

Toward a unified model of developmental timing

A “molting” approach

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Abbreviations: NR, nuclear hormone receptor; RNAi, RNA-interference; GFP, green fluorescent protein; UTR, untranslated region; *let-7s*, *let-7* family of microRNAs; 20-E, 20-hydroxy-ecdysone; HLH, helix-loop-helix

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Animal development requires temporal coordination between recurrent processes and sequential events, but the underlying timing mechanisms are not yet understood. The molting cycle of *C. elegans* provides an ideal system to study this basic problem. We recently characterized LIN-42, which is related to the circadian clock protein PERIOD, as a key component of the developmental timer underlying rhythmic molting cycles. In this context, LIN-42 coordinates epithelial stem cell dynamics with progression of the molting cycle. Repeated actions of LIN-42 may enable the reprogramming of seam cell temporal fates, while stage-specific actions of LIN-42 and other heterochronic genes select fates appropriate for upcoming, rather than passing, life stages. Here, we discuss the possible configuration of the molting timer, which may include interconnected positive and negative regulatory loops among *lin-42*, conserved nuclear hormone receptors such as NHR-23 and -25, and the *let-7* family of microRNAs. Physiological and environmental conditions may modulate the activities of particular components of this molting timer. Finding that LIN-42 regulates both a sleep-like behavioral state and epidermal stem cell dynamics further supports the model of functional conservation between LIN-42 and mammalian PERIOD proteins. The molting timer may therefore represent a primitive form of a central biological clock and provide a general paradigm for the integration of rhythmic and developmental processes.

Introduction

Once-in-a-lifetime changes, such as metamorphosis, and repeated processes, such as cell division, are both essential for animal development. However, the timing mechanisms that coordinate sequential and recurrent events in developing organisms are not well understood.

Studies of developmental timing have focused on hormones and gene regulatory cascades that trigger specific chronological events. One prominent example is the regulation of insect metamorphosis by the steroid hormone 20-hydroxy-ecdysone (20-E) and related transcriptional cascades.¹ Specific cytokines and transcription factors that promote the stepwise maturation of mammalian B-cells from pluripotent hematopoietic stem cells have also been well-characterized.² The use of *C. elegans* as a model system led to the seminal discovery of conserved microRNAs (miRNAs) and protein-coding genes that program the successive temporal fates of the stem cell-like lateral epithelial seam cells.^{3–5} These cells divide asymmetrically early in every larval stage; undergo one additional symmetric division in the L2 stage; and ultimately terminally differentiate, by fusing with one another and exiting the cell cycle, at the larval-to-adult transition.^{5,6} Collectively, the genes that control these stage-specific patterns of seam cell division and differentiation make up the heterochronic gene regulatory network.^{7,8}

Relatively little is known about biological timers that drive temporally reiterated processes in the context of metazoan

development. One of the best-characterized developmental oscillators is the segmentation clock of vertebrates, which is composed of interconnected positive and negative regulatory interactions among components of the Notch and Wnt signaling pathways. The segmentation clock drives the rhythmic expression of master transcription factors, which in turn program spatial and temporal cell fates.⁹ The clock operates in somite precursors but not mature tissues. In *C. elegans*, most epithelial cells and syncytia of juveniles exhibit rhythmic gene expression profiles associated with the four larval molts, as we shall describe.^{10,11}

The best-characterized biological timers are the circadian clocks of mature animals. These clocks synchronize daily behavioral, hormonal and metabolic rhythms with predictable fluctuations in environmental and physiological conditions.¹² The mammalian clock consists of interlocked positive and negative feedback loops among the transcription co-factor PERIOD (PER), the basic helix-loop-helix (HLH) transcription factors CLOCK and BMAL, the nuclear hormone receptors (NRs) ROR α and REV-ERB and other ancillary components.¹² PER and other core clock proteins also regulate the cell cycle, and disruption or misalignment of the circadian clock leads to tumor progression in mouse models.^{13,14} However, the extent to which the canonical circadian clock or any other PER-based oscillator coordinates cell cycles with developmental transitions had not been examined.

To address these basic questions, we investigated the molecular mechanism that times the molting cycles of *C. elegans*.¹⁵ The larval molts involve distinctive cellular and organism behaviors that together enable the rapid and repeated reconstruction of cuticle, which is a collagen-rich extracellular matrix (ECM) (Fig. 1).^{16,17} Briefly, the underlying hypoderm detaches from the preexisting cuticle (apolysis), and generates a new cuticle underneath the old one. Various cell-ECM adhesive complexes that collectively tether the cuticle to the hypoderm, the underlying basement membrane (BM) and body wall muscles are also remade during the molts.^{11,18} Notably, the seam cells repeatedly switch between proliferative and

quiescent states in phase with the periodic molts. As described, these cells undergo stem cell-like asymmetric divisions early in every larval stage. The posterior daughters retain pluripotency and proliferative potential. In contrast, the anterior daughters fuse with the hypodermal syncytium (*hyp7*), increasing the size of the body.^{19,20} The pluripotent seam cells are largely quiescent and contribute to the synthesis of cuticles during the molts.⁶ The mechanisms that coordinate stem cell and ECM dynamics in this context are not well understood, but are essential for viability. Indeed, both genetic mutations that prevent seam cell specification and drugs that delay seam cell division cause aberrant, fatal molts.²¹⁻²⁴

The rhythmic behaviors associated with molting cycles include lethargy and idiosyncratic movements used to escape the old cuticle (ecdysis). Lethargus has been used to model mammalian sleep, as this reversible quiescent state is characterized by the cessation of food-intake and locomotion as well as decreased responsiveness to external stimuli.^{25,26} Communication among neurons, muscles and epithelial cells likely coordinates these behavioral routines with progression of the molting cycle.^{15,25-27} Larvae molt four times, once every 8–10 h under favorable culture conditions. Although this periodicity resembles a harmonic of the circadian clock, the anticipated pacemaker had not been characterized.

Using a candidate gene approach, we identified *lin-42*, which is related to *PERIOD*,^{28,29} as a key component of the molting clock that operates in the epithelium of juveniles.¹⁵ This LIN-42-based timer sustains the rhythm of the molting cycle and synchronizes seam cell dynamics with the larval molts.¹⁵ In addition, LIN-42 acts in the heterochronic pathway to program the L3-stage, and possibly other, seam cell temporal fates.^{15,30,31}

Here, we summarize our recent findings and further discuss the possible molecular configurations, environmental and physiological inputs and systemic outputs of the molting clock. Several lines of evidence now support the model of functional conservation between *C. elegans* LIN-42 and mammalian PER proteins. The use of both reiterated and consecutive functions

of LIN-42/PER to regulate developmental timing in *C. elegans* may therefore provide a general paradigm for the integration of rhythmic and sequential processes in biology (Fig. 1). Our ongoing studies of the molting timer will likely uncover novel mechanisms by which conventional and unconventional PER-based clocks regulate biological rhythms throughout the life of metazoans.

Identifying Components of the Molting Timer

The genetic basis of the circadian clock was established by the characterization of three distinct alleles of *Drosophila melanogaster period* that shortened, lengthened or abolished daily rhythms in locomotor activity.³² Two hallmarks of core clock components have since emerged. The first is that either inactivation or constitutive expression of the corresponding genes disrupts the clock-controlled biological rhythm.¹² We found that *lin-42(ok2385)* mutants molted at unpredictable times.¹⁵ Strikingly, individual *lin-42(ok2385)* mutants completed the fourth molt up to 24 h apart, whereas wild-type animals completed the molts virtually in synchrony (Fig. 2A). Unusually long periods of time typically elapsed between ecdyses in *lin-42(ok2385)* mutants. However, new molts occasionally began prior to the completion of earlier ecdyses. These unnaturally short cycles became evident when larvae shed two cuticles within an hour, or became trapped within two incompletely shed cuticles. Further, forced expression of *lin-42a* from an inducible promoter led to anachronistic and fatal molts, in which the hypoderm detached from the cuticle prior to stage-specific developmental benchmarks.¹⁵

We also found that *lin-42* was essential for proper seam cell dynamics throughout larval development.¹⁵ The seam cells were inappropriately detached from one another and misshapen in *lin-42(ok2385)* mutants undergoing molts. At the L3-to-L4 transition, some seam cells fused prematurely and secreted alae, a phenotype that was previously observed in other *lin-42* mutants.^{30,31} However, at the L4-to-adult transition, some seam cells failed to exit the cell cycle and subsequently underwent

supernumerary divisions (Fig. 2B). Thus, *lin-42(ok2385)* animals exhibited defining features of both “precocious” and “retarded” heterochronic mutants. Taken together, our findings suggest that a LIN-42-based timer synchronizes seam cell dynamics with larval molting cycles. The *lin-42* gene regulates both the periodicity and the quality of the molts, as *lin-42* also functions in the heterochronic pathway to program the L3, and possibly other, seam cell temporal fates.²⁸ In this context, reiterated functions of LIN-42 may enable the reprogramming of seam cell temporal fates, while sequential actions of LIN-42 and other heterochronic genes select the fates appropriate for upcoming, rather than passing, life stages.

Oscillatory activity is the second distinctive feature of core clock components. Both transcriptional and post-transcriptional regulatory mechanisms contribute to these oscillations, which occur in phase with the clock-controlled biological rhythm.¹² The *lin-42* locus is complex and encodes three major transcripts with both shared and unique cis-acting regulatory elements.²⁹ The overall abundance of *lin-42* transcripts and proteins rises and falls in phase with the larval molts.²⁸ To better define the temporal expression profiles of *lin-42a*, *b* and *c*, we separately combined both the unique promoter of *lin-42a* and the shared promoter of *lin-42b* and *c* with the *gfp-pest* reporter. Expression of the corresponding fusion genes in *hyp7* showed that activity of the *lin-42b* promoter peaked during the intermolts, as previously reported.²⁹ However, the *lin-42a* promoter was most active toward the end of every larval stage; GFP was robustly expressed in lethargic larvae undergoing molts, and was barely detectable about two hours after ecdysis.¹⁵ Expression of *lin-42a* restored both rhythmic molts and proper epidermal development to *lin-42* mutants, underscoring the significance of the independent transcription of *lin-42a*.^{15,29}

Taken together, our findings provide substantial evidence that LIN-42A functions as a central component of the molting clock. The isolation of missense alleles of *lin-42* associated with consistently shorter or longer, rather than unpredictable, molting cycles would further support this model. In theory, the molecular

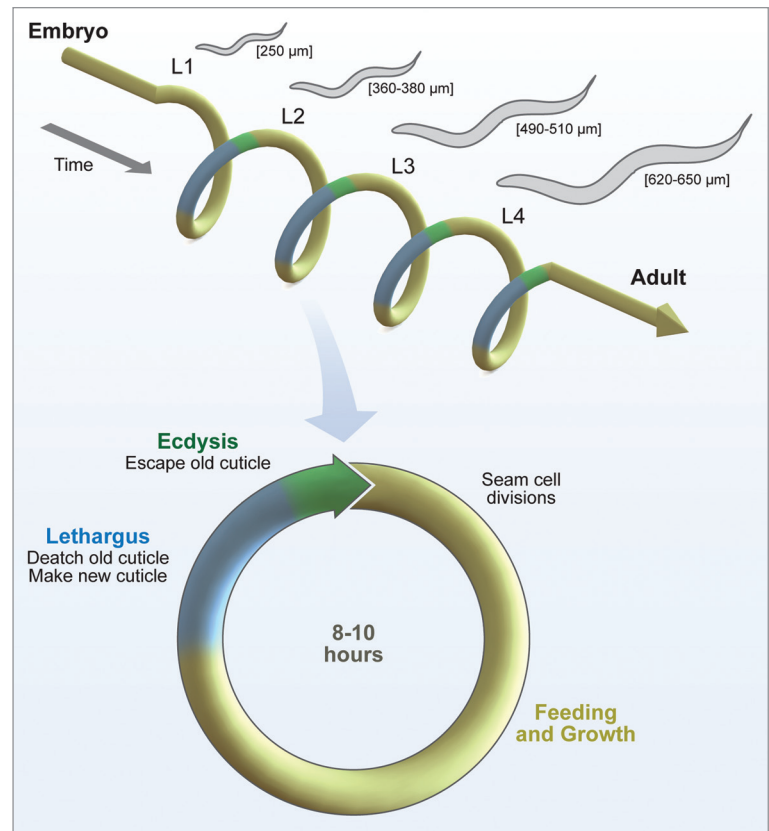


Figure 1. Molting is a reiterated process in development. Each molt involves specialized cellular programs and animal behaviors. Epithelial cells and syncytia detach from the preexisting cuticle and secrete a new cuticle underneath the old one. Larvae are quiescent for approximately 2 h while the cuticle is remade (lethargus). Larvae then execute a series of idiosyncratic movements to escape the old cuticle (ecdysis); this step takes about 20 min. The entire process is repeated four times, every 8–10 h under favorable culture conditions. The lateral epithelial seam cells undergo stem cell-like asymmetric divisions early in every larval stage, but contribute to the synthesis of new cuticles during the molts. Reprogramming of the successive, stage-specific temporal fates of the seam cells occurs around the time of the molts (not shown).

identities of related substitutions might identify motifs essential for the activity of LIN-42A, and possibly sites of post-translational modifications that affect the abundance or intracellular distribution of LIN-42A. Similar approaches uncovered pivotal roles for the phosphorylation and O-glycosylation of PER in the circadian clock.^{33–36} Identifying the transcriptional regulators and targets of LIN-42 would also improve our understanding of the molting timer. This task is challenging, in part, because the genome of *C. elegans* encodes scores of HLH transcription factors, none of which are clearly homologous to CLOCK or BMAL.

Particular conserved NRs are excellent candidates for additional components of the molting clock: namely, NHR-23, which is homologous to both mammalian

ROR α and *Drosophila* DHR3, NHR-25, which is homologous to both mammalian SF-1 and *Drosophila* FTZ-F1, and DAF-12, which is related to the Vitamin D receptor.³⁷ Inactivation of either *nhr-23* or *-25* prevents completion of the molts, and overexpression of either gene causes larval lethality.^{38–41} NHR-23 and *-25* also directly or indirectly activate the expression of many genes involved in the process of molting.^{11,39,42–45} These genes encode various intercellular signaling molecules, matrix modification enzymes and ECM proteins, including collagens and MLT-10.⁴⁶ NHR-25 also regulates seam cell dynamics.^{23,24} In addition, we found that RNAi of either *nhr-23* or *-25* exacerbated the molting defects of *lin-42(ok2385)* mutants.¹⁵ DAF-12 regulates the decision to undergo rapid development or

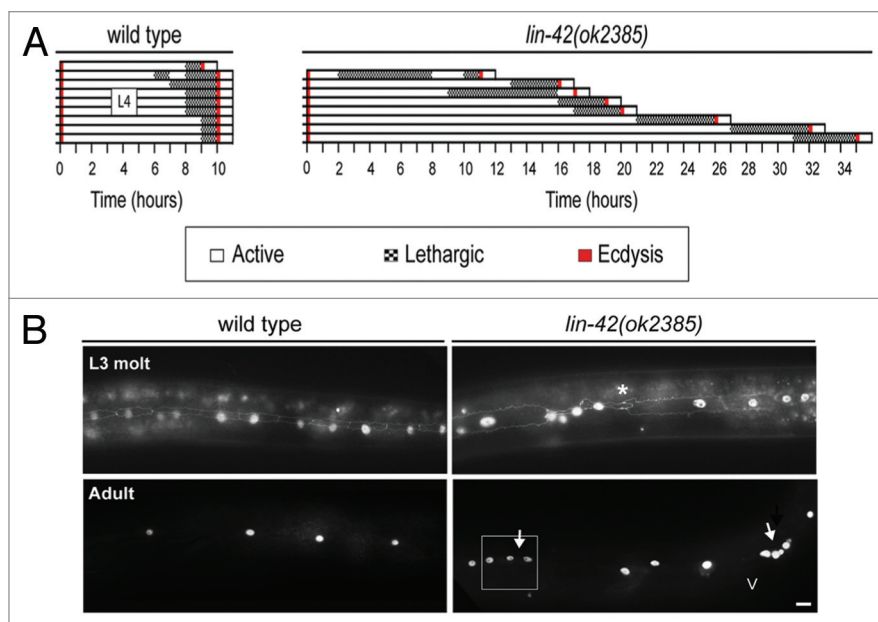


Figure 2. Molting cycles and seam cell dynamics are temporally misaligned in *lin-42* mutants. **(A)** Chart shows the asynchronous execution of the fourth molt in *lin-42(ok2385)* mutants, compared with wild-type larvae. Each bar represents the behavior of a single animal monitored from the third to the fourth molt. Larvae were observed for 30 sec of every hour and behavior scored by visual inspection. The average duration of the L4 stage was 21.8 ± 3.1 ($C_v = 0.4$) hours in *lin-42(ok2385)* mutants, compared with 9.9 ± 0.1 ($C_v = 0.05$) in wild-type animals. **(B)** Seam cells were visualized in *lin-42(ok2385)* mutants and wild-type animals by fluorescence microscopy, using the *AJM-1::GFP* marker for adherens junctions and the *scm::gfp* reporter for seam nuclei. Seam cells of *lin-42* mutants often underwent precocious homotypic fusion during the third molt (asterisk). However, some seam nuclei underwent supernumerary divisions in reproductively mature animals (arrows). The white box indicates one region where alae were detected on the cuticle. "V" indicates the position of the vulva. Scale bar corresponds to $10 \mu\text{M}$. This figure was adapted from Monsalve et al., LIN-42/PERIOD Controls Cyclical and Developmental Progression of *C. elegans* Molts, *Current Biology* 21, 2033–2045 (2011).

facultative diapause in L2 larvae.³⁷ In this context, *lin-42* interacts with *daf-12* and ligand-activated DAF-12 induces the expression of *lin-42a* when conditions favor rapid development.^{47,48} However, *daf-12* is expressed throughout development and may regulate the progression of additional life stages.³⁷

The abundance of *nhr-23* and *-25* transcripts also oscillates in phase with the molting cycle.^{39,49} Overall levels of *nhr-23* transcripts peak midway through the intermolt, but the temporal expression patterns of the six distinct isoforms of *nhr-23* curated in Wormbase229 have not yet been examined. Levels of *nhr-25 α* transcripts, which encode the full-length receptor, peak during the molts, whereas levels of *nhr-25 β* transcripts, which encode a truncated receptor, peak after ecdysis.⁴⁹ Once made, NHR-25 β might interfere with transcriptional activation

by NHR-25 α , as the former isoform lacks a DNA binding domain. Based on the timing of peak *nhr-23* expression and the annotations of confirmed targets of the receptor, we hypothesize that transcriptional activation by NHR-23 enforces a commitment to molt. NHR-23 might directly promote the expression of NHR-25, given that *Drosophila* DHR3 activates the transcription of *FTZ-F1*.⁵⁰ In theory, the expression of various targets of NHR-25 may then promote completion of the molt and the start of the next life stage. The precise temporal expression pattern of *daf-12* has not yet been characterized.³⁷

The conserved *let-7* family of miRNAs has been predicted to target and downregulate both *lin-42* and *nhr-25*,^{4,51} and may thereby fine-tune the periodicity of larval molting cycles. The best-characterized function of *let-7* is to promote terminal differentiation of the lateral hypoderm by

downregulating additional protein-coding genes in the heterochronic pathway.⁴ The seam cells fail to terminally differentiate at the larval-to-adult transition in *let-7* mutants, and instead undergo extra divisions in reproductively mature animals.⁴ Mutations in *let-7* are also associated with supernumerary molts, which can be suppressed by knocking-down either *nhr-23* or *-25*.^{4,51} The fact that *let-7* mutants undergo supernumerary molts at predictable, rather than sporadic, times indicates that the core molting timer operates in this background. However, the extent to which *let-7* or related miRNAs affect the duration of specific larval stages or the synchronicity of the molts has not yet been determined. Notably, mutations in *lin-42* partially suppress the phenotypes of *let-7* mutants and vice-versa,^{29,31} but the mechanism of this co-suppression has not yet been defined. One interesting possibility is that *lin-42* negatively regulates the expression of mature *let-7* miRNAs.

Primary transcripts of *let-7* are also expressed during each of the four molts, even though mature *let-7* miRNAs are only produced in late stage larvae and adults.⁵² However, additional members of the *let-7* family act during earlier larval stages,^{53,54} and the expression of some, if not all, paralogs of *let-7* might oscillate in phase with the molts. Although the cyclical expression of *lin-42*, *nhr-23*, *nhr-25* and *let-7* is critical for larval development,^{15,55} the transcriptional and post-transcriptional regulatory mechanisms that account for these oscillations have not yet been described.

Modeling the Molting Timer

As depicted in **Figure 3A**, we postulate that interlocked positive and negative regulatory loops involving LIN-42, conserved NRs and the *let-7* family of miRNAs compose a developmental oscillator that drives rapid molting cycles. In the positive limb of the proposed timer, specific NRs activate the expression of *lin-42a* and *let-7* family miRNAs, and levels of the corresponding gene products begin to rise. The precise time of gene activation within each larval stage may be determined by the abundance of the receptors, affiliated co-factors and

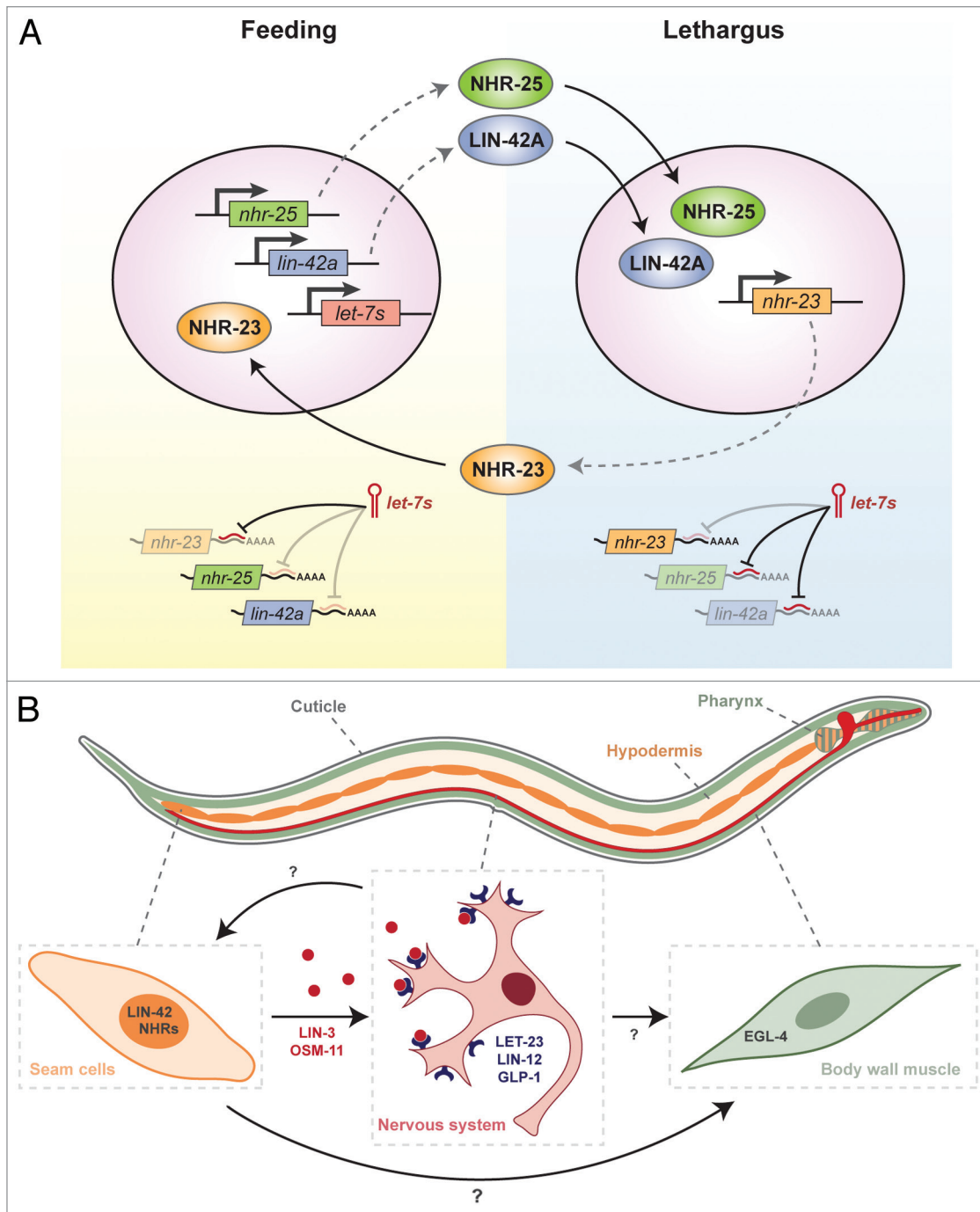


Figure 3. Modeling the molting timer. **(A)** We propose that interconnected positive and negative regulatory interactions among LIN-42, certain NRs, and *let-7* family miRNAs compose a developmental oscillator that operates in the epidermis of juveniles and drives rhythmic molting cycles. This diagram depicts hypothetical molecular events that might occur in seam cells as animals enter and exit the larval molts. **(B)** The proposed molting timer may also regulate the rhythmic production of endocrine cues that affect animal behavior. Additional signals among the epidermis (orange), the nervous system (red), and body wall muscles (green) are expected to regulate lethargus and ecdysis. See text for additional information about this model.

small-molecule ligands. Consistent with this hypothesis, there are several half-sites for NR binding⁵⁶ in the experimentally defined promoters of *lin-42a* and *let-7*.^{15,29,52,57} Further, DAF-12 is known to induce the expression of *lin-42a* and

let-7 under favorable conditions.^{47,48,58,59} In addition, the mammalian NR NF- κ B activates expression of *let-7* in tissue culture.⁶⁰ Although a high-throughput screen for transcriptional regulators of the *let-7* family did not uncover NHR-23 or -25,⁶¹

the possibility that one or both of these receptors periodically activate the expression of *let-7* family miRNAs in larvae has not been evaluated. In the negative limb of the proposed timer, *let-7* family miRNAs target *lin-42* and *nr* transcripts for

translational inhibition or degradation, contributing to a decline in levels of the corresponding proteins. Consistent with this model, *let-7* is known to directly target *daf-12* transcripts.^{58,59} The extent, if any, to which mammalian *let-7* miRNAs downregulate *per* or other components of the circadian clock is not known.

The molecular function of LIN-42A has not yet been defined, but the protein is thought to act as a transcriptional co-factor, similar to larger PER proteins. Consistent with this model, several sequence motifs are conserved between LIN-42A and mammalian PER proteins, although LIN-42A lacks a Period-Ant-Sim (PAS) domain.²⁹ Further, LIN-42A was detected in the nuclei of epithelial cells.¹⁵ Per2 was recently found to bind the NR REV-ERB through an LXXLL motif as part of the circadian clock.⁶² Various co-activators of Drosophila FTZ-F1 and Mammalian SF-1 also use LXXLL motifs to bind the receptors.^{63,64} LIN-42A and B contain two LXXLL motifs, one or both of which might enable physical interactions with NHR-23 or -25. Consistent with this general idea, initial studies have suggested that LIN-42B interacts with DAF-12 in a manner that does not depend on the PAS domain.⁴⁸ If LIN-42 does in fact associate with these particular NRs, then the abundance of related complexes in epithelial cells might fluctuate during every larval stage and contribute to the rhythmic expression of downstream target genes. Protein-protein interactions between LIN-42 and these particular NRs may therefore play a role in timing molting cycles.

In summary, LIN-42A functions in a developmental oscillator underlying rhythmic molting cycles. In this context, high levels of LIN-42A coincide with lethargy, ECM remodeling and stem cell quiescence, whereas low levels of LIN-42A coincide with physical activity, stable matrices and stem cell divisions. Rising levels of LIN-42 may allow the reprogramming of seam cell temporal fates; falling levels of LIN-42 may license the subsequent round of cell division. Reprogramming of seam cell temporal fates may be achieved, in part, by repressing the expression of preexisting miRNAs, including *lin-4* in L1 larvae and the *let-7*

family in older larvae. The timer would start to tick in the epithelium of late-stage embryos, around the time of synthesis of the L1-stage cuticle, and cease to operate when the epithelium terminally differentiates at the larval-to-adult transition. Anticipated clock-controlled genes include some components of the heterochronic network. In theory, the 8-h rhythm of the molting cycle may represent a harmonic of a circadian oscillator adapted to support the rapid growth of nematode larvae.²⁰

Finding that individual *lin-42* mutants complete the larval stages at variable times underscores the need to account for progression of the molts, in addition to the passage of time, when characterizing mutations that affect developmental timing and the expression profiles of corresponding genes. The heterochronic mutants of *C. elegans* have been classified based on the relative timing of either the symmetric divisions or the terminal differentiation of the seam cells, compared with development of the reproductive system.⁶⁵ It has since become standard practice in the field to synchronize hatchlings by starvation-induced L1-stage diapause, cultivate larvae on food for pre-selected periods of time, and then collect animals for visual inspection or the extraction of nucleic acids. Interpretations of related data often assume that all larvae of a given genotype developed at a consistent pace, similar to wild-type animals. Some heterochronic phenotypes may therefore be attributable to slower, faster or arrhythmic molting cycles, rather than the misspecification of temporal cell fates.

Potential Inputs to the Molting Timer

The circadian clocks of mammals beneficially synchronize rhythms in behavior and metabolism with daily fluctuations in sunlight, temperature and the availability of food.^{12,66,67} These environmental factors are typically held constant when nematodes are cultivated in the lab, but almost certainly vary in the natural habitat of the soil, and may impact the periodicity of molting cycles in that context. Indeed, cooler cultivation temperatures reduce the speed of larval development, and rhythmic fluctuations in ambient temperature

produce oscillations in the expression of many *C. elegans* genes.⁶⁸ Further, both the quality and abundance of food affect the pace of larval development.⁶⁹

Fluctuating levels of specific nutrients or metabolites and thresholds in body size may also provide physiologic inputs to the molting timer. In general, energy reserves should increase as larvae feed. However, because the volume of the body increases continuously, whereas the volume of the mouth only increases during molts, the ratio of food intake to energy expenditure likely drops when larvae reach a critical size.²⁰ The process of molting then requires substantial work, at a time when larvae are unable to feed. Consistent with the idea that metabolic states influence the progression of molting cycles, signaling through the insulin pathway promotes rapid larval development.^{70,71} Mechanotransduction pathways coupled to stress on the cuticle may also promote molting when larvae attain critical sizes. These concepts are consistent with the observation that the average size of shed cuticles increases saltationally from one larval stage to the next.⁶⁹ We further anticipate that “checkpoints” on the status of the new cuticle regulate the transition from lethargus to ecdysis, although the corresponding circuits have not yet been described.

Fluctuations in the abundance of particular steroid hormones may also affect the periodicity of the molts, by modulating the activity of NRs including NHR-23, -25 and DAF-12. *C. elegans* cannot synthesize cholesterol *de novo*, and an exogenous supply of cholesterol is required for completion of the molts.^{72,73} Cholesterol is generally thought to serve as a precursor for the biosynthesis of essential steroid hormones,⁷⁴ although 20-E has not been detected in any free-living nematode.⁷⁵ The natural ligand of human ROR α is a derivative of cholesterol,⁷⁶ and a related molecule might serve as a physiologic ligand for NHR-23. Recent findings have shown that FTZ-F1 functions as a constitutive transcriptional activator without a small molecule ligand,⁶³ and NHR-25 might operate in a similar manner. The endogenous ligand for DAF-12 was the first steroid hormone characterized in *C. elegans*.⁷⁷ Both insulin and

TGF- β signaling promote production of this hormone.³⁷

Endocrine and neuroendocrine cues coupled to any or all of the aforementioned environmental and physiological factors may converge on the regulation of LIN-42 and other components of the molting timer. In natural habitats, such interactions might align the molts with favorable extrinsic and intrinsic conditions, and thereby decrease the possibility of injury or death during the molting process.

Systemic Outputs of the Molting Timer

As described, molting cycles include the distinctive behaviors of lethargy and ecdysis, which are regulated by endocrine and neuroendocrine circuits.^{25,27} Both the onset and duration of lethargus were irregular in *lin-42(ok2385)* mutants, and ecdyses were often aberrant.¹⁵ Remarkably, seam cell-specific expression of *lin-42a* restored normal cycles of physical activity and quiescence to *lin-42(ok2385)* mutants, in addition to successful ecdyses.¹⁵ This particular finding implies that activity of LIN-42 in the seam cells promotes the rhythmic production or release of intercellular signals that directly or indirectly modulate the functions of various neurons and muscles throughout the body (Fig. 3B). Several recent reports have confirmed that neuroendocrine cues produced by epithelial cells either initiate or maintain the behavioral quiescence associated with larval molts. Secretion of the DOS protein OSM-11 from the seam cells induces quiescence by activating the Notch receptors LIN-12 and GLP-1 in the nervous system.²⁷ In a parallel pathway, the EGF-like molecule LIN-3 induces quiescence by activating the EGF receptor LET-23 in the ALA interneuron.²⁵ The relevant source of endogenous LIN-3 has not yet been defined. However, the *lin-3* gene is expressed in the pharynx, the hypoderm and the intestine, and expression of *lin-3* in some epithelial cells is activated by NHR-25.^{78,79} The ectodomain of the Amyloid Precursor-Like protein APL-1, which may be released from both seam cells and neurons, might also regulate behavior during the molts.⁸⁰⁻⁸² Moreover,

the expression of an *apl-1* fusion gene in the seam cells of L4 larvae is induced by *nhr-25*, and indirectly repressed by the *let-7* family of miRNAs.^{45,83} Another key component of the signaling network that regulates lethargus is the conserved cyclic GMP protein kinase EGL-4, which is expressed in body wall muscles, the hypodermis and head neurons.^{26,84,85} Loss-of-function mutations in *egl-4* are associated with increased physical activity and partly suppress the behavioral quiescence triggered by forced expression of *lin-42a*, *osm-11* or *lin-3*.^{15,25,27} Although the events downstream of EGFR and Notch Receptor activation have not yet been characterized, these pathways likely converge on the regulated secretion of neurotransmitters at neuromuscular junctions. In theory, the molting timer might control behavioral rhythms by directly or indirectly modulating the activities of OSM-11, LIN-3 or APL-1. Additional outputs of the molting timer may affect osmoregulation, metabolism and other systemic processes that differ between molting and non-molting larvae.

The Molting Timer as a Primitive Biological Clock

In mammals, a master circadian clock operates in the suprachiasmatic nucleus (SCN) of the brain. The central clock resets and synchronizes peripheral clocks in vital organs, and responds to feedback from peripheral oscillators.¹² The ontogeny of this system is not well understood.⁸⁶ When viewed in this context, the LIN-42/PER-based molting timer, which operates in epithelial cells and affects rhythmic behaviors of several other tissues, may represent a primitive form of a central clock. Interestingly, the eclosion of insects and the rhythmic growth of plants are also regulated by the circadian clock.⁸⁷⁻⁸⁹ The use of PER-based clocks as developmental timers may therefore represent an ancient adaptation of multicellular organisms.

The regulation of sleep-wake cycles is perhaps the best-characterized function of human PER proteins, and mutations in the corresponding genes are associated with various inherited disorders of sleep.⁹⁰⁻⁹³ Finding that *lin-42* regulates the timing of lethargus further supports the view that

nematode lethargy and human sleep share an ancient evolutionary origin. Homologs of several additional molecules that regulate lethargus also affect the sleep-wake cycles of mammals. ROR α , which is related to NHR-23, regulates sleep as a component of the circadian clock.⁹⁴ The mammalian EGF receptor and the EGL-4 homolog PRKG1 also influence the timing and quality of sleep.^{95,96} Further, levels of *let-7* increase in the hippocampus of sleep-deprived rats, suggesting that the activity of *let-7* correlates with sleep-wake cycles.^{97,98}

Another striking similarity between the function of *C. elegans* LIN-42 and mammalian PER is that both proteins regulate epidermal stem cell dynamics.^{15,99} The epidermal stem cells of mammalian hair follicles also undergo cycles of proliferation and quiescence,¹⁰⁰ and PER was recently found to regulate the overall size of this stem cell population and the propensity of cells therein to enter the cell cycle.^{99,101} This particular function of human PER may be analogous to the role of LIN-42 in regulating seam cell proliferation and temporal specification.¹⁵

Recent studies have uncovered the clinical significance of clock misalignment or dysfunction in modern epidemics including obesity, diabetes and age-related declines in bodily function.^{12,102-105} Further basic research on the molting timer may therefore uncover novel but conserved aspects of PER-based oscillators relevant to human development as well as metabolic syndromes and sleep disorders.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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