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REVIEWS

Heterochronic genes and the temporal control of *C. elegans* development

VICTOR AMBROS AND ERIC G. MOSS

The heterochronic genes of Caenorhabditis elegans encode part of a regulatory system that controls the temporal component of cell fates in development. The genes have been characterized genetically and molecularly, and their study has so far revealed a genetic hierarchy that specifies sequences of developmental events, a novel RNA-mediated mechanism of gene regulation and a reprogramming phenomenon associated with arrested development.

Although genetic analysis has revealed much about positional information and sex determination in animal development, little is known about the systems that regulate the succession of stage-specific developmental events and ensure synchrony among developing tissues. The nematode *Caenorhabditis elegans* is a convenient animal for the analysis of developmental timing: the worm's development is simple and well characterized, and genes have been identified that appear to act exclusively to control the timing of stage-specific events[1,2]. Mutations in the genes *lin-4*, *lin-14*, *lin-28* and *lin-29* cause precocious or retarded development, with precocious animals skipping developmental patterns specific to particular stages and retarded animals reiterating such patterns (Fig. 1). These genes have been termed 'heterochronic' by analogy with the evolutionary concept of heterochrony, which describes changes in the relative timing of developmental events as an important mechanism for evolutionary variation[3].

Stage-specific transformations in cell fate

After hatching from the egg, the worm develops through four larval stages (termed L1, L2, L3 and L4) separated by molts, and becomes a sexually mature adult. During these stages, more than 400 somatic cells are added to the 558 cells of a newly hatched larva, and cell division and differentiation events occur in virtually invariant patterns at specific times[4]. Heterochronic mutations cause stage-specific transformations in larval developmental patterns such that cells adopt fates characteristic of cells that normally occur earlier or later in the same lineage. For example, a null mutant of *lin-14* [*lin-14(0)*] fails to execute L1-specific developmental patterns in lateral hypodermal lineages, and instead precociously executes L2-specific patterns in the L1, followed in subsequent stages by precocious patterns specific to the L3, L4 and adult stages[2,5] (Fig. 2). A semidominant gain-of-function *lin-14* mutation [*lin-14(gf)*] causes retardation of events in the same lineage, so that L1-specific patterns are reiterated at each stage after the L1 (Fig. 2). Similarly, development of the vulva, which is required for the animals to mate and lay eggs, occurs one stage early in *lin-14(0)* mutants, and is blocked in *lin-14(gf)* mutants (Fig. 2). The nature of these cell fate transformations suggests that cells of equivalent developmental potential are generated at successive stages and that heterochronic genes determine the temporal component of their fates. The known heterochronic genes regulate development in a variety of tissues, including hypodermis, muscle, neuron and intestine, but do not appear to affect the somatic gonad and germ line.

Temporal patterning via a decrease in *lin-14* activity

Genetic analysis of the heterochronic gene *lin-14* has shown that a temporal decrease in *lin-14* activity is required for the normal succession of stage-specific developmental patterns[5]. *lin-14(gf)* mutations that result in the reiteration of L1-specific patterns cause an elevated level of *lin-14* activity in the L2 and later stages, when normally *lin-14* activity would be low or completely absent. Temperature-shift experiments with temperature-sensitive alleles of *lin-14* indicate that a high level of *lin-14* activity is required early in the L1 stage for L1-specific patterns to occur in certain lineages: if at that time *lin-14* activity is too low, L2-specific patterns result. These experiments also show

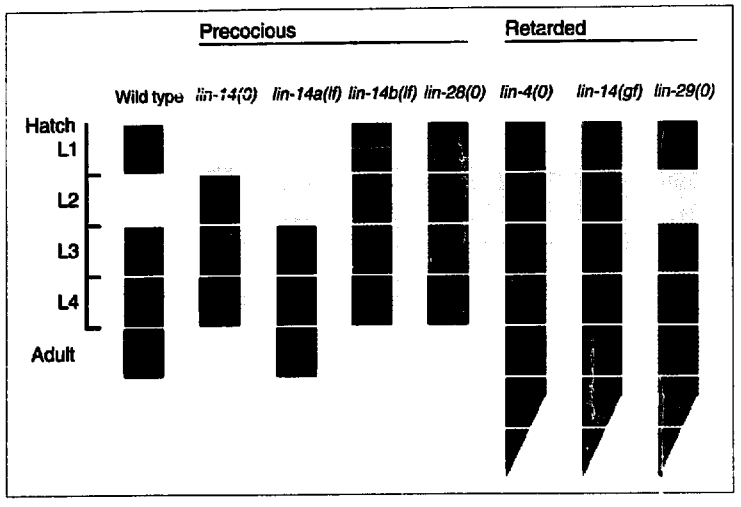

FIGURE 1. Temporal patterns of development in *C. elegans* heterochronic mutants compared with wild-type animals. Colors identify developmental events that are characteristic of each larval stage in the wild type, indicated to the left. Stage-specific transformations of cell fates occur in a variety of lineages and tissues. Molts punctuate the stages. Certain precocious mutants have fewer molts, because of precocious terminal differentiation of the hypodermis. Retarded mutants have extra molts, because the hypodermis fails to undergo terminal differentiation. *0*, null mutation; *lf*, loss-of-function mutation; *gf*, gain-of-function mutation[2,5,10].

that *lin-14* acts just before cells adopt L1-specific fates, indicating that *lin-14* may be a direct effector of cell fate at this stage.

Molecular analysis confirms that the level of *lin-14* gene product is temporally regulated. The *lin-14* gene has been cloned and sequenced and encodes multiple protein products of unknown function[6,7]. Lin-14 protein can be detected in the nuclei of those cells of the newly hatched larva that are affected by *lin-14* mutations, a finding consistent with the idea that Lin-14 is a direct regulator of gene expression in the L1 larva[7,8]. The level of Lin-14 protein declines between the early L1 stage, when it is abundant, and the L2 stage, when it is almost undetectable[8,9]. Because the abundance of the *lin-14* message remains relatively unchanged throughout development, this downregulation of *lin-14* appears to occur mostly at the post-transcriptional level[7,9]. Interestingly, two *lin-14(gf)* alleles that cause constitutive production of the Lin-14 protein contain rearrangements that remove part of the long 3' untranslated region (3'UTR) of the *lin-14* mRNA, indicating that downregulation of *lin-14* occurs through its 3'UTR (Refs 6, 7).

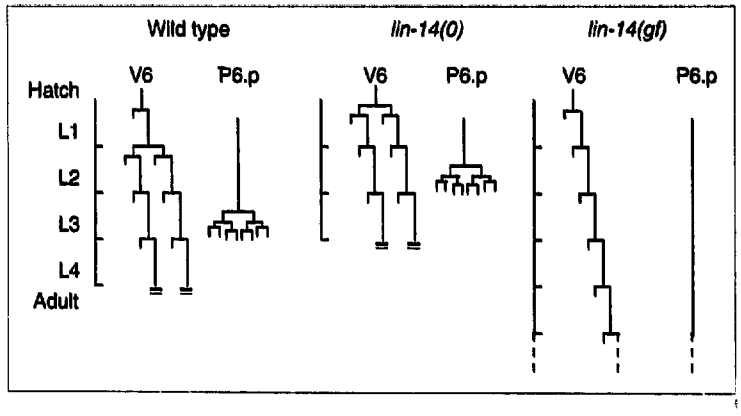

FIGURE 2. Examples of the precocious and retarded cell lineage phenotypes seen in *lin-14* mutant animals[2,5]. Mutations in *lin-14* cause temporal transformations of the fates of lateral hypodermal cells, such as V6, and in vulval precursor cells, such as P6.p. *lin-14(0)* mutations cause precocious cell lineage patterns at all stages of the V6 lineage, and a shortening of the cell cycle in the P6.p lineage. *lin-14(gf)* mutations cause the reiteration of L1-specific patterns of V6, and prevent normal cell division in P6.p.

lin-4 regulates *lin-14* activity by an antisense RNA

Genetic and molecular experiments indicate that the heterochronic gene *lin-4* is a negative regulator of *lin-14*, and that it plays a critical role in *lin-14* downregulation[9–11] (Fig. 3a). A *lin-4(0)* mutation causes a retarded phenotype that is essentially identical to that caused by *lin-14(gf)* mutations[1,5] (Fig. 1). Epistasis experiments indicate that *lin-4* acts upstream of *lin-14* in a regulatory pathway[10,12] (Fig. 4). Furthermore, *lin-4* activity is required for the normal temporal decrease in Lin-14 protein: in *lin-4(0)* animals, the level of Lin-14 protein remains abnormally high during later development[13].

The *lin-4* gene has recently been characterized molecularly and, surprisingly, has been found to encode two very small RNAs of 22 and 61 nucleotides[11]. Four *lin-4* homologs isolated from different members of the genus *Caenorhabditis*, each of which can complement a *lin-4(0)* mutation in *C. elegans*, share no conserved open reading frames, despite having extensive nucleotide sequence conservation. Thus it appears that the active gene product of *lin-4* is not protein but RNA, among the smallest active RNAs so far identified. Remarkably, the *lin-4* transcript is complementary to seven short sequences in the 3'UTR of the *lin-14* transcript (Fig. 3b; Refs 9, 11). A *lin-4* RNA is therefore likely to interact directly with the 3'UTR of the *lin-14* mRNA. Because the downregulation of *lin-14* appears to be post-transcriptional, these observations suggest that the *lin-4* RNA negatively affects translation of the *lin-14* mRNA. This is the first known case in animals of a small complementary RNA that appears to regulate the translation of a specific mRNA.

lin-4 transcripts accumulate late in the L1 stage, several hours after hatching, and around the time that the level of Lin-14 protein begins to decline (R. Feinbaum and V. Ambros, unpublished). When normal embryos hatch in the absence of food, they can delay

the initiation of development, for days if necessary, until food is encountered. During the period of starvation, *lin-4* transcripts are undetectable and the level of Lin-14 protein remains high (Ref. 13; R. Feinbaum and V. Ambros, unpublished). After feeding, the embryos commence larval development and Lin-14 protein levels decrease appropriately[13]. Thus, the downregulation of *lin-14* is generated by a temporally controlled increase in the activity of *lin-4*, which may in turn be triggered by a 'food signal' that initiates postembryonic development.

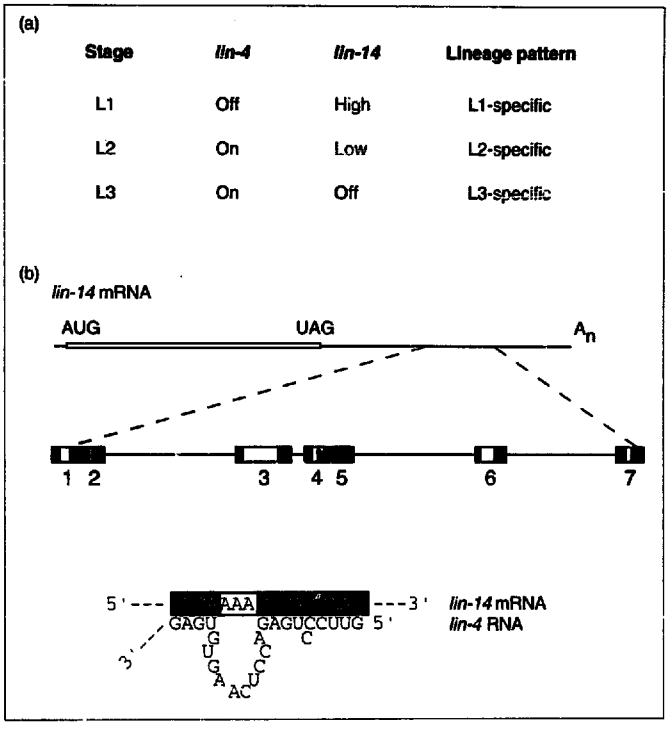

FIGURE 3. Post-transcriptional regulation of *lin-14* by *lin-4* RNA[9–11]. (a) *lin-4* is expressed late in the L1 and downregulates *lin-14*. The succession of stage-specific lineage patterns reflects the decreasing activity of *lin-14*. (b) It appears that a *lin-4* RNA negatively regulates translation of the *lin-14* mRNA by a novel mechanism. The *lin-4* RNA is complementary to seven sites (boxed) in the 3'UTR of the *lin-14* mRNA; this complementarity (filled boxes) is discontinuous and is slightly different for each of the seven sites. A proposed interaction at the second site is shown at the bottom of the diagram, with complementary sequences in the *lin-14* mRNA shown in filled boxes.

lin-14 has two distinct activities

Genetic evidence indicates that there are two components of *lin-14* activity[5]: one that is required before downregulation occurs, and another that acts during, or as a consequence of, downregulation. These two component activities, referred to as *lin-14a* and *lin-14b*, are defined by experiments with temperature-sensitive alleles, which show that *lin-14* is required at two distinct times in the L1 for normal development, and by certain alleles of *lin-14*, which cause developmental phenotypes that correspond to the loss of *lin-14* during either of these two temperature-sensitive periods. *lin-14a* activity is important for normal L1 development: in its absence, affected cells execute L2-specific patterns precociously in the L1, although developmental patterns executed in later stages are unaffected (Fig. 1). In the absence of *lin-14b* activity, the L1 is normal, but L3-specific patterns are executed precociously in the L2, demonstrating that *lin-14b* is important for normal L2 development (Fig. 1). Unlike *lin-14a*, *lin-14b* also affects development in subsequent stages, probably acting through other temporal regulators (see below). Interestingly, *lin-14b* activity is not required for the execution of L2-specific patterns *per se*, since those patterns are executed, albeit precociously, in *lin-14(0)* mutants, which lack both *lin-14b* and *lin-14a* activities (Figs 1, 2).

The two activities of *lin-14* may reflect two different levels of a temporal gradient of *lin-14* gene product, where the *lin-14a* activity occurs at a high level and the *lin-14b* activity at an intermediate level[5]. In addition to the downregulation of *lin-14* by *lin-4*, the switch in activities may reflect modification of the Lin-14 protein or the expression of different products from the gene. Several different *lin-14* transcripts have been identified from cDNA clones, and these appear to encode proteins that differ at their amino termini[7]. However, these Lin-14 proteins might be tissue-specific, not necessarily stage-specific, products.

Interaction between *lin-28* and *lin-14*

The *lin-28* mutant phenotype is similar to that caused by mutations that reduce *lin-14b* activity[2,5] (Fig. 1), indicating that the products of these genes probably interact to influence developmental events in the L2. Precisely how they might interact, however, is unclear. It appears that *lin-28* activity is not absolutely dependent on *lin-14* activity since the development of a worm that is doubly mutant, carrying both the *lin-28(0)* and *lin-14(0)* mutations, is more precocious than that of a *lin-14(0)* mutant. Therefore, unlike *lin-14b*, *lin-28* is required for the execution of L2-specific patterns in the L1 stage of larvae that lack *lin-14a* activity[10]. On the other hand, there is evidence to suggest that *lin-28* may in some way positively regulate *lin-14* or stabilize its product: specifically, *lin-28* mutations can partially suppress the effects of elevated *lin-14* activity in a *lin-4* mutant[10]. Consistent with this idea is the observation that *lin-28* mutants seem to have an abnormally low level of Lin-14 protein late in the L1 stage[13].

Other genes that might interact with *lin-14* and *lin-28* in regulating developmental timing in the L2 have also been identified. A mutation in *lin-42* enhances the *lin-14b* defect of a weak allele of *lin-14*, but not the *lin-14a* defect (Z. Liu and V. Ambros, unpublished). Mutations in a newly discovered heterochronic gene, *lin-46*, eliminate the requirement for *lin-28* for normal developmental timing; this indicates that, like *lin-14*, *lin-28* does not directly specify L2-specific patterns, rather, it permits their expression by inhibiting later programs. The elucidation of the control of developmental timing in the L2 and later stages

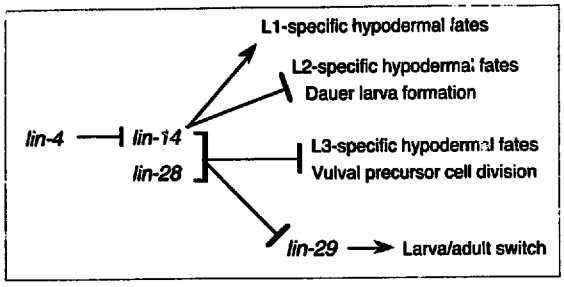

FIGURE 4. A hierarchy of heterochronic genes[10–12]. The best-characterized genes and developmental events are indicated. *lin-4* negatively regulates *lin-14*. *lin-14* might be a positive regulator of L1-specific programs or a negative regulator of L2-specific programs, or both. *lin-14* and *lin-28* appear to interact to influence developmental events in the L2 by inhibiting L3 specific programs. The evidence suggests that the negative regulation of *lin-29* by *lin-14* and *lin-28* is indirect. The normal succession of stage-specific events may be controlled by the sequential activation and deactivation of these genes or their products.

awaits the molecular characterization of *lin-28* and these other genes.

The hierarchy of heterochronic genes

The heterochronic genes can be placed in a regulatory hierarchy on the basis of their phenotypes and epistasis relationships (Fig. 4). *lin-14*, specifically the *lin-14a* activity, might be a positive regulator of L1-specific programs or a negative regulator of L2-specific programs (or both), and *lin-4* is a negative regulator of *lin-14*. Because *lin-14* and *lin-28* do not themselves specify L2-specific developmental patterns, these genes probably act during the L2 stage as negative regulators of L3-specific programs (Fig. 4). The normal succession of developmental events appears to result from the stage-specific activity of these genes.

Besides skipping L2-specific developmental events, animals that lack *lin-14* or *lin-28* activity also show precocious development in later stages (Figs 1, 2). Experiments with temperature-sensitive *lin-14* mutations demonstrate that the developmental defects that occur in the L2 and later stages result from the absence of *lin-14b* activity. Thus, it appears that *lin-14* affects the later stages of development indirectly. Direct targets of *lin-14* may include not only genes that implement cell fates in early larval development, such as regulators of cell division and cell differentiation, but also genes that regulate the temporal component of cell fates in later stages.

An example of this is seen with *lin-14* and *lin-28*, both of which act through the heterochronic gene *lin-29* to regulate the adult-specific terminal differentiation of certain hypodermal cells[10]. Terminal differentiation, referred to as the 'larva-to-adult switch', involves fusion of lateral hypodermal blast cells, synthesis of adult-specific cuticle and cessation of the molting cycle[4,10]. *lin-29* is required for the switch: in a *lin-29* mutant animal, the molting cycle continues after the L4 stage, and at each additional cycle, hypodermal stem cells divide and a larval cuticle is synthesized[10] (Fig. 1). *lin-29* encodes a transcription factor of the Cys_2–His_2 zinc finger type, and is likely to be a direct regulator of differentiation in hypodermal cells (Ref. 14; A. Rougvie and V. Ambros, unpublished). In *lin-14* and *lin-28* mutant animals that show precocious development, the number of molts is reduced as the result of *lin-29* being activated one stage earlier than usual[10,15]. This observation indicates that *lin-14* and *lin-28* are negative regulators of *lin-29*, although it is not yet known whether *lin-29* is a direct target of these genes (Fig. 4).

Another event that is regulated by *lin-14* and *lin-28* (but is independent of *lin-29*) is the execution of vulval development. The vulval precursor cells (VPCs) arise in the L1 and normally undergo multiple rounds of division that begin in the mid L3 stage[4]. In precocious *lin-14* and *lin-28* mutants, these cell divisions occur in the L2, because of a specific shortening of the cell cycle in VPCs (Ref. 2; S. Euling and V. Ambros, in preparation) (Fig. 2). In general, heterochronic mutations do not affect rates of cell division, but may directly or indirectly regulate components of the cell cycle regulatory machinery in a way that is specific to the vulval precursor cells.

A third well-studied event that is controlled by *lin-14* (but is independent of *lin-28* and *lin-29*) is dauer larva arrest. In response to crowding and starvation, development of the *C. elegans* larva may be arrested after the L2 stage in a diapause state called the dauer larva[16]. Dauer larvae are resistant to harsh environments for long periods; upon exposure to more favorable growth conditions, they recover and resume development. The stage at which an animal may arrest as a dauer larva is affected by the level of *lin-14* activity[12]. Animals that have reduced *lin-14* activity may form precocious dauer larvae after the L1 stage, and those with increased *lin-14* activity may form retarded dauer larvae after the L3. Both types of mutants can arrest development

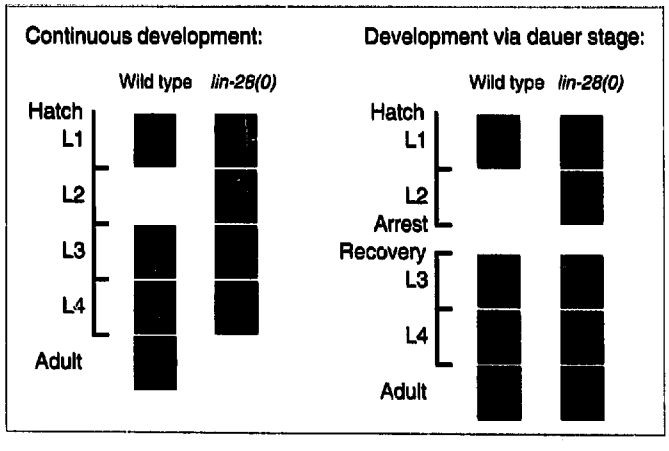

FIGURE 5. Reprogramming of precocious development after dauer larva arrest[15]. In continuous development, a *lin-28(0)* mutant fails to execute L2-specific patterns in the hypodermal lineage, and precociously executes patterns specific to the L3, L4 and adult stages. When *lin-28(0)* mutants develop by way of the dauer larva stage, the L3 and later stages develop in the same way as wild-type animals.

after the L2, as wild-type animals might do. Therefore, the targets regulated by *lin-14* might include previously identified genes that are required to initiate dauer development in response to environmental stimuli or to trigger differentiation into the dauer larva[17,18].

Reprogramming of cell fates after arrested development

Further evidence that the early-acting genes *lin-14* and *lin-28* indirectly regulate late developmental events comes from work showing that, provided the animals develop through the dauer larva stage, developmental timing after the L2 is independent of these genes. Precocious *lin-14* or *lin-28* mutants and retarded *lin-14* mutants that have recovered from dauer larva arrest after the L2 develop in the post-dauer stages like wild-type animals[15]. For example, whereas a continuously developing *lin-28* mutant executes the larva-to-adult switch at the end of the L3, a *lin-28* animal recovering from the dauer state resumes development with normal L3-specific patterns of cell lineage, followed by normal L4 development, and executes the larva-to-adult switch at the appropriate time (Fig. 5). Thus, there seems to be a regulatory event or signal during dauer larva arrest or recovery that reprograms the temporal identities of cells, regardless of their previous temporal identities, and instructs the animal to execute cell lineage patterns appropriate to the L3. This reprogramming seems to act globally, since heterochronic defects in cell lineages other than the lateral hypodermis (such as the vulva) are corrected after recovery from dauer larva arrest (S. Euling and V. Ambros, in preparation).

Other heterochronic genes

The known heterochronic genes do not affect all the aspects of postembryonic development that are likely to be temporally regulated. At least one new gene was identified by screening for mutations that cause the persistence throughout larval development of a cuticle surface antigen that is normally specific to the L1 stage[19]. Because this antigen is not affected by mutations in *lin-4* or *lin-14* (S. Politz, pers. commun.) and does not appear to affect any other aspect of development, its altered temporal regulation might result from mutation of a gene that specifically affects the structure or synthesis of the antigen.

Heterochronic mutants that have developmental defects analogous to those in *C. elegans* have been identified in other organisms, including plants[20], slime molds[21] and fungi[22]. The maize *Teopod* mutations (*Tp1*, *Tp2* and *Tp3*) appear to be dosage-sensitive, gain-of-function lesions in genes that control a juvenile signal. *Tp* mutations cause retarded development[20], and thus seem to be analogous, genetically and phenotypically, to *lin-14(gf)* mutations. The *anachronism* (*ana*) locus of *Drosophila* controls the time at which certain neuroblasts proliferate: in this, the activity of *ana* is analogous to that of *lin-14* and *lin-28* in controlling the timing of vulval development[23].

It's all in the timing

The study of heterochronic genes in *C. elegans* has begun to reveal a complex system that specifies and coordinates the temporal component of cell fates in development. Although in defining cell fates, this system must act in concert with regulators of positional information and sex determination, it is also likely that the system includes regulatory features that are unique to the control of developmental timing. Indeed, the staging of development, which ensures the succession and synchrony of developmental events, might require that events in disparate tissues be linked through interdependent regulatory pathways that depend on checkpoints analogous to those in the cell cycle[24]. For example, at the outset, developmental patterns are synchronized by the action of a food signal, followed by activation of the temporal regulator, *lin-4*. The workings of both the temporal regulators themselves and their interconnections must be rigid enough to ensure reproducible outcomes, but must also be flexible enough to allow for developmental contingencies, such as variation in growth rates and arrested development.

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