenorhabditis elegans*

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Studies of the nematode *Caenorhabditis elegans* have identified genetic and molecular mechanisms controlling temporal patterns of developmental events. Mutations in genes of the *C. elegans* heterochronic pathway cause altered temporal patterns of larval development, in which cells at certain larval stages execute cell division patterns or differentiation programs normally specific for other stages. The products of the heterochronic genes include transcriptional and translational regulators and two different cases of novel small translational regulatory RNAs. Other genes of the pathway encode evolutionarily conserved proteins, including a homolog of the *Drosophila* Period circadian timing regulator, and a member of the nuclear receptor family of proteins. These regulators interact with each other to elaborate stage-specific regulatory switches and act through downstream effectors to control the timing of cell-type-specific developmental events.

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Abbreviations

gf	gain-of-function
lf	loss-of-function
UTR	untranslated region
VPC	vulva precursor cell

Introduction

Multicellular organisms develop with astonishing precision, considering the enormous complexity of development, involving processes that occur on the level of cells, tissues and the whole organism. Normal development depends on the accurate timing of cell division, cell-fate commitment and differentiation. Insights about the genetic and molecular mechanisms controlling developmental timing have come from studies of amphibian and insect development, revealing a central role for hormonal signals [1,2], and from studies of plants, where the timing of developmental phase transitions has been shown to be influenced by specific genes [3,4]. Unlike the control of spatial patterns of development, for which evolutionarily conserved molecular genetic pathways have been identified [5], however, the control of developmental timing in diverse species is understood only rather superficially.

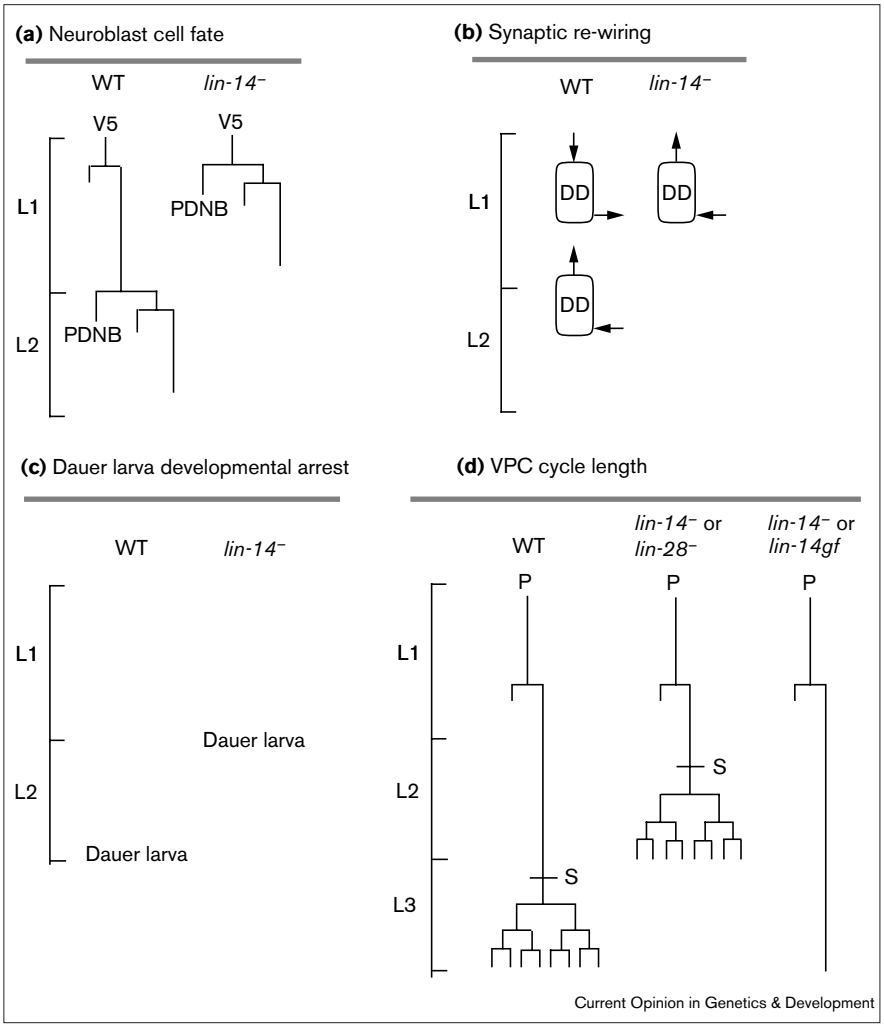
One of the better-characterized genetic pathways involved in the temporal control of development is the *Caenorhabditis elegans* heterochronic gene pathway, which controls the schedule of many developmental events that occur as the worm develops through its larval stages [6,7]. In this review, I focus on recent findings regarding the *C. elegans* heterochronic gene pathway that further illuminate the regulatory mechanisms at work in controlling developmental timing in this animal. The cloning of new genes of the pathway has provided fresh perspectives on the possible relationship of heterochronic genes to other systems. Specifically, there are now two heterochronic genes that are known to encode small antisense translational regulatory RNAs [8,9••]. This novel class of regulatory molecule, first identified in the *C. elegans* heterochronic gene pathway, could also lie as yet undiscovered in other pathways or other organisms. Furthermore, other heterochronic gene products have been identified recently that are similar to developmental or circadian timing regulators in other animals, suggesting common evolutionary origins of certain elements of biological timing mechanisms.

The schedule of *C. elegans* larval development

C. elegans development follows two major phases: embryogenesis, which results in the generation of a 550 cell larva, and postembryonic development, which comprises the four larval stages, and produces the reproductively competent adult. Numerous precursor cells set aside at the end of embryonic development divide according to a schedule associated with progression through the four larval stages (L1–L4). The postembryonic cell lineages derived from these precursor cells exhibit stage-specific patterns of cell division and cell-fate determination [10]. For example, certain cell lineages, such as the V5 hypodermal cell lineage (Figure 1a), exhibit patterns of cell division that distinguish the first larval stage from the second. Similarly, cell-fate determination in the vulval cell lineages, accompanied by a burst of cell proliferation and differentiation, occurs during a specific temporal window in the third larval stage (Figure 1d).

The heterochronic genes identified to date [7,8,9••,11••,12••] are part of a regulatory pathway whereby general temporal control genes act via more specialized regulators to control the timing of specific postembryonic developmental events in *C. elegans* larvae. These stage-specific events include cell division patterns [6], cell cycle progress [13], synaptic rewiring [14], developmental arrest [15] (Figure 1), stage-specific transcriptional activation and repression [16], and other differentiation and morphogenetic processes [6,17,18]. The pathway includes a set of early acting genes that acts as a timer for progression from hatching through the beginning of the L3, and a set of later-acting genes that specify the completion of L3 and L4 development (Figure 2). These genes do not affect embryonic development, even as null alleles [6,7,19], suggesting that separate genetic mechanisms control postembryonic and embryonic developmental timing.

Figure 1



Examples of heterochronic developmental defects. **(a)** The postdeirid neuroblast (PDNB), the anterior grand-daughter of V5, is generated in the wildtype (WT) at the second larval stage. In *lin-14(lf)* animals, V5 itself produces the PDNB in the L1 stage [6]. **(b)** In newly hatched wild-type L1 larvae, the ventral cord DD motoneurons receive their synaptic inputs dorsally, and send their outputs ventrally. In the L2, the DD neurons remodel their synaptic polarity such that they then receive their inputs ventrally and send their outputs dorsally. In *lin-14(lf)* animals, the DD neurons remodel precociously, in the L1 [14]. **(c)** Wild-type animals can adopt the optional developmentally-arrested larval form, the dauer larva, only at the second larval molt. In *lin-14(lf)* mutants, the dauer larva can form one stage earlier than normal, at the L1 molt [15]. **(d)** VPCs are the posterior daughters of P cells, and divide during the L3 stage after an extended G_1 phase of the cell cycle. In *lin-14(lf)* animals, the G_1 phase of the VPC cell cycle is shortened, and VPCs divide one stage earlier than normal, in the L2; in *lin-4(lf)* or *lin-14* gain-of-function *(gf)* mutants, which over-express *lin-14* late in development, the VPC cycle is lengthened [13].

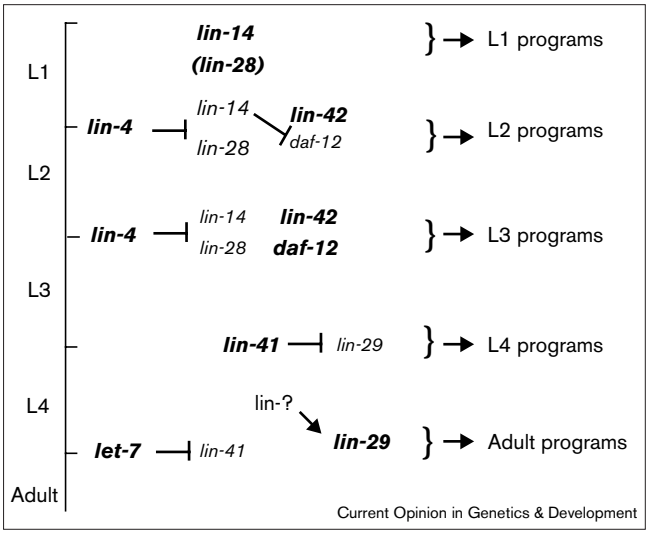
Specifying events of the early larval stages: translational repression by *lin-4* RNA causes a temporal decrease in LIN-14 and LIN-28

The genes *lin-14* and *lin-28* play a central role in the timing events of the first three larval stages. A progressive developmental decrease in LIN-14 and LIN-28 protein occurs during the L1–L3 stages [20,21]. As different levels of these gene products elicit distinct outcomes in terms of stage-specific gene expression [7,19], their decrease specifies the progressive execution of L1 and L2 developmental events, followed by L3 events (Figure 2).

Postembryonic development is initiated by feeding of the larva after hatching, which triggers larval growth and cell division, and the downregulation of *lin-14* and *lin-28* during the late L1 and L2 stages. Feeding leads to the expression of *lin-4*, a key repressor of *lin-14* and *lin-28* [8,21,22,23•,24]. The product of *lin-4* is a small antisense RNA translational regulator [8] that is complementary to sequences in the 3′ untranslated regions (UTRs) of *lin-14* [8,24] and *lin-28* [21] mRNAs. In *lin-4* mutants, *lin-14* and *lin-28* activities remain high throughout development, and the animal reiterates early larval developmental programs [21,25,26]. Studies of protein and RNA levels indicate that this progressive downregulation of *lin-14* and *lin-28* during postembryonic development occurs at the level of translation, and requires 3′ UTR sequences of the target mRNAs [21,25].

lin-14 and *lin-28* are subject to other regulatory inputs besides *lin-4*. Specifically, LIN-14 or LIN-28 protein levels seem to be downregulated in the absence of *lin-4* — provided that either *lin-28* or *lin-14* activities are either reduced or inactivated by mutation [21,26]. One explanation of this observation is that a positive feedback between *lin-14* and *lin-28* acts in opposition to downregulation [21,26]. Although *lin-4* is essential to repress *lin-14* and *lin-28* in the wildtype — where they reinforce each other by positive feedback — it would appear that a *lin-4*-independent repressive mechanism can downregulate either *lin-14* or *lin-28* alone, when the positive feedback is disengaged [21]. *lin-4* may act in parallel to, or in conjunction with this other repressive activity. It is not yet known

Figure 2



Stage-specific activities of genes of the heterochronic pathway. Gene names are shown in a smaller font at stages where they have been shown to be repressed by one or more upstream gene in the pathway. Genes are in a large, bold font where they are active and required for the normal expression of the corresponding stage-specific programs. The time axis at left starts at hatching at the top. Horizontal marks on the time axis indicate the molts that separate the four larval stages (L1–L4) and the adult stage. Developmental programs specific for each larval stage (e.g. see Figure 1) refer to events that occur at the corresponding larval stages. A key element of this genetic regulatory pathway is the progressive decrease in *lin-14* and *lin-28*. *lin-14* acts in L1 to specify L1 developmental programs. *lin-28* is in parentheses in L1 to indicate that *lin-28* is normally nonessential at that stage, but acts redundantly with *lin-14* to prevent expression of L3 programs in L1. A reduced level of *lin-14* (after partial repression by *lin-4*) acts in opposition to *daf-12* [34] and in conjunction with *lin-28* [21,26] and *lin-42* [12••,36] to specify L2 developmental programs. *lin-14* and *lin-28* levels seem to be coupled through mutual positive feedback [21,22], that may serve to modulate their response to *lin-4* repression. In the L3, *lin-14* and *lin-28* are further reduced by the action of *lin-4* [21]; at this stage, *daf-12*, perhaps together with *lin-42*, specifies L3 programs [12••,34]. lin-42 could also have undiscovered roles at other stages [12••]. (L4 programs correspond to the condition where *lin-41* represses *lin-29* [11••].) As L4 progresses, *let-7* is expressed [9], triggering the de-repression of *lin-29* and hence adult-specific differentiation [16–18,37,39]. As the *let-7* and *lin-41* phenotypes are weaker than what would be expected if these genes were the only regulators of *lin-29*, another pathway, represented by 'lin-?', is proposed to work in parallel with *lin-41* and *let-7* to regulate *lin-29*.

if, like *lin-4*, these other regulatory inputs affect translation via *lin-14* and *lin-28* 3′ UTR sequences.

Analysis of the *in vivo* fate of *lin-14* mRNA late in development, when LIN-14 translation is repressed, indicates that the block to LIN-14 translation occurs at a step after initiation. Specifically, the polyribosome profile of *lin-14* mRNA is unchanged during development [27•]. The polyribosomal profile of *lin-28* mRNA is similarly unchanged (H Seggorson, E Moss, personal communication), suggesting a similar mechanism for both *lin-14* and *lin-28*. A key question arises as to whether such post-initiation regulation of translation is either the rule or the exception among cases of translational control via 3′ UTR sequences. Although some well-studied translational control mechanisms have been shown to act at the level of translation initiation [28], in certain other cases, mRNAs have been reported to associate with polyribosomes without producing detectable protein, similar to the situation for *lin-14* mRNA under *lin-4* repression [29–31]. For example, the translational repression of unlocalized *nanos* mRNA in *Drosophila* embryos, which is mediated by *nanos* 3′ UTR sequences [32], does not result in an altered polyribosome profile of *nanos* mRNA, indicating a block after initiation of translation (I Clark, E Gavis, personal communication). Although these examples seem to involve translational control after initiation, the precise point(s) of regulation have not yet been identified, and only in the *C. elegans* system have small regulatory RNAs been implicated.

The *daf-12* gene plays a significant role in the control of L3 developmental events. *daf-12* encodes a member of the nuclear hormone receptor gene family [33••], and certain *daf-12* mutations (class 1 alleles) that affect the putative ligand binding domain of the encoded protein [33••], display strong heterochronic phenotypes [34]. Epistasis experiments with *daf-12* class 1 mutations [34] suggests that *daf-12* may function between *lin-14* and *lin-28*. However, the class 1 alleles are not equivalent to *daf-12* null [33••,34], and because epistasis experiments with non-null alleles are difficult to interpret, it is possible that *daf-12* could also act more directly to control the onset of L3 events (Figure 2). *daf-12* null mutations do not have a particularly strong heterochronic phenotype compared to *daf-12* class 1 alleles, indicating that the class 1 alleles may result in a gain-of-function. Perhaps a class 1 *daf-12* mutation results in ligand-unresponsive DAF-12 product that interferes with a redundant gene activity [34].

The possibility that DAF-12 activity could be modulated by a ligand suggests a way for extracellular signaling to participate in the coordination of developmental events throughout the animal, in particular the advancement from the L2 to L3 developmental program [34]. *daf-12* affects other developmental processes, including dauer larva arrest, gonadal morphogenesis [34] and aging [35]. These diverse roles for DAF-12 in *C. elegans* and the fact that steroid hormone receptors similar to DAF-12 play roles in developmental progression in insects and vertebrates [1,2], suggest that DAF-12 may be part of a relatively ancient mechanism for the coordination of diverse aspects of life history.

lin-42 is one of the most intriguing genes of the heterochronic pathway [12••,36]. In *lin-42* loss-of-function (*lf*) animals, mutations cause precocious adult differentiation in the hypodermis and can also enhance weak *lin-14(lf)* alleles to cause precocious L3 programs, mimicking a *lin-28(lf)* phenotype [12••,36]. Thus, *lin-42* may act at more than one stage of development. *lin-42* mRNA abundance cycles with the larval stages [12••], consistent with such a repeating role for *lin-42*. The oscillation of *lin-42* mRNA

Table 1

***Caenorhabditis elegans* heterochronic genes and their gene products.**

Gene	Product	Predicted activity*	Reference
lin-4	22 nt RNA	Translational repressor	[8]
let-7	21 nt RNA	Translational repressor	[9••]
lin-14	Novel nuclear protein	Unknown	[20,40•]
lin-28	Cold shock/Zn finger	RNA binding, translation factor	[21]
daf-12	Nuclear receptor	DNA binding, transcription factor	[33••,34]
lin-41	RBBC-RING finger	RNA binding? Protein–protein?	[11••]
lin-42	PAS domain	Protein–protein?	[12••]
lin-29	Zn finger	DNA binding, transcription factor	[37]

*From evolutionarily conserved protein structure, or, in the case of LIN-29, *in vitro* biochemical assays. For *lin-4* and *let-7* RNAs, predicted activity is deduced from their complementarity to, and affects on, apparent target mRNAs. nt, nucleotide.

is in stunning concordance with the fact that the gene product LIN-42 has strong similarity to the *Drosophila* Period protein [12••]. Period and LIN-42 both contain a PAS domain, a protein structure likely to mediate protein–protein interactions within multiprotein complexes [37]. Period controls circadian behavior in flies, and *Period* mRNA and protein levels fluctuate in a circadian cycle [37]. LIN-42 could represent an evolutionary link between circadian and developmental timing mechanisms [12••]. Perhaps the fundamental cyclic nature of the worm larval molts involves a regulatory context analogous to circadian clocks, and represents the preservation of some common molecular components in both processes. LIN-42 protein levels have not been examined yet to determine whether the protein also cycles, and if so, with what relationship to the molting cycle.

L3–adult development: a pathway involving another small antisense RNA

Once the L1–L3 sequence of events is executed, a somewhat distinct developmental timer then controls subsequent larval events. This later timing mechanism involves LIN-29 (a transcription factor [38,39]) LIN-41 (a RBBC (ring finger, B box, coiled coil) protein [11••]) and the product of *let-7* (a small RNA analogous to, but different in sequence than, *lin-4* RNA [9••]). Properties of the L3–adult timer parallel the early gene set. In particular, in the L4 stage, a small antisense RNA, *let-7*, accumulates and brings about the translational repression of a pre-existing mRNA, *lin-41*. The timing of L3 and L4 developmental events is influenced by the timing of LIN-41 downregulation [9••,11••]. It is not known whether *let-7* RNA works by a translational repression mechanism similar to the post-initiation effect of *lin-4* [27•].

Although LIN-14 and LIN-28 are not expressed at appreciable levels after the L2, mutations in *lin-14* and *lin-28* affect L3–adult events. Thus, it is clear that the L3–adult timer is coupled to the L1–L3 pathway. Perhaps transcription of *let-7* occurs as a (direct or indirect) consequence of the earlier downregulation of LIN-14 and LIN-28. A major unresolved issue is how the *lin-4* and *let-7* small RNAs are developmentally regulated to bring about the timely repression of their targets.

Timing development post-transcriptionally

Post-transcriptional control of gene expression seems to be a prominent theme in the heterochronic gene pathway. Numerous heterochronic gene products are predicted to interact with RNA and/or are themselves translationally controlled (Table 1). Besides the *let-7* and *lin-4* antisense RNA translational repressors, heterochronic gene products include LIN-28, with two putative RNA-binding motifs [21] and LIN-41, whose RBBC-RING finger domains could have numerous potential functions, including perhaps RNA binding [11••]. By epistasis, *lin-41* represses *lin-29* activity and, indeed, *lin-29* seems to be translationally controlled; the *lin-29* mRNA level is constant after the L1, yet in many cell types LIN-29 accumulates in the L4 stage [39]. The L3–adult timer must contain additional genes and gene products that have not yet been identified. We know this because the *let-7* null retarded phenotype is significantly weaker than the *lin-29* null phenotype, and the *lin-41* null precocious phenotype is also weak compared to that of either *lin-14* or *lin-28* [9••,11••]. Thus, *let-7* and *lin-41* cannot alone couple the early L1–L3 timer to the activation of LIN-29 expression in the L4, and may act in parallel with other inputs acting at the level of *lin-29* mRNA or protein (Figure 2).

One of the intriguing unresolved issues about the *let-7* and *lin-4* RNAs is why there seem to be so many putative complementary sites for these RNAs in the 3′ UTR of members of the heterochronic gene pathway [9••,11••]. For certain genes, the presumed *lin-4* or *let-7* binding sites are predicted to mediate significant regulation, whereas for other genes, these sites seem either secondary or irrelevant. For example, *let-7* complementary sequences occur in the 3′ UTR of *lin-41* mRNA and epistasis data support a role for *let-7* in the temporal control of *lin-41*. However, *let-7* complementary sites also appear in the 3′ UTR of *lin-14*, yet epistasis and developmental expression of *let-7* suggest that *lin-14* is not significantly regulated by *let-7* [9••,11••]. A challenge for the future is to determine which of the putative complementary sites for these small RNA regulators actually mediate a significant functional interaction with either *lin-4* or *let-7* regulatory RNA *in vivo*.

The existence of at least two genes in *C. elegans* that encode small regulatory RNAs (*lin-4* and *let-7*) indicates that this class of small RNAs represents a translational control mechanism that may be relatively common in *C. elegans* and perhaps in other organisms as well. The difficulty of

identifying small regulatory RNA genes by standard biochemical, genetic or informatic approaches requires new methods to comprehensively survey other organisms for regulators like *lin-4* and *let-7* RNAs.

Stage-specific programs: downstream targets of the heterochronic genes

The diversity of developmental events the expression of which is controlled by genes of the heterochronic pathway suggests that the ultimate regulatory targets of the pathway are cell-type-specific genes required to implement the stage-specific programs of particular cells. The culmination of the L3–adult pathway is the activation of *lin-29*, which controls the expression of adult-specific terminal differentiation of the hypodermis [6,16], and the proper morphogenesis of the hermaphrodite vulva [18] and male tail [17]. LIN-29 is a zinc-finger transcription factor whose regulatory targets include stage-specific collagen genes [16,37], and probably also genes involved in cell fusion and cell-cycle exit — behaviors associated with hypodermal differentiation.

LIN-14 is a novel nuclear protein [20,40•] but whether it regulates gene expression through DNA binding, RNA binding or some other activity is still an open issue. As LIN-14 is expressed in L1, it could control the fates of L1 cells through the direct regulation of L1-specific differentiation genes. But LIN-14 also affects events of the L3 and L4 stages, and probably does so indirectly by controlling downstream regulators. In the vulva precursor cells (VPCs), *lin-14* controls the length of the G_1 phase of the cell cycle and also controls the competence of VPCs to respond to intercellular signals that determine their fates [13,41]. *cki-1*, which encodes a cyclin-dependent kinase inhibitor of the p27/p21 class [41–43], is a downstream effector of *lin-14* in the VPCs, inhibiting G_1/S during L2 [41]. Specifically, *cki-1* transcription in VPCs depends on *lin-14* activity, and loss of *cki-1* leads to precocious VPC division. Downregulation of *lin-14* leads ultimately to the downregulation of *cki-1* specifically in the VPCs during late L2, approximately when VPCs complete G_1 and enter S phase [41]. The fact that certain *daf-12* mutations rescue the precocious VPC division defect of *lin-14* null [34] suggests that *lin-14* regulates VPC division indirectly, perhaps via *daf-12*.

Conclusions and future directions

These recent findings about *C. elegans* heterochronic genes raise many interesting questions: how are the early acting regulators *lin-14* and *lin-28* coupled to later regulators, including *let-7*? What other genes and gene products collaborate with *let-7* RNA and *lin-41* to bring about the timely activation of *lin-29*? What is the precise molecular mechanism of translational control by *lin-4* and *let-7* RNAs, and is this mechanism employed in systems besides worms? In general, what molecular features of the heterochronic gene pathway represent truly conserved timing mechanisms, and what features are adaptations specific to *C. elegans* development? Another question concerns the fidelity of development: what regulatory inputs and feedback circuits function to ensure the precise and essentially invariant schedule of larval developmental events, despite the inevitable challenges presented by varying physiological, temperature and nutritional stresses that a larva experiences? Finally, what is the genetic basis for heterochronic differences between *C. elegans* and other nematode species, such as *Pristionchus pacificus*, which develops through only three larval stages (like *C. elegans lin-14* and *lin-28* mutants) and displays other aspects of overt heterochrony compared to *C. elegans* [44••]? The answers to these and other exciting questions about the heterochronic gene pathway will undoubtedly reveal additional interesting regulatory molecules and contribute to our understanding of the general principles underlying the behavior of dynamic regulatory networks.

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