Toward a unified model of developmental timing
A “molting” approach

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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. Specific cytokines and transcription factors that promote the stepwise maturation of mammalian B-cells from pluripotent hematopoietic stem cells have also been well-characterized. 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. 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. Collectively, the genes that control these stage-specific patterns of seam cell division and differentiation make up the heterochronic gene regulatory network.

Relatively little is known about biological timers that drive temporally reiterated processes in the context of metazoan
The larval molts involve distinct cellular and organism behaviors that together enable the rapid and repeated reconstruction of cuticle, which is a collagen-rich extracellular matrix (ECM) (Fig. 1). Briefly, the underlying hypoderm detaches from the preexisting cuticle (apoposis), 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. 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. The pluripotent seam cells are largely quiescent and contribute to the synthesis of cuticles during the molts. 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, lethal mutations.  

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. The mammalian clock consists of interlocked positive and negative feedback loops among the transcription factor PERIOD (PER), the basic helix-loop-helix (HLH) transcription factors CLOCK and BMAL, the nuclear hormone receptors (NRs) RORα and REV-ERB and other ancillary components. 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. 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. 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). Briefly, the underlying hypoderm detaches from the preexisting cuticle (apoposis), 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. 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. The pluripotent seam cells are largely quiescent and contribute to the synthesis of cuticles during the molts. 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, lethal mutations.

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. 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. We found that lin-42(ok2385) mutants molted at unpredictable times. 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-42 from an inducible promoter led to anachronistic and fatal molts, in which the hypoderm detached from the cuticle prior to stage-specific developmental benchmarks.

We also found that lin-42 was essential for proper seam cell dynamics throughout larval development. 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. 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.\textsuperscript{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.\textsuperscript{12} The lin-42 locus is complex and encodes three major transcripts with both shared and unique cis-acting regulatory elements.\textsuperscript{29} The overall abundance of lin-42 transcripts and proteins rises and falls in phase with the larval molts.\textsuperscript{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.\textsuperscript{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 ec dysis.\textsuperscript{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.\textsuperscript{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 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.\textsuperscript{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 \textit{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 RORα 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.\textsuperscript{37} Inactivation of either \textit{nhr-23} or -25 prevents completion of the molts, and overexpression of either gene causes larval lethality.\textsuperscript{38-41} NHR-23 and -25 also directly or indirectly activate the expression of many genes involved in the process of molting.\textsuperscript{31,39,42-45} These genes encode various intercellular signaling molecules, matrix modification enzymes and ECM proteins, including collagens and MLT-10.\textsuperscript{46} NHR-25 also regulates seam cell dynamics.\textsuperscript{23,24} In addition, we found that RNAi of either \textit{nhr-23} or -25 exacerbated the molting defects of \textit{lin-42(ok2385)} mutants.\textsuperscript{15} DAF-12 regulates the decision to undergo rapid development or

![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 epidermal 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).](image-url)
facultative diapause in L2 larvae.\textsuperscript{37} In this context, \textit{lin-42} interacts with \textit{daf-12} and ligand-activated DAF-12 induces the expression of \textit{lin-42a} when conditions favor rapid development.\textsuperscript{67,68} However, \textit{daf-12} is expressed throughout development and may regulate the progression of additional life stages.\textsuperscript{37}

The abundance of \textit{nhr-23} and -25 transcripts also oscillates in phase with the molting cycle.\textsuperscript{39,49} Overall levels of \textit{nhr-23} transcripts peak midway through the intermolt, but the temporal expression patterns of the six distinct isoforms of \textit{nhr-23} curated in Wormbase\textsuperscript{229} have not yet been examined. Levels of \textit{nhr-25\alpha} transcripts, which encode the full-length receptor, peak during the molts, whereas levels of \textit{nhr-25\beta} transcripts, which encode a truncated receptor, peak after eclosion.\textsuperscript{49} Once made, NHR-25\beta might interfere with transcriptional activation by NHR-25\alpha, as the former isoform lacks a DNA binding domain. Based on the timing of peak \textit{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 \textit{FTZ-F1}.\textsuperscript{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 \textit{daf-12} has not yet been characterized.\textsuperscript{37}

The conserved \textit{let-7} family of miRNAs has been predicted to target and downregulate both \textit{lin-42} and \textit{nhr-25},\textsuperscript{4,51} and may thereby fine-tune the periodicity of larval molting cycles. The best-characterized function of \textit{let-7} is to promote terminal differentiation of the lateral hypoderm by downregulating additional protein-coding genes in the heterochronic pathway.\textsuperscript{4} The seam cells fail to terminally differentiate at the larval-to-adult transition in \textit{let-7} mutants, and instead undergo extra divisions in reproductively mature animals.\textsuperscript{4}

Mutations in \textit{let-7} are also associated with supernumerary molts, which can be suppressed by knocking-down either \textit{nhr-23} or -25.\textsuperscript{4,51} The fact that \textit{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 \textit{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 \textit{lin-42} partially suppress the phenotypes of \textit{let-7} mutants and vice-versa,\textsuperscript{29,51} but the mechanism of this co-suppression has not yet been defined. One interesting possibility is that \textit{lin-42} negatively regulates the expression of mature \textit{let-7} miRNAs.

Primary transcripts of \textit{let-7} are also expressed during each of the four molts, even though mature \textit{let-7} miRNAs are only produced in late stage larvae and adults.\textsuperscript{32} However, additional members of the \textit{let-7} family act during earlier larval stages,\textsuperscript{53,54} and the expression of some, if not all, paralogs of \textit{let-7} might oscillate in phase with the molts. Although the cyclical expression of \textit{lin-42}, \textit{nhr-23}, \textit{nhr-25} and \textit{let-7} is critical for larval development,\textsuperscript{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 \textit{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 \textit{lin-42\alpha} and \textit{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
small-molecule ligands. Consistent with this hypothesis, there are several half-sites for NR binding in the experimentally defined promoters of lin-42a and let-7 under favorable conditions. In addition, the mammalian NR NF-κB activates expression of let-7 in tissue culture. Although a high-throughput screen for transcriptional regulators of the let-7 family did not uncover NHR-23 or -25, 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

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.
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.

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. 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. 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. Further, both the quality and abundance of food affect the pace of larval development.

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 larval reach a critical size. 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. Mechano-transduction 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 saliently from one larval stage to the next. 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.

Cholesterol is generally thought to serve as a precursor for the biosynthesis of essential steroid hormones, although 20-E has not been detected in any free-living nematode. The natural ligand of human RORα is a derivative of cholesterol, 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, and NHR-25 might operate in a similar manner. The endogenous ligand for DAF-12 was the first steroid hormone characterized in C. elegans. Both insulin and...
TGF-β signaling promote production of this hormone. 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 ecdisis, which are regulated by endocrine and neuroendocrine circuits. Both the onset and duration of lethargus were irregular in lin-42(ok2385) mutants, and ecdisis were often aberrant. 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 ecdisis. 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 Drosophila protein OSM-11 from the seam cells induces quiescence by activating the Notch receptors LIN-12 and GLP-1 in the nervous system. In a parallel pathway, the EGFR-like molecule LIN-3 induces quiescence by activating the EGFR receptor LET-23 in the ALA interneuron. 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. 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. Moreover, the expression of an apl-1 fusion gene in the seam cells of L4 larvae is induced by nhbr-25, and indirectly repressed by the let-7 family of miRNAs. 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. 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. 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. The ontogeny of this system is not well understood. 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. 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. 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. The mammalian EGF receptor and the EGL-4 homolog PRKG1 also influence the timing and quality of sleep. 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.

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

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. 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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