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Heterochronic Genes and the Nature of Developmental Time

Review

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Timing is a fundamental issue in development, with a range of implications from birth defects to evolution. In the roundworm *Caenorhabditis elegans*, the heterochronic genes encode components of a molecular developmental timing mechanism. This mechanism functions in diverse cell types throughout the animal to specify cell fates at each larval stage. MicroRNAs play an important role in this mechanism by stage-specifically repressing cell-fate regulators. Recent studies reveal the surprising complexity surrounding this regulation — for example, a positive feedback loop may make the regulation more robust, and certain components of the mechanism are expressed in brief periods at each stage. Other factors reveal the potential for important roles of steroid hormones and targeted proteolysis. Investigation of the heterochronic genes has revealed a mechanism composed of precisely timed switches linked to discrete developmental stages. Timing is a dimension of developmental regulation that may be difficult to witness in vertebrates because developmental stages are not as discrete as in *C. elegans*, each tissue is likely to be independently regulated. Homologs of certain heterochronic genes of vertebrates show temporally regulated expression patterns, and may ultimately reveal timing mechanisms not previously known to exist.

Introduction

Genetic differences in developmental timing, even when subtle, can cause catastrophic birth defects or a novel morphology that confers an evolutionary advantage [1,2]. Each scale of development — the cycle of cell divisions, the growth of tissues, the emergence of patterns, the formation of organs, and even postembryonic life — requires proper timing. Does timing merely emerge from other aspects of developmental regulation, or is it explicitly governed by molecular mechanisms? Where they do exist, do timing mechanisms involve the same kinds of regulators as spatial patterning, or do they require specialized factors? How are such factors organized in pathways to achieve the synchrony and succession of events? Answers to these questions are emerging from a variety of studies, many involving experimental genetics. Through these studies a timing mechanism has been outlined — the heterochronic pathway in *Caenorhabditis elegans* — that may have broadly conserved components and, in general, sheds light on mechanisms that have arisen to solve the problem of regulated timing in animal development.

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Heterochrony and Developmental Timing in Evolution

Changes in developmental timing have long been believed to be a major force in the evolution of morphology [1]. A variety of changes is encompassed by the concept of 'heterochrony' — differences in the relative timing of developmental events between two closely related species. A classic example of heterochrony is the axolotl. This salamander reaches sexual maturity without undergoing metamorphosis, such that its non-gonadal tissues retain larval features of other salamanders. Different species of axolotls exhibit genetic differences in the production or activity of thyroid hormones that trigger metamorphosis from aquatic juvenile to land-living adult [3]. In these cases, relatively few genetic changes in the endocrine regulation of metamorphosis have led to profound morphological consequences.

Other cases of evolutionary heterochrony are not so simply explained. For example, despite their genetic relatedness, humans and chimpanzees exhibit distinct differences during early development, particularly in skull shape and brain growth [4–6]. Genetic changes appear to have altered the relative timing of developmental events, but the events affected are numerous and occur over a long span of developmental time. Although differences in size and shape can be precisely measured, the underlying molecular mechanisms are difficult to define.

Two types of evolutionary heterochrony have been generally distinguished: sequence heterochrony, or changes in the order of developmental events, and growth heterochrony, or developmental changes in size compared to shape. Smith has re-examined our understanding of heterochrony in evolution and emphasized the importance of developmental sequences [7–9]. Such sequences include ordered events underlying morphogenetic development within tissues, cell proliferation, stages of differentiation, the appearance of structures, or even the induction of specific genes. Analyzing changes in sequences is thought to add significant power to the analysis of heterochrony because such sequences may be independent of specific developmental stages, the size of the embryo, and even the overall rate of development. Importantly, such changes may reflect discrete developmental regulatory mechanisms operating at the cellular level.

But a change in timing does not necessarily reflect a change in a distinct timing mechanism. Normal developmental timing may emerge from other developmental processes, such as growth, induction and differentiation. Altering a regulatory pathway controlling differentiation, for example, may delay or accelerate the formation of tissues [10–12]. Evolutionary heterochrony may therefore arise from changes in all

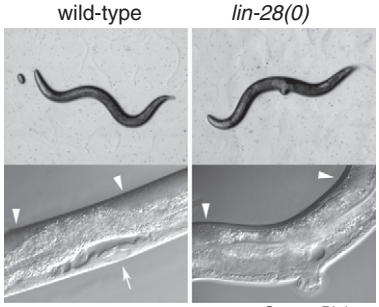

Figure 1. Failure of proper developmental timing.

Light micrographs of *C. elegans* adults at low power (upper panels) and the mid-body of larvae at high power, showing internal structures (lower panels). The positions of the two gonad arms (arrowheads) indicate that the two larvae are at the same stage of development. In a wild-type animal, the gonad and the vulva (arrow) develop synchronously and the two connect at maturity. In a mutant lacking the heterochronic gene *lin-28*, the vulva completes development one stage early, and fails to connect to the gonad at the proper time. Whereas the wild-type vulva is invaginated and still developing, the premature structures of the mutant protrude from the animal, preventing mating and egg-laying.

sorts of developmental mechanisms that do not explicitly govern the timing of specific events. Therefore, how development within an individual is timed may not be easily revealed by interspecies comparisons. But a number of distinct timing mechanisms have been identified through experimental approaches using model organisms.

Diversity of Developmental Timing Mechanisms

The cell division cycle is the basic unit of development and is regulated by a well understood molecular mechanism involving a repeating cascade of phosphorylation and proteolysis [13]. In early embryonic development, major developmental events such as the mid-blastula transition and gastrulation are linked in some way to the number of cell cycles starting from fertilization [14]. The cell cycle itself is not the essential feature of the timing, but a change in the nuclear-cytoplasmic ratio which appears to affect the abundance of transcription factors relative to their target sites [15]. In other instances, cell cycle regulators, including cyclins, are key to the molecular mechanism.

Components of timing mechanisms have been identified that regulate the generation of specific cell types during the development of the central nervous system [16,17]. In these cases, cells of different types arise in a stereotypical sequence from dividing progenitor cells, and the fate of each cell depends on when it is born. These events also involve cell-cycle components and cell-signaling factors involved in differentiation. In *Drosophila*, cell-cycle events and cell-intrinsic signals ensure that neuroblasts express four transcription factors in a series, a process which in turn determines the fates of neurons based on their birth order [18,19].

Another type of developmental timing gives rise to vertebrae and other segmental structures through the process of somite formation [20,21]. Oscillations of gene expression in somite precursor cells slow as the cells move from their origin, until the oscillations arrest and the cells differentiate based on which cycling genes are expressed. The oscillations themselves are driven primarily by members of the Notch signaling pathway, and are coupled to an additional important aspect of somite developmental timing — the growth of the axis along which the somites form, which depends on additional developmental signals [22,23].

Timing is also the hallmark of the remarkable colinearity of vertebrate Hox gene expression, in which genes are expressed in time according to their order along the chromosome [24,25]. Diverse developmental signals underlie the temporal order of Hox gene expression. Properly timed expression of specific genes is an outcome of any developmental timing mechanism, although these genes are not necessarily components of the timing mechanism itself.

Hormones play a critical role in timing the major transitions in the development of *Drosophila* and other insects [26,27]. The steroid hormone ecdysone in particular is responsible for molting in the larva and for its metamorphosis into the adult. Ecdysone binds to and activates nuclear hormone receptors that directly regulate target genes, which in turn direct developmental events that establish the duration of each larval stage and the temporal boundaries at molts and pupation. How the pulses of ecdysone in *Drosophila* are produced is not yet known, but they likely depend on other hormones.

These developmental timing mechanisms exemplify the extensive integration of developmental timing with various regulatory mechanisms. Some of these mechanisms appear to be specialized to solve specific timing problems, but general themes also emerge, such as the importance of oscillating factors. As studies of these mechanisms advance, further principles are likely to be revealed. Another well-studied mechanism appears to be explicitly involved in timing separately from other fate regulation. This mechanism, composed of the heterochronic genes of the roundworm *C. elegans*, also provides insight into developmental timing generally. Furthermore, the conservation of these genes in vertebrates may allow us to witness developmental timing mechanisms where they have gone unobserved.

The Heterochronic Genes

When heterochronic mutants of *C. elegans* were first described, they were unique [28]. Like other developmental mutations, the worm heterochronic defects alter cell fate — instead of dividing or differentiating in one way, a cell does something else. However, rather than altering spatial identity, sexual identity, or cell type, they disrupt the temporal component of a cell's fate. As a result, the succession and synchrony of developmental events is altered in many different tissues (Figure 1). These mutants, therefore, revealed a developmental timing mechanism that functions independently of other types of developmental regulation.

C. elegans develops rapidly from a fertilized egg to a larva that resembles the sexually mature adult, except that it is much smaller and lacks reproductive organs. Four larval stages each end with a molt in which a new cuticle is synthesized and the old one shed. During these stages, the reproductive organs develop and

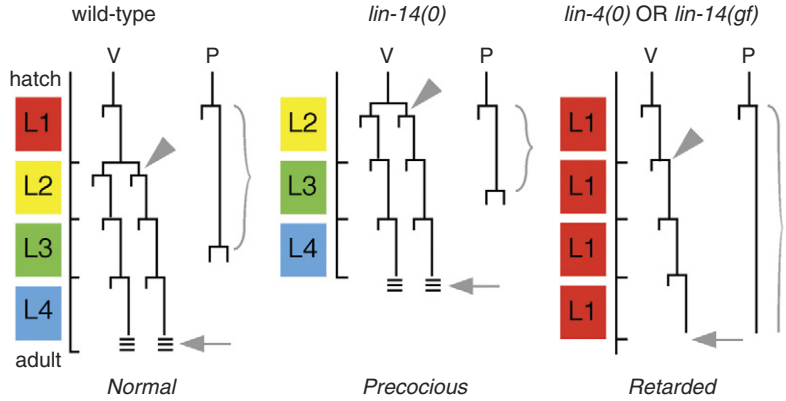

Figure 2. Heterochronic phenotypes of some *C. elegans* mutants.

Two representative cell lineages, V and P, show the transformations caused by the absence (0) or continuous activity (gf) of two heterochronic genes, *lin-4* and *lin-14*. V cells normally divide twice in the L2 stage (arrowhead) and differentiate at the end of the L4 stage (arrow), but these events occur one stage early in a precocious mutant and not at all in the retarded mutants. P cells show a completely different overall pattern from the V lineage, but their fates are likewise changed in the heterochronic mutants — in this case, through alteration of cell-cycle length (gray bar).

the overall size of the animal increases. This roundworm is extremely small and comparatively simple: a first stage larva has approximately 600 cells, only about 30 of which are blast cells that divide and further differentiate. Despite its simplicity, the worm possesses conserved molecular regulators, including Hox genes and Wnt and Ras signaling pathways, that comprise the core developmental toolkit of animals [29].

The simplicity and accessibility of *C. elegans* development was key to recognizing the phenotypes of the heterochronic mutants as timing defects. The blast cells of the larva divide and differentiate in known patterns at each stage, producing neurons, epidermal cells, muscles, and other cell types in a stereotypic manner. All of these events can be witnessed in living specimens through the transparent cuticle. The cell division patterns may be depicted as lineages of cells from birth, through further divisions, to terminal differentiation. The mutant lineages show differences from the normal patterns (Figure 2).

Two general phenotypes are seen in heterochronic mutants — 'precocious,' in which developmental events are skipped, and 'retarded,' in which they are repeated. The affects are both global, occurring throughout the animal, and stage-specific. A heterochronic mutation may affect different tissues (intestine, epidermis, muscle, and neurons), and different kinds of developmental events (a pattern of cell division, a cell cycle lengths, and differentiation). Furthermore, the effects are generally delimited by the larval stages: the events that normally occur during a particular stage are skipped or repeated (Figure 2).

For example, the *lin-4* mutant passes normally through embryonic development and the first larval stage (L1), but beginning in the second larval stage (L2), it reiterates cell lineage patterns of the first stage. However, because each tissue displays its own patterns of cell division and differentiation, the effects of the mutation differ tissue by tissue. For example, intestinal nuclei, which normally divide only in the L1, divide at subsequent stages in the *lin-4* mutant. By contrast, ventral epithelial cells that are normally quiescent until the L3, when they proliferate, remain permanently quiescent in the mutant (Figure 2). As in other heterochronic mutants, the gonad produces mature germ cells at the right time. Thus, the loss of *lin-4* produces a severely deformed adult with a retarded heterochronic phenotype.

The key to interpreting the *lin-4* mutant phenotype was a second mutation that arose spontaneously in a culture of *lin-4* mutant animals. This mutation, which defined the gene *lin-14*, acts as a genetic suppressor, causing *lin-4* mutant animals to develop essentially normally [30]. On its own, the *lin-14* mutation causes an abnormal phenotype that is the opposite of that of *lin-4*: instead of repeating developmental events, it skips them. (Both genes are named '*lin*' for 'lineage abnormal' — although many *lin* mutants have been identified, only a few are heterochronic.) A comparison of the cell lineages of the *lin-4* and *lin-14* mutants reveals that they affect events of the L1 in opposite ways: the *lin-4* mutant repeats events of the L1 in subsequent stages and the *lin-14* mutant skips the L1 events (Figure 2).

The identification of additional heterochronic mutants followed, including the isolation of two extraordinary alleles of *lin-14* that displayed the same retarded phenotype as the *lin-4* mutant (Figure 2). Through the use of elegant genetic analysis alone, and without knowledge of the nature of the molecules encoded by these genes, Ambros and Horvitz deduced that the *lin-14* gene specifies developmental events of the L1, then is down-regulated by *lin-4* to allow the subsequent events of L2 and beyond [30,31]. The special *lin-14* alleles appeared to be unresponsive to *lin-4* regulation. These deductions have since been confirmed by molecular analysis [32–36].

The microRNA–Target Paradigm

lin-4 is now famous for being the first gene found to encode a microRNA [34,37]. MicroRNAs, typically only about 22 nucleotides long, are the smallest genetically encoded regulatory molecules, and are involved in a variety of biological processes [38,39]. *lin-14* is a more typical regulatory gene, encoding a transcription factor [32,40]. The *lin-4* microRNA regulates *lin-14* through specific sequences in the 3′ untranslated region (3′ UTR) of the *lin-14* mRNA. These sites are deleted in the unusual *lin-14* mutant alleles mentioned above [34,35]. As is the case for many other microRNAs, the *lin-4* microRNA, when base-paired to the *lin-14* message, brings to the target a complex of proteins that inhibit translation or mRNA stability [41–46]. Thus, upon *lin-4* expression, *lin-14* protein levels are reduced. Although transcription from the *lin-14* gene still occurs, it is of no consequence [36].

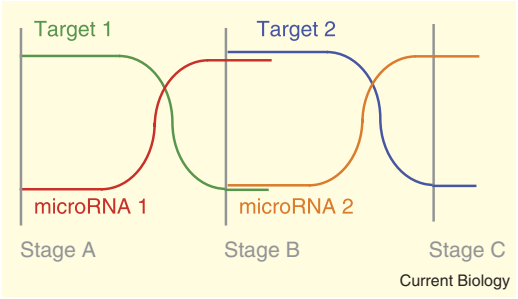

Figure 3. The microRNA–target paradigm.

MicroRNAs increase in abundance at each stage and repress specific targets that encode developmental regulators. The change in regulators at each stage leads to a succession of developmental events. This has been a useful paradigm for understanding the heterochronic gene pathway of *C. elegans*. But it should be noted that it is an oversimplification and does not account for some key features of the pathway.

The *lin-4* microRNA first appears during the L1 stage and reaches its peak as development nears the molt [33]. Experiments using a temperature-sensitive allele of *lin-14* showed that *lin-14* acts near the end of the L1 to affect developmental events of the L2 [31], coincident with the time *lin-4* expression peaks. Therefore, with *lin-4* acting to repress *lin-14* at the end of the L1, repetition of L1 developmental events is prevented, and L2 events proceed normally.

lin-4 and *lin-14* epitomize the dominant paradigm of the *C. elegans* heterochronic pathway: a regulator of cell fates is repressed by a microRNA prior to a molt, the temporal boundary between two stages (Figure 3). Other heterochronic genes are similarly repressed by microRNAs (Table 1). *lin-28* is also repressed by *lin-4* to affect fates of the L2 [47], and *lin-41* and *hbl-1* are repressed by a second microRNA, encoded by the gene *let-7*, in the last larval stage [48–52].

What is appealing about this paradigm is that it suggests a type of developmental module — a molecular mechanism that can be implemented in different versions at different stages [53]. It is tempting to depict the heterochronic pathway as a series of switches in a microRNA-target cascade: key protein regulators act to specify events at a particular stage, then specific microRNAs are expressed at various times to shut them off, allowing a transition to the next stage (Figure 3). This could be a powerful scheme for understanding developmental timing in animals other than *C. elegans*. But how accurate is this paradigm?

The expression of both *lin-4* and *let-7* are transcriptionally controlled, consistent with the idea that their timed expression constitutes a timing switch [54–57]. Also supporting this idea is the finding that a mutation in a regulatory region of *mir-48,* a *let-7* microRNA family member, causes premature expression of the *mir-48* microRNA and a precocious phenotype [55]. Unfortunately, we do not yet know the factors that mediate the transcriptional control of the microRNA gene. In general, microRNA accumulation may also be post-transcriptionally regulated [58,59], although currently there is no evidence for this in the heterochronic pathway.

As it happens, microRNAs in the heterochronic pathway accumulate over a series of many hours, sometimes beginning entire stages prior to the down-regulation of their target. For example, as methods for detecting small RNAs have improved, it has been shown that the *let-7* microRNA starts to accumulate as early as the L2 [56,60], rather than late in larval development, as first reported [50]. Thus, accumulation of the individual microRNAs appears insufficient to cause a switch in the expression of their targets.

Table 1. Targets of microRNAs in the heterochronic gene pathway.

gene	product	microRNA family[1]
lin-14	Novel transcription factor	*lin-4*, *let-7*
lin-28	CSD and CCHC domains	*lin-4*, *let-7*
lin-41	TRIM-NHL	*lin-4*, *let-7*
hbl-1	Hunchback homolog	*lin-4*, *let-7*
daf-12	Nuclear hormone receptor	*let-7*
lin-42	Period homolog	*lin-4*, *let-7*

[1] Sites for microRNAs of *lin-4* and *let-7* families present in the 3′ UTR. Sites for other microRNAs may be predicted based on bioinformatics methods. Underlined are those sites with at least some experimental evidence. *lin-4* includes *lin-4* and *mir-237*; *let-7* includes *let-7*, *mir-48*, *mir-84*, and *mir-241* (see Figure 4).

It is remarkable that of the hundred or so microRNAs encoded in the *C. elegans* genome (mirBase, http://microrna.sanger.ac.uk), only *lin-4*, *let-7* and their four close relatives are implicated in developmental timing (Table 1; Figure 4). When *lin-4* and *let-7* microRNAs were the only such small RNAs known, they were named stRNAs for “small temporal RNA” [50,61]. Although many genes are predicted targets of regulation by the *let-7* microRNA, not all are involved in the timing mechanism [62–64]. Furthermore, *lin-4* and *let-7* and some of their targets have been linked to insulin signaling and lifespan regulation, suggesting they have some role outside developmental timing [40,65,66].

Further confusing the issue is the observation that many of the heterochronic genes have predicted sites for both *lin-4* and *let-7* family members (Table 1) [48–50,62]. Single microRNA binding sites are typically insufficient to cause repression, and multiple microRNAs frequently cooperate to regulate a single target [67,68]. In *lin-14* there are seven *lin-4* sites and three predicted *let-7* sites. In *lin-41* there are two *let-7* sites and one predicted *lin-4* site [50,52,69]. Sometimes, however, experiments contradict the predictions. Despite having a conserved *let-7* site, the regulation of *lin-28* appears unaffected by deletion of the three *let-7* family members that repress *hbl-1* [60]. Why multiple microRNA sites are present in the targets is not yet known.

In an important study, Abbott and colleagues showed that three *let-7*-like microRNAs (*mir-48*, *mir-84*, and *mir-241*) control proper timing at the L2/L3 transition by repressing *hbl-1*, which encodes a transcripton factor and homolog of *Drosophila* Hunchback [60,70]. Deletion of any one of these microRNAs does not cause a strong developmental effect, but animals lacking all three exhibit a severe retarded phenotype and altered *hbl-1* expression. This is a vivid example of functional redundancy among related microRNAs — they all target the same gene, despite slight sequence differences (Figure 4). However, because *hbl-1* was originally predicted to be repressed by *let-7* at a later stage, it is unclear whether *hbl-1* is targeted by

C. elegans

let-7	UGAGGUAGUAGGUUGUAUAGUU
mir-48	UGAGGUAGGCUCAGUAGAUGCGA
mir-84	UGAGGUAGUAUGUAAUAUUGUA
mir-241	UGAGGUAGGUGCGAGAAAUGA
lin-4	UCCCUGAGACCUCAAGUGUGA
mir-237	UCCCUGAGAAUUCUCGAACAGCUU

mammals

let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7b	UGAGGUAGUAGGUUGUGUGGUU
let-7c	UGAGGUAGUAGGUUGUAUGGUU
let-7d	UGAGGUAGUAGGUUGCAUAGU
let-7e	UGAGGUAGGAGGUUGUAUAGU
let-7f	UGAGGUAGUAGAUUGUAUAGUU
let-7g	UGAGGUAGUAGUUUGUACAGU
let-7i	UGAGGUAGUAGUUUGUGCUGU
mir-98	UGAGGUAGUAAGUUGUAUUGUU
mir-125a	UCCCUGAGACCCUUUAACCUGUG
mir-125b	UCCCUGAGACCCUAACUUGUGA

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Figure 4. "Small temporal RNAs" and homologs.

MicroRNAs known to be involved in developmental timing in *C. elegans,* and their mammalian homologs. MicroRNAs are grouped according to homology to *let-7* and *lin-4.* The mature functional form of each microRNA is shown; these range from 21 to 24 nucleotides. The "seed" sequence, important for target recognition, is underlined.

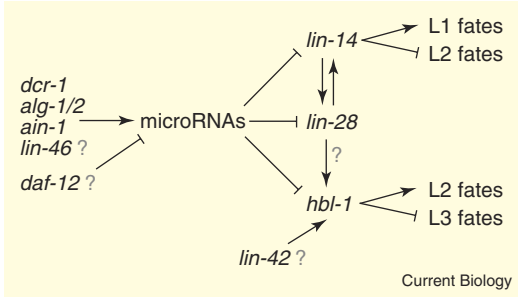

Figure 5. The heterochronic gene pathway from L1 to L3.

A pathway model for the control of timing of *C. elegans* larval development by the heterochronic genes. Additional genes are listed that are generally required for microRNA biogenesis and function (*dcr-1*, *alg-1/2*) or that may support microRNA activity (*ain-1*). The microRNAs of the *lin-4* and *let-7* families are grouped together for simplicity, since they all potentially target the same genes. The question marks indicate good guesses, but require testing. Several known heterochronic genes are not listed because they either act later in development or their placement is uncertain.

different *let-7* family members as part of two timing switches. Nor is it clear why some *let-7* family members act redundantly, and another, *let-7* itself, apparently does not.

A Feedback Loop

lin-14 and *lin-28* fit the microRNA-target model in that their stage-specific repression depends on the *lin-4* microRNA. When *lin-4* is deleted, both *lin-14* and *lin-28* remain highly expressed throughout larval development, causing a severe retarded phenotype. However, when either *lin-14* or *lin-28* is also removed, the retarded phenotype is repressed and the expression of the remaining gene is down-regulated *at the normal time* [47,71,72]. This finding implies the existence of additional repressors acting simultaneously with *lin-4*. Somehow, *lin-14* and *lin-28* each oppose the repression of the other, thus forming a positive feedback loop (Figure 5).

This positive feedback loop, which is critical to the timing mechanism, shows that *lin-4* is not the whole story. The additional repression may be the action of microRNAs [72], and it is especially interesting that both *lin-14* and *lin-28* contain potential binding sites for *let-7* family members. However, the three *let-7*-like microRNAs that down-regulate *hbl-1* do not appear to affect *lin-28* expression [60]. The identity of the relevant regulators remains unknown.

The feedback loop has an important implication: *lin-4* is insufficient to repress *lin-14* and *lin-28* completely without the help of the additional repressors [72]. Yet deletion of *lin-4* alone causes a 10–20-fold change in protein levels for *lin-14* and *lin-28*, and a severe retarded mutant phenotype [36,72,73]. Why? The up-regulation of *lin-14* and *lin-28* that results from deletion of the *lin-4* gene must increase their ability to resist the other repression. Thus, the affect of removing *lin-4* is further amplified by the positive feedback loop. This arrangement may make the timing switch more robust — a high threshold of microRNA activity must be reached before the switch is thrown and the targets are completely repressed. It also underscores the observation that microRNA-target interactions removed from their natural context may not be as potent [67].

Periodic Factors in the Heterochronic Pathway

If the simple paradigm of a microRNA-target cascade is not sufficient to explain the timing mechanism, then the heterochronic genes that fall outside this paradigm become particularly interesting. One such gene is *lin-42*. This gene encodes a homolog of Period, an oscillating component of the circadian-rhythm mechanism of *Drosophila* [74,75]. Most of the genes of the microRNA-target paradigm show continuous expression with a single developmental switch: either on-then-off, like *lin-14*, or off-then-on, like *lin-4*. However, *lin-42* is expressed periodically, once each larval stage [74]. This is reminiscent of the daily pattern of *Period*, except it occurs at each of the four larval stages, about every 12 hours at normal growth temperature.

Like other heterochronic genes, *lin-42* influences developmental events in multiple tissues [75]. Its mutant phenotype is precocious, similar to that of *lin-28*, notably causing differentiation of epidermal cells one stage too early. Interestingly, it has no known effects on circadian rhythms in *C. elegans* [76]. Two other genes that are homologs of *Drosophila* circadian rhythm regulators, *tim-1* and *kin-20*, may also play a part in the developmental timing mechanism in *C. elegans*, although their effects are significantly weaker than that of *lin-42* [77].

lin-46 also departs from the paradigm. Like *lin-42*, it is expressed in brief pulses at each stage ([78]; K. Kemper and E.G. Moss, unpublished). However, in contrast to *lin-42*, the *lin-46* mutants show signs of retarded development, with repetition of epidermal lineage

patterns and postponed differentiation. This finding indicates that, despite both being periodically expressed, *lin-46* and *lin-42* must have different roles in the pathway. We do not yet know whether they are expressed exactly coincidently because their expression patterns have not been assayed together. However, genetic interactions suggest that *lin-42* functions downstream of *lin-46* [75].

The *lin-46* mutant was discovered for its ability to reverse the precocious phenotype of *lin-14* and *lin-28* mutants [78]. Interestingly, another mutation that partially suppresses the *lin*-28 mutant phenotype is in *ain-1*, which encodes a protein that interacts with the microRNA silencing machinery, and can localize to cytoplasmic processing bodies [79]. *lin-46* encodes a homolog of a scaffolding protein which might interact physically with other pathway components. Although their precise molecular functions are unknown, *lin-42* or *lin-46* may fit into the microRNA paradigm as bona fide targets or co-factors like *ain-1*.

The periodic expression patterns of *lin-42* and *lin-46*, which distinguish them from the other heterochronic genes, underscore the important link between the heterochronic genes and the molting cycle. *daf-12* encodes a nuclear hormone receptor with several roles in *C. elegans* biology, including developmental timing, developmental diapause and longevity [80–82]. Certain alleles of *daf-12* encoding altered hormone-binding domains have very strong retarded heterochronic phenotypes, suggesting that *daf-12* can regulate the expression of one or more heterochronic genes in response to hormone signals [81]. Exit from the molting cycle at the last stage is under the control of nuclear hormone receptor genes which are themselves regulated by two let-7 family members, demonstrating a link between the heterochronic fate regulators and molting itself [83]. In an intriguing study, application of an acetylcholine receptor agonist named DMPP was found to uncouple the molting cycle from fate patterning [84]. Little is known about the hormonal control of molting; however, recently, steroid ligands of the DAF-12 protein have been identified [85,86]. It remains to be determined whether these hormones rise and fall with the cycle of molts and how they might link progression of larval stages with the succession of developmental events [26].

Finally, targeted protein degradation may have an important role in the *C. elegans* developmental timing mechanism. Two genes, *lin-41* and *dre-1*, may encode E3 ubiquitin ligases that could target specific proteins for ubiquitin modification and, ultimately, proteolysis [87,88]. The possible involvement of heterochronic genes in protein modification and degradation pathways reminds us how much more there is to learn about the heterochronic pathway.

Principles of the Heterochronic Gene Pathway

What do the *C. elegans* heterochronic genes tell us about ways in which animal developmental timing can be regulated? From a temporal standpoint, *C. elegans* larval development is segmented. The "temporal segmental boundaries" are the molts and the intermolt period is the unit of pattern — a fact immediately recognized from the cell lineage patterns of mutants of the original heterochronic mutants (Figure 2) [26,28]. Certain genes, such as *lin-14*, *hbl-1* and *lin-29*, encode transcription factors that act stage-specifically to affect cell fates. Because these genes control only the temporal component of cell fates, they must work with a host of other developmental regulators to effect stage-appropriate fates in each cell lineage.

Attempts to summarize genetic and molecular data concerning the heterochronic genes into formal relationships have generated a variety of pathway models [89–94]. Some key players have been added over time, and others have shifted position or remain difficult to place. A summary of part of the pathway based on some recent advances is shown in (Figure 5) [60,62,75,78]. Some of the depicted relationships have been firmly established, while others are inferred and require critical tests. At the center are the *lin-4* and *let-7* family microRNAs. They are grouped together for simplicity, and because the three downstream genes all have conserved binding sites for both families (Table 1). Upstream of the microRNAs are general components required for microRNA biogenesis and activity, and factors that might play a pathway-specific role. Downstream are the microRNA targets, whose functional inter-relationships are complex. The targets may be aided in their function by additional factors. Several genes are not shown either because they act later in development or they await further genetic positioning: *lin*-29, *lin-41*, *lin-66*, *kin-20, tim-1*, *dre-1*, and *puf-9* [51,77,88,95–97]. It is not yet clear how the early-acting regulators, which are shown, interact with the later-acting regulators, such as *lin-41* and *lin-29*, that directly control the transition to adulthood [51].

In his classic work on heterochrony, Gould postulated that a hormone gradient could be responsible for development and the underlying source for heterochronic changes [1]. Although hormones such as those that activate DAF-12 are likely involved, so far there is no evidence for a gradient in the heterochronic pathway. A gradient theory was one of two possibilities originally proposed for *lin-14* activity based on genetic data [31]. Through insightful experiments, Ambros and Horvitz demonstrated that *lin-14* acts at two different times, first to affect L1 fates, then later to affect L2 fates. One possibility was that *lin-14* produces two products to carry out these roles, but this theory has been essentially eliminated [98,99]. A second possibility was that *lin-14* acts akin to a morphogen that elicits different fates from cells depending on its concentration—the gradient model. This idea does not yet have any molecular verification, although there is no direct evidence against it either [40]. However, the two activities of *lin-14* can be explained in light of the feedback loop with *lin-28*. *lin-14* first acts alone in the heterochronic pathway to control L1 fates. Later, as microRNAs accumulate, it enters a positive feedback loop with *lin-28* [72], the more direct regulator of L2 fates [78]. *lin-28* in turn affects genes further downstream in the pathway [60]. The succession through the first three larval stages does not depend on a gradient of *lin-14* activity, but is a consequence of the action of microRNAs and the positive feedback between *lin-14* and *lin-28*.

Taking these observations together, key features of a theoretical timing mechanism based on the

C. elegans heterochronic genes include components of switches (such as the microRNAs and their targets) and periodic factors (Figure 6). Multiple microRNAs become activated and then repress multiple target mRNAs, some of which may encode factors that govern cell fates directly. The slow accumulation of microRNAs suggest that mechanisms in addition to their biogenesis may be critical for defining their key targets and limiting the time when their presence is significant. For example, the combined activities of multiple microRNA may reach a threshold for repressing sensitive targets. In addition, opposition to or enhancement of microRNA function may occur generally or on specific targets. Periodically active factors are coordinated with the molting cycle, which is controlled at least in part by an independent mechanism. These factors may influence the accumulation or activity of the microRNAs, or participate in fate specification. The likely explanation for the complexity of the timing system is the need to link the fate switches with the oscillations of the molting cycle and make the mechanism robust — stable to stochastic fluctuations, particularly variations in the accumulation of the critical repressors, the microRNAs.

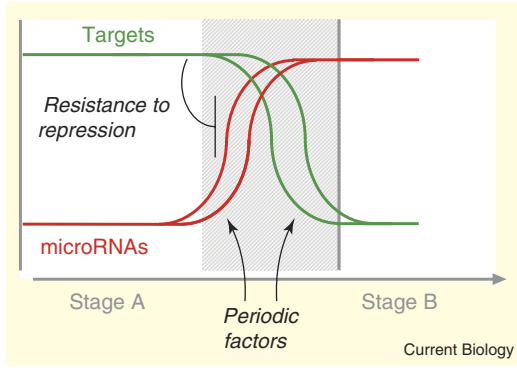

Figure 6. A developmental timing mechanism.

Multiple microRNAs repress multiple targets at each stage. Some of these targets are direct regulators of cell fates. More than one of the targets has the role of inhibiting the repression, but eventually the microRNAs succeed in repressing all of the targets at each stage. Periodic factors are active during a particular period (gray box) which may be defined by the same mechanism that defines the temporal boundary of the stage itself. The periodic factors may assist the microRNA repression, or assist the targets in resisting repression or in specifying the stage-specific developmental events.

Conservation of Heterochronic Genes in Vertebrates

Some *C. elegans* heterochronic genes have clear homologs in mammals and other vertebrates, such as the microRNA genes homologous to *lin-4* and *let-7* (Figure 5) [100,101], and genes related to *lin-28* and *lin-41*, which encode unique domain combinations [51,102]. Others belong to conserved gene families (*daf-12, hbl-1, lin-29, lin-42*). Others are significantly different from their closest relatives (*lin-46*) or have no homologs in vertebrates (*lin-14*). Still, an important question is: Are any heterochronic gene homologs involved in developmental timing in vertebrates or other animals?

The *let-7* homologs were the first microRNAs recognized in a wide range of bilaterian animals [103]. The *let-7* homologs are widely expressed, and their presence is generally associated with development. Expression of *let-7* appears temporally regulated in a number of species, rising over time, as in *C. elegans*; however, a more precise analysis shows a complex picture, especially when the different *let-7* family members are accounted for [104]. The *lin-4* homolog (named *mir-125*) was originally found to be more abundant in the nervous system of vertebrates than in other cell types [101].

No definitive insight into function has yet come from expression analysis of these microRNAs — genetic analysis would seem needed to define their developmental roles in vertebrates. Although mis-expression of *let-7* in zebrafish produces developmental effects, they are difficult to interpret [105]. Mammalian *lin-4* and *let-7* homologs have been linked with regulation of cell proliferation in cultured cells, but there are no reported mutants [106,107]. Eliminating all microRNAs during development by knocking out the Dicer enzyme, which is primarily responsible for processing the precursor microRNA into its mature form, causes a catastrophic failure of morphogenesis [108–112].

A remarkable feature of vertebrate *lin-28* and *lin-41* homologs is that they are both predicted targets of *lin-4* and *let-7* family microRNAs [102,107,113–117]. Although most microRNA–target pairings are based on predictions, in this case, they are experimentally supported [117,118]. Such deep conservation of microRNA–target pairs is uncommon [119]. These findings might suggest that an important relationship has persisted between the microRNAs since the common ancestors of *C. elegans* and vertebrates, or they may reflect a case of convergent evolution [120].

The vertebrate homologs of *lin-28* and *lin-41* also show temporally regulated expression patterns [102,115–117,121]. This regulation may be viewed on three different scales. At the gross level, western blot analysis of whole embryos shows a general decrease in expression over time. This is roughly the inverse of the expression of the *let-7* homolog [103]. In a more anatomical view, whole-mount *in situ* hybridization shows the expression of both genes in a variety of developing tissues, but particularly the limbs, where the genes exhibit unmistakable temporal regulation [115–117] (K.S. Choi and B. Harfe, pers. comm.). But cellular-level examination of the mouse Lin28 protein in tissue sections reveals that the protein is expressed in a variety of embryonic tissues and self-renewing tissues of the adult where cells are progressing through stages of differentiation [121]. A recent report demonstrates a timing role for Lin-28 in the differentiation of muscle, which is only one of the many tissue types in which it is expressed [122].

The Nature of Developmental Time

The expression of the mouse Lin-28 protein in the adult intestinal epithelium represents an additional instance where a developmental timing mechanism may be at work [115] (Figure 7). This epithelium is a continuously self-renewing tissue, and Lin28 protein is present where cells transition from proliferating to differentiated cells. Because clusters of cells are developing synchronously, the time of Lin-28 expression is

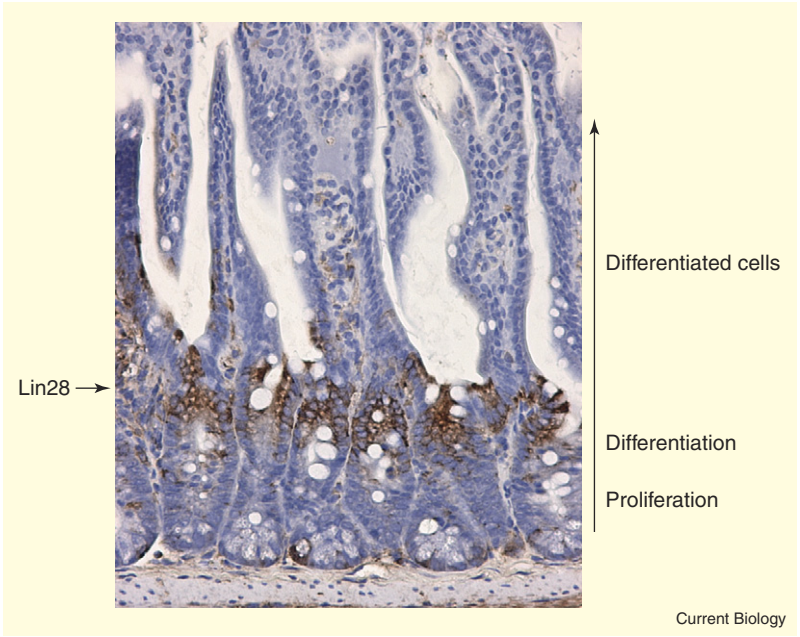

Figure 7. Expression of Lin-28 protein in epithelium of an adult mammal.

The intestinal epithelium is a continuously self-renewing tissue of adult mammals. In the crypt, stem cells divide and form so-called transit amplifying cells that further divide and differentiate. Once fully differentiated, these cells move farther up to form the villus. The arrow indicates Lin-28 expression in developing villus cells. If mammalian Lin-28 is a conserved developmental timing regulator, then it appears to function at the scale of individual cells, acting tissue-by-tissue, throughout the animal [115].

readily observed. If the development were not synchronous, or if the expression were viewed from any lower resolution (by whole mount hybridizations, for example) its precise temporal expression in this tissue might be missed.

This example illustrates both the potential and the challenge of identifying developmental timers in vertebrates and other complex animals. In *C. elegans*, each heterochronic gene acts simultaneously throughout the animal to control developmental timing. This happens in part because the animal is anatomically simple: a single epithelium surrounding muscles, intestine and gonad. A vertebrate has far more cells, proliferating and differentiating in different places in different tissues. Beyond the earliest stages of development, we may not expect timing regulation to be global. If Lin-28 and the other heterochronic gene homologs are indeed conserved developmental-timing regulators, then this timing is happening in each developing tissue according to its own needs.

The many studies of developmental timing that have used comparative and experimental approaches reveal the nature of developmental time to operate at many scales, from individual cells to whole organisms. What the heterochronic genes of *C. elegans* have shown us is that an explicit timing mechanism can function separately from — or orthogonally to — other aspects of developmental regulation. In addition, these genes have prompted the identification of a set of interacting regulators whose homologs may lead us to the discovery of developmental timing mechanisms where they are not yet known to exist.

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