REVIEW

Molting in C. elegans

Vladimir Lažetić and David S. Fay

Department of Molecular Biology, College of Agriculture and Natural Resources, University of Wyoming, Laramie, WY, USA

ABSTRACT

Molting is an essential developmental process for the majority of animal species on Earth. During the molting process, which is a specialized form of extracellular matrix (ECM) remodeling, the old apical ECM, or cuticle, is replaced with a new one. Many of the genes and pathways identified as important for molting in nematodes are highly conserved in vertebrates and include regulators and components of vesicular trafficking, steroid-hormone signaling, developmental timers, and hedgehog-like signaling. In this review, we discuss what is known about molting, with a focus on studies in Caenorhabditis elegans. We also describe the key structural elements of the cuticle that must be released, newly synthesized, or remodeled for proper molting to occur.

KEYWORDS

C. elegans; cuticle; extracellular matrix; intracellular trafficking; molting; signaling

Introduction

The majority of animal species undergo molts during their life cycles. This includes all arthropods (insects, arachnids, and crustaceans), nematodes (roundworms), and other members of the Ecdysozoa superphylum.1,2 Molting is the process by which animals replace their old exoskeleton, also termed the cuticle, with a new one. Depending on the species and the stage of development, molting is essential for normal growth or developmental progression, or for both processes. Molting is regulated by a combination of genetic and environmental factors and must be closely coordinated with other developmental events. Interest in the mechanisms controlling molting is due in part to the significant medical and economic consequences caused by parasitic Ecdysozoa species. As such, agents that interfere with molting, such as drugs or pesticides, have been a focus of pharmacological research.3-6 In addition, the analysis of molting provides a good opportunity for understanding a variety of conserved developmental processes. For example, molting can reveal details about ECM remodeling, developmental timing, steroid hormone functions, signal transduction pathways, intracellular trafficking, and other fundamental cellular mechanisms that are evolutionarily conserved. Moreover, molting can be studied in well-developed and streamlined genetic systems such as the fruit fly, Drosophila melanogaster, and the roundworm, Caenorhabditis elegans.

Functions of molting

Although molting is required for development in a wide range of species, its primary functions are likely to vary across different groups of animals. In arthropods, which contain a rigid chitin-based cuticle, molting is essential for body size expansion. In the case of most insects, molting occurs prior to adulthood, whereas crustaceans can undergo periodic molts, coupled with continued growth, as adults. In contrast to arthropods, the nematode body is covered with a collagen-based cuticle that allows growth and expansion between molting periods. In fact, some authors reject the premise that molting is required for growth in nematodes, especially in parasitic species.7,8 This is supported by the observation that infective L2 larvae of plant parasites from the nematode genus Meloidogyne can grow substantially before reaching the L3 molt.9,10 Moreover, the L3 and L4 stages in Meloidogyne occur without intervening feeding or growth. In such cases, molting may be important for the development of anatomical structures in the mouth region, which are required for normal feeding and growth at the adult stage.
Another example in which molting is not essential for nematode growth was observed in the parasite species *Ascaris lumbricoides*. The adult animal increases in length from 1–2 cm after the final molt to 25–35 cm after living inside the host’s intestine for several months. Furthermore, the cuticle becomes thicker as the animal grows during its adult life, suggesting that, in at least some nematode species, cuticle components are actively synthesized after the final molt. Based on these observations in parasitic species, a proposed function of nematode molting is to change the surface composition and structure of the organism to best match the different environments that it inhabits at different life stages. Interestingly, all members of the Nematode phylum undergo four larval molts, regardless of whether or not they are parasitic or free-living.

In the case of many free-living nematodes, such as *C. elegans*, their ecological niche is unlikely to change dramatically during the course of their life cycle. Given this, environmental adaptation may not be a driving force behind molting or changes in cuticle composition in many free-living nematode species. Rather, molting may serve as a means for body-size expansion and may trigger periods of rapid growth. Consistent with this, *C. elegans* growth rates, based on volume, are linear between molting cycles but are exponential during the periods surrounding molts. Thus, slow linear growth during each larval stage may be permitted by the elastic properties of the cuticle, whereas rapid exponential growth may be enabled by the process of molting. Consistent with this model, the new cuticle is initially highly convoluted after each molt in *C. elegans*, suggesting that it has a larger surface area than the previous cuticle and may therefore accommodate a rapid increase in size at the end of each molt. This accordion-like structure of the nascent cuticle is facilitated by the presence of regularly spaced circumferential actin bundles, which reside at the apical epidermal surface during molting periods. These actin bundles may serve as attachment points for the new cuticle during synthesis, thereby allowing for the addition of excess cuticle material at the folds in between the bundles.

Although molting may serve primarily to promote the rapid growth of free-living nematodes, it is notable that the structural composition of the cuticle does vary somewhat between larval stages, with distinct types of collagen being expressed at specific life stages. These different cuticle compositions could serve several functions. Cuticle composition may affect expansion rates or the formation of surface structures that are specific to developmental stages. For example, L1 larvae and adult-stage *C. elegans*, along with environmental stress–induced dauer larvae, contain cuticle striations termed alae, which have been suggested to aid in locomotion. Nevertheless, with the exception of dauer larvae, the cuticle is fairly similar in composition among the four larval stages and in adults, consistent with their sharing the same environment. As would be expected, a number of genes encoding both structural components of the cuticle and enzymes that modify cuticular proteins have been identified in screens for molting-defective mutants. These components, as well as the gross structure of the cuticle, are discussed in more detail below (Table 1).

**Structure and composition of the *C. Elegans* cuticle**

The *C. elegans* cuticle is a complex apical ECM containing proteins, lipids, and sugars. It serves several important functions including protection from the environment and pathogens. In addition, through its attachment to muscle cells, the cuticle functions as an exoskeleton to facilitate locomotion. As worms progress through their life cycle, the cuticle becomes somewhat thicker and more complex in its organization. As shown in Fig. 1, the adult cuticle is organized into five layers and is ~0.5 μm thick. From the outside in, these layers are referred to as the surface coat; the epicuticle; and the cortical, medial, and basal zones. In contrast to the adult cuticle, larval cuticles lack a medial zone, and the appearance of the basal zone differs between life-cycle stages. Furthermore, dauer larvae contain expanded basal and epicuticle layers, which may aid in their survival under stressful conditions.

The surface coat, also termed the glycocalyx, is rich in carbohydrates and mucins. Unlike other cuticle layers, which are produced by epidermal cells, the glycocalyx is likely derived from the secretions of excretory gland cells that maintain connections to the animal’s surface. Glycocalyx surface layers are found in many organisms ranging from bacteria to vertebrates and serve several important functions, including roles in development and immunity. Relatively little is known about the carbohydrate components, complex structures, or enzymatic activities required for the formation of the *C. elegans* glycocalyx. Recently, it was shown that cycles of rhamnose biosynthesis are in phase with *C. elegans* molting cycles, suggesting that
Table 1. *C. elegans* genes implicated in molting that are discussed in this review.

| Functional class | Gene name | Description | Reference |
|------------------|-----------|-------------|-----------|
| Cuticle structural elements | bli-1 | Collagen, specific for the last molt; strut formation in medial zone | 15 |
| | col-1 | Intermediate collagen | 72 |
| | col-12 | Late collagen | 72 |
| | col-14 | Late collagen | 72 |
| | col-19 | Collagen – specific for the last molt | 15,73 |
| | cut-1 | Dauer alae-specific cuticlin | 29,30,73 |
| | cut-2 | Cuticlin; present in all developmental stages | 29,30,73 |
| | cut-3 | L1 alae-specific cuticlin | 29,30,73 |
| | cut-4 | Adult-specific cuticlin | 29,30,73 |
| | cut-5 | L1 and dauer stage-specific cuticlin | 29,30,73 |
| | cut-6 | Cuticlin in dauer larvae | 31,73 |
| | dpy-2 | Early collagen | 73,74 |
| | dpy-3 | Early collagen | 73,74 |
| | dpy-5 | Intermediate collagen | 73,74 |
| | dpy-7 | Early collagen | 72-74,96 |
| | dpy-8 | Early collagen | 73,74 |
| | dpy-10 | Early collagen | 73,74 |
| | dpy-13 | Intermediate collagen | 72-74 |
| | fbn-1 | Fibrillin | 32,33 |
| | noah-1 | PAN, ZP domain | 32 |
| | noah-2 | PAN, ZP domain | 32 |
| | sqt-1 | Intermediate collagen | 72-74 |
| Regulators of cuticle synthesis, maintenance, remodeling and degradation | abu/pqn genes | Prion-like glutamine and asparagine rich proteins; pharyngeal cuticle synthesis | 48 |
| | bah family | Glycolytic transferase; surface coat production | 22,24 |
| | bli-3 | H₂O₂-generating dual oxidase; collagen modifier | 36,37 |
| | bli-5 | Protease inhibitor; possible apolysis suppressor | 32,67 |
| | bus family | Glycolytic transferase; surface coat production | 22,23,25 |
| | cgz-1 | Cathepsin Z-like cysteine protease; promotes apolysis | 66 |
| | gsr-1 | Glutathione reductase; promotes apolysis | 68 |
| | mlt-7 | Collagen modifying peroxidase; collagen modifier | 37 |
| | mlt-10 | Nematode-specific; potential collagen modifier | 39 |
| | mlt-11 | Protease inhibitor; possible apolysis suppressor | 32 |
| | nas-36 | Metalloprotease; promotes apolysis | 32,63,64 |
| | nas-37 | Metalloprotease; promotes apolysis | 32,64,65 |
| | pdi-2 | Disulfide isomerase; collagen modifier | 34,35 |
| | phy-1 | Prolyl 4-hydrolase; collagen modifier | 35 |
| | phy-2 | Prolyl 4-hydrolase; collagen modifier | 35 |
| | srf family | Nucleotide sugar transporter; surface coat production | 20-23 |
| | trxr-1 | Selenoprotein thioredoxin reductase; promotes apolysis | 32,55 |
| Attachments components | let-805 | Myotactin; basal HDLS component | 32,46 |
| | muo-3 | Fibrillin, transmembrane protein; apical HDLS component | 41,44 |
| | mup-4 | Transmembrane protein; apical HDLS component | 32,43,45 |
| | pan-1 | Transmembrane protein, possible component of cuticle attachments | 82 |
| | pat-3 | Beta-integrin subunit; muscle dense body component | 42 |
| | rnf-5 | E3 ligase; muscular attachment modifier | 42 |
| | unc-52 | Perlecan; component of basement membrane | 32 |
| | unc-95 | Paxillin-related; muscle dense body component | 42 |
| Heterochronic regulators | let-7 | microRNA, suppression of supernumerary molts | 79,83,86 |
| | lin-14 | Protein coding, suppression of premature molting termination | 75,79,80,83 |
| | lin-28 | Zinc finger protein, suppression of premature molting termination | 75,79,83 |
| | lin-29 | Zinc finger transcriptional regulator, promotes final molt | 16,60,83 |
| | lin-41 | NHL protein, suppression of premature molting termination | 75,79,83 |
| | lin-42 | PERIOD protein ortholog, suppression of premature molting termination | 50,84,85 |
| | mir-84 | microRNA, suppression of supernumerary molts | 79,83,86,114 |
| Hormonal regulation of molting | daf-9 | Cytochrome P450; might stimulate daphachronic acid biosynthesis | 89,90,115,116,132 |
| | daf-12 | Nuclear hormone receptor – dauer-specific | 89,92,113,114,132 |
| | nhr-23 | Nuclear hormone receptor; general molting regulator | 50,91,92,94,96,97,100 |
| | nhr-25 | Nuclear hormone receptor; general molting regulator | 91,92,96,99,100 |
| | nhr-41 | Nuclear hormone receptor – dauer-specific | 91,92 |
| | nhr-67 | Nuclear hormone receptor; general molting regulator | 91,92 |
| | nhr-85 | Nuclear hormone receptor – dauer-specific | 91,92 |
| | sdf-9 | Protein tyrosine phosphatase; DAF-9 activator | 116 |

(Continued on next page)
rhamnose may be incorporated into the new cuticle.\textsuperscript{18} Consistent with this, rhamnose is the most abundant sugar in the glycocalyx of certain parasitic amoebae.\textsuperscript{19} Sugars such as rhamnose may be linked to lipid or protein cuticular components by members of the \textit{srf}, \textit{bus}, and \textit{bah} gene families. In support of this, \textit{srf}, \textit{bus}, and \textit{bah} mutants show changes in anti-carbohydrate antibody labeling and lectin binding at the cuticle surface.\textsuperscript{20-23} Interestingly, mutations in \textit{srf}, \textit{bus}, and \textit{bah} genes change the susceptibility to pathogens, indicating that the glycocalyx is important for protection against pathogen invasion.\textsuperscript{22-25}

\textbf{Table 1. (Continued).}

| Functional class | Gene name | Description | Reference |
|------------------|-----------|-------------|-----------|
| Intracellular trafficking components and regulators | \textit{chc-1} | Clathrin heavy chain; endocytosis | 62,118 |
| | \textit{dab-1} | Cytoplasmic adaptor protein; endocytosis | 120,121 |
| | \textit{hgrs-1} | Vps27 ortholog; recruits ESCRT machinery to endosomes | 119 |
| | \textit{let-767} | Steroid dehydrogenase; intestinal sterol absorption | 122 |
| | \textit{lp-1} | Low-density lipoprotein receptor; epidermal sterol absorption | 61,63,118-121 |
| | \textit{mlt-2} | Ankyrin repeat proteins; possible molecular anchors for NEKL-2 and NEKL-3 | 62 |
| | \textit{mlt-3} | | 62 |
| | \textit{mlt-4} | | 62 |
| | \textit{myrf-1} | Myelin regulatory factor-like; part of secretory machinery during cuticle synthesis | 83 |
| | \textit{neki-2} | NIMA kinases; intracellular trafficking regulation | 61,62 |
| | \textit{neki-3} | | 61,62 |
| | \textit{ncr-1} | Transmembrane glycoprotein with predicted sterol-sensing domain; sterol trafficking | 128,132 |
| | \textit{ncr-2} | Contains sterol-sensing domain | 132 |
| | \textit{sec-23} | Component of COPII; ER to Golgi transport | 117 |
| Components of hedgehog-like signaling | \textit{che-14} | Dispatched ortholog; contains sterol-sensing domain; exocytotic secretion | 138,139 |
| | \textit{pcr-1} | Patched orthologs; contains sterol-sensing domain | 133,135 |
| | \textit{pcr-2} | | 133 |
| | \textit{pcr-3} | | 133 |
| | \textit{qua-1} | Hedgehog-like protein; predicted signaling ligand | 32,134 |
| | \textit{wnt-5} | Contains wnt domain; predicted signaling ligand | 142 |
| Other functions | \textit{acn-1} | Angiotensin-converting enzyme-like; seam cell development | 32,100 |
| | \textit{dat-1} | Dopamine transporter; lethargus promotion | 54,59 |
| | \textit{lev-11} | Tropomyosin, muscular contraction | 32 |

\textbf{Figure 1.} Structural organization of the cuticle in adult \textit{C. elegans}. Indicated are the five layers of the cuticle together with the apical part of epidermis (right side), and composition of cuticle layers (left side). The upper enlarged region shows the surface coat (glycocalyx), whereas the lower enlarged region indicates the presence of hemidesmosome-like structures (HDLSs) at the interface between the cuticle and epidermis. © WormAtlas. Adapted by permission of WormAtlas. Permission to reuse must be obtained from the rightsholder.\textsuperscript{15}
Lying directly beneath the glycocalyx is the relatively thin layer of the epicuticle. The epicuticle is composed primarily of lipids and glycolipids. This layer is most pronounced in dauer larvae and may enable their survival in unfavorable conditions. Because of its lipid content, the epicuticle is thought to function as a surface hydrophobic barrier. In addition, the epicuticle is likely to serve as a substrate for glycosylation pathways in the formation of the glycocalyx.

In contrast to the glycocalyx and epicuticle, the cortical, medial, and basal zones are composed largely of proteins. Differences among these three layers are indicated by differences in their amino acid composition, their susceptibility to different proteases, and their solubility in sulfhydryl reducing agents. Because the outermost portion of the cortical zone is not susceptible to digestion by collagenases, this region is thought to be composed mainly of non-collagen proteins and is potentially rich in cuticlins, which are an integral part of the external layers of the *Ascaris lumbricoides* and *Ascaris suum* cuticles. Characterized cuticlins in *C. elegans* include CUT-4, which is required only at the adult stage and is important for cuticle assembly. Another cuticlin, CUT-2, is a component of cuticles at all four larval stages. mRNA levels for cut-2 exhibit an oscillatory pattern, peaking before each molt. In addition, cut-2 mRNA is also present in adults, suggesting that new CUT-2 may be incorporated into the cuticle of aging adults. Other cuticlins are specifically involved in the formation of alae at the L1 (CUT-3 and CUT-5) and dauer (CUT-1 and CUT-5) stages, or are important for dauer body shape (CUT-6).

The non-collagen components of the cortical layer, most notably cuticlins, are thought to be cross-linked via non-reducible covalent bonds. This is supported by the finding that CUT-2 represents a good substrate for *in vitro* cross-linking reactions, leading to the formation of insoluble complexes of high molecular weight. Other non-collagen-based components of cuticle, for which layer-specific localization has not been described, include a fibrillin-like protein, FBN-1, and two *Drosophila* NompA orthologs, NOAH-1 and NOAH-2. In addition to producing molting defects, mutations in *fbn-1* cause embryonic morphogenesis defects, which are due to the role of FBN-1 in the embryonic precursor to the cuticle, termed the sheath.

Analogous to mammalian skin, the cuticle in nematodes includes collagen as its main protein fraction. Multiple types of collagen are present in the inner parts of the cortical zone, in the struts of the medial zone, and in the basal cuticular zone. As discussed below, certain collagens (e.g., COL-1 and DPY-2) are incorporated in cuticles at every postembryonic life stage, whereas others (e.g., BLI-1 and COL-19) are stage specific. Cuticle collagens are extensively modified after secretion and are cross-linked by disulfide bonds. Notably, a disulfide isomerase enzyme, PDI-2, is required for normal function of the collagen-modifying prolyl 4-hydroxylase complex, which is encoded by *phy-1* and *phy-2*. Consistent with their proposed function in cuticle biosynthesis, *phy-1, phy-2*, and *pdi-2* mRNA levels oscillate during larval development, reaching a maximum several hours before molting, and a similar expression pattern is observed for some collagens.

Another collagen-modifying enzymatic complex is proposed to be formed by BLI-3, a hydrogen peroxide–generating NADPH dual oxidase, and MLT-7, a peroxidase. Together, these activities are thought to be essential for the normal crosslinking of cuticular collagens. BLI-3 catalyzes the crosslinking of free tyrosine ethyl esters *in vitro* and the formation of dityrosines and trityrosine *in vivo*. Downregulation of *bli-3* or *mlt-7* leads to molting defects and to changes in the *in vivo* expression patterns of the cuticle collagens DPY-13 and COL-12. Furthermore, *bli-3(RNAi)* causes extensive structural abnormalities in the medial zone, including blister formation caused by excessive liquid accumulation and collagen-strut breakage. Collagen organization is also changed following depletion of SURO-1, a metalloproteinase, and by loss of MLT-10, a protein with oscillatory expression pattern of unknown function.

**Linkage of the cuticle, epidermal cells, and muscle cells**

Collagens and most other components of the cuticle are synthesized by underlying lateral epidermal cells (seam cells) and epidermal syncytia (hyp1–hyp11), which are directly adjacent to the basal zone of the cuticle. The epidermis and cuticle are attached to each other in part through hemidesmosome-like structures (HDLSs), which are part of larger pillar-like structures termed fibrous organelles. Fibrous organelles contain both apical and basal HDLSs that are connected by intermediate filaments, which span the width of the epidermis (Fig. 2). At the basal surface, HDLSs form attachments
that connect the epidermis to the basement membrane. Likewise, muscle cells attach to the basement membrane through structures termed dense bodies, which contain integrins and are homologous to vertebrate focal adhesions. Together, these linkages provide critical connections between the various layers but must also be remodeled extensively during molts.

Candidate HDLS proteins include MUA-3 and MUP-4, which are related transmembrane proteins important for the establishment (MUP-4) and maintenance (MUA-3 and MUP-4) of attachments between the epidermis and the cuticle. MUA-3 may help to link the epidermal cytoskeleton to collagens in the basal zone. Mutations in mua-3 typically cause developmental arrest during the final molt, which can be suppressed by RNAi depletion of the DPY-17 collagen. Although both MUA-3 and MUP-4 are expressed from late embryonic stages, only MUP-4 expression is required during embryogenesis, whereas both proteins are necessary during postembryonic development. This could be because of increasing muscle activity, which may put additional stress on the interconnections between the cuticle and epidermis. However, because these two proteins are believed to be expressed only at the apical epidermal membrane, other components within basal epidermal HDLSs may be necessary to connect the epidermis to the underlying basement membrane. One protein that may be specific to basal HDLSs is LET-805/myotactin. In addition, myotactin may be involved in intercellular signaling, which may guide remodeling of basement membrane attachments during molting.

Muscle cells are also connected to the basement ECM through dense bodies (Fig. 2). Expression of two dense-body proteins, PAT-3 and UNC-95, is decreased during initial stages of molting (lethargus, see below). UNC-95 is ubiquitinated by the RING finger E3-ligase RNF-5, which may promote its degradation. Consistent with this, RNF-5 is expressed during molting, and inhibition of rnf-5 leads to molting defects. Thus, regulated proteolysis may be a key mechanism for modulating dense bodies, as well as other structural components that are linked to the cuticle, during molting periods. Furthermore, downregulation in the expression of LEV-11/tropomyosin in muscles or UNC-52/perlecan at the basement membrane, leads to molting defects, which are likely caused by an inability to modify muscle-epidermal attachment points. Taken together, these findings indicate that attachments between the cuticle, epidermis, basement membrane, and muscle cells must be dynamically regulated to allow for release of the old cuticle and proper synthesis of a new one.

**Internal cuticles**

In addition to covering the external portions of the *C. elegans* body, a cuticle also lines internal epithelial surfaces that are directly connected to the body surface. Internal cuticles line the mouth (buccal cavity), pharynx (foregut), rectum, vulva, excretory duct, and excretory...
pore. Most of these internal cuticles appear to be simple in structure and are collagen-based. The exception is the pharyngeal cuticle, which cannot be digested by elastase or collagenase but can be degraded by bacterial pronase, suggesting that collagen is not its main protein component. In addition, the pharyngeal cuticle contains chitin, which forms specialized anatomical elements that are important for proper food maceration. Because chitin does not allow for shape changes, the pharynx can grow only during the molting period when the old cuticle is released. Limitations in adequate food intake caused by a pharynx that is static in size and small relative to the rest of the body could be one of the triggers for molting. Release and partial digestion of the pharyngeal cuticle may be assisted by secretions from pharyngeal gland cells, based on the observed accumulation of secretory granules in g1 gland cells during molting. Furthermore, transcriptional profiling revealed that genes involved in chitin metabolic processes are upregulated during molting cycles.

The specialized structure of the pharyngeal cuticle necessitates that molting within the pharynx be partially independent of molting by the epidermis. In pharyngeal epidermal cells, molting-specific expression of abu/pqn genes, which encode prion-like glutamine and asparagine-rich proteins, is necessary for normal molting and development. Furthermore, the exoribonuclease XRN-2, which is expressed in myoepithelial pharyngeal cells, is required for cuticle formation. Notably, xrn-2(RNAi) causes molting defects in the pharyngeal region as well as more general defects in cuticle shedding. In summary, nematodes synthesize additional ECMs that cover several internal epithelia that are closely connected to the body surface. These matrices must also be shed during the molting process and, in the case of the pharynx, may have distinct properties and requirements for molting.

Molting stages and ECM remodeling

Molting can be separated into two behavioral phases: lethargus and ecdysis. Lethargus was named after the lethargic behavior exhibited by animals that have commenced molting and is most obvious during the first half of this phase. Decreased locomotion, slower pharyngeal pumping, and loss of seam cell granulation are some of the main hallmarks of lethargus. These behavioral and morphological changes can be used to distinguish molting from non-molting animals but are labor intensive, as they require careful observations on individual animals. Simplified methods for identifying molting animals have been developed, including fluorescent reporters for genes that are expressed specifically during molts. In addition, fluorescent-bead oral uptake assays have recently been described that take advantage of reduced pharyngeal pumping exhibited by molting animals.

Although lethargus is usually described as a phase of behavioral quiescence lasting from 2 to 3 hours, it encompasses alternating bouts of quiescence and activity, which last from 2 to 100 seconds. The first half of lethargus, quiescent periods are longer, whereas during later stages of lethargus the motile behavior becomes dominant. Studies of the quiescent phase of molting in C. elegans indicate that it could be a useful model for understanding hibernation and sleep in higher eukaryotes. For example, the dopamine transporter encoded by dat-1 is expressed in an oscillatory manner and is upregulated during molting, consistent with the role of dopamine transporters in controlling sleep in mammals.

During lethargus, animals begin to release their old cuticle, which eventually detaches from the underlying epidermis. This process, known as apolysis, starts in the head region and next occurs in the tail region and within the buccal cavity; the cuticle in the central body region is released last. Hypomorphic mutations in the nekl–mlt gene network halt the molting process after the release of the cuticle from the head and tail regions, resulting in central body entrapment within the old cuticle (the corset phenotype). In contrast, null alleles of genes within the nekl–mlt network lead to complete encasement within the old cuticle. These observations suggest that there may be somewhat different physiological or mechanistic requirements for release of the cuticle from different portions of the body.

The process of apolysis is not well understood but is thought to require the activity of proteases to degrade the old cuticle. Notably, the conserved metalloproteases NAS-36 and NAS-37, as well as a cathepsin Z-like cysteine protease, CPZ-1, are implicated in the process of old-cuticle degradation at each molt in both free-living and parasitic species. Expression of nas-37 cycles in phase with the first three molts in epidermal syncytia, but it is expressed specifically in the lateral seam cells during the final molt. Interestingly, several predicted protease inhibitors are involved in proper molting as well. For example, a cuticle blistering phenotype and molting defects are
observed following depletion of BLI-5, a serine protease inhibitor.32,67 Similarly, inhibition of another protease inhibitor, MLT-11, which shares a pancreatic trypsin inhibition domain with BLI-5, also causes molting defects. MLT-11 is expressed in both the main epidermal syncytia and in lateral seam cells and shows an oscillating pattern of expression, which is highest during intermolting periods.32 This suggests that some protease inhibitors may suppress the activity of cuticle-degrading enzymes to prevent unwanted apolysis in periods between two molts. In addition to proteases, several other proteins with enzymatic activity are implicated in cuticle release during molting, including the selenoprotein thioredoxin reductase, TRXR-1, and GSR-1/glutathione reductase, which may facilitate removal of the old cuticle by promoting the reduction of disulfide groups in the cuticle.68

Prior to complete removal of the old cuticle, however, it is essential for a new cuticle to be synthesized. Thus, during lethargus, production of the new cuticle begins while the worm is still protected by the partially detached old cuticle. Outer protein layers of the cuticle are synthesized first, whereas the basal zone components are synthesized last. As stated above, the main source of components for the new cuticle is the underlying epidermal tissue, although some superficial components are secreted from the gland cells.15 It is notable that most described molting defects and molting mutants appear to affect cuticle shedding rather than cuticle synthesis. One explanation for this is that many of the components and enzymes involved in new cuticle synthesis may be required during embryogenesis for the formation of the embryonic sheath, which is essential for normal morphogenesis.33,69 In addition, loss of individual structural components may have only mild effects on phenotypes, such as abnormal movement or greater susceptibility to injury or environmental toxins. Finally, it is possible that failure to synthesize a functional new cuticle could result in a failure to release the old cuticle, which could manifest as a shedding defect.

After completing synthesis of the new cuticle at the end of the lethargus phase, the partially detached old cuticle must be removed. This phase, known as ecdysis, commences with worms spinning around their long axis, a behavior believed to aid in the release of the old cuticle.70 Several distinct types of motions have been observed including longitudinal contractions and expansions and forward thrusts. Foregut contractions are used to break the internal pharyngeal cuticle, so that the back portion is ingested, whereas the front part is expelled through the mouth and ultimately shed with the outer body cuticle. The old body cuticle usually breaks in the head region, after which the worm crawls out, facilitated by a series of quick body motions.70

Failure to properly shed old cuticle results in a range of molting defects as shown in Fig. 3. The most severe

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**Figure 3.** Schematic representation of the molting process and molting defects with representative micrograph examples. The normal physiological molting process is shown on the left. Schematic and micrograph examples of molting defects are shown on the right side, including (from top to bottom) the complete encasement phenotype of a ml-3(fd72) mutant, the partially released cuticle in the head region of a qua-1(RNAi) larva, the corset phenotype of a nekl-3(sv3) mutant, a narrow constriction caused by the old cuticle in a nekl-3(sv3) mutant, and an old cuticle attached to the body surface after nekl-2(RNAi). White arrowheads indicate the presence of the old cuticle. Scale bars: 25 μm.
phenotype is complete encasement, in which the whole body is covered with the tightly adhering old cuticle. Examples of mutants with this phenotype include null alleles in members of the NEKL–MLT kinase network. In cases where ecdysis is initiated but not completed, morphologically less severe defects can be observed. For example, after qua-1(RNAi), detachment of the old cuticle can be observed in the head region, but because the cuticle does not break, the animal remains trapped within the old cuticle. In other mutants, such as fbn-1, animals may become fully detached from the old cuticle but are unable to break through in a timely manner. Hypomorphic alleles of nekl-2, nekl-3, and mlt-4 lead to a corset phenotype, which consists of a characteristic constriction within the central portion of the body and release of the old cuticle in the head and tail regions only. Some molting-defective animals are largely successful in escaping from the old cuticle. In these cases, ecdysis fails within a small region of the body. For example, the old cuticle can form a thin constrictive ring wrapped around the body, or it can be loosely attached to the new cuticle. These less severe phenotypes can be lethal or viable, depending on the location of the old cuticle attachment, as the external constrictions can cause internal organ malfunctions or can be an obstacle for normal physiological functions, including feeding and excretion. Phenotypic differences between different genotypes may be due in some cases to the strength of individual alleles or because certain genes are required for spatially and/or temporally restricted processes during molting.

**Oscillations in the expression of molting genes**

As molting phases are cyclical and temporally regulated, it might be expected that genes involved in molting and cuticle biogenesis would exhibit oscillatory expression patterns. To address this, Hendriks and colleagues used RNA sequencing, ribosome profiling, and RT-qPCR to analyze transcriptional and translational oscillations during *C. elegans* larval development. An independent study, using microarray approaches, identified 520 genes that showed oscillations with molting cycles. The majority of these genes encoded proteins predicted to be associated with cellular membranes, with 113 being upregulated and 68 being downregulated during molting. As expected, 19 of the identified genes that encode structural components of cuticle were expressed at higher levels during molting. The most abundant components of the cuticle are collagens, many of which show oscillatory expression patterns. Whereas some collagens are incorporated into the cuticle at each developmental stage, others, such as BLI-1 and COL-19, are stage specific. Oscillating collagens that are expressed at each molt, can be classified into three temporal groups—termed early (DPY-2, DPY-3, DPY-7, DPY-8, DPY-10), intermediate (COL-1, DPY-5, DPY-13, SQT-1), and late collagens (COL-12, COL-14)—with mRNA levels that peak ~4 hours prior, 2 hours prior, and during cuticle synthesis, respectively. Interestingly, mutations or deletions of specific oscillating collagens affect the structural organization of other collagens from the same temporal group, but not from other groups, providing strong support for functional connections within temporal groups. Moreover, additive effects in the severity of cuticle-defective phenotypes are observed only in double mutants of collagens belonging to different groups, suggesting that strong defects result from the perturbation of multiple layers of the cuticle strata.

**Heterochronic genes in molting and development**

Underlying both the timing of molting and the orderly progression of larval developmental events are the heterochronic genes (Table 1). Heterochronic genes, which encode a number of proteins as well as micro-RNAs, constitute an intrinsic developmental timer. Mutations in heterochronic genes lead to two general classes of phenotypes: precocious, in which developmental events are skipped, and retarded, in which developmental events are repeated. Because the heterochronic gene network is coupled to molting cycles during larval development, both phenotypic classes of heterochronic mutants are associated with abnormal molting, such that precocious mutants undergo fewer molts than wild type, whereas retarded mutants undergo supernumerary molts. In the case of precocious mutants, such as *lin-14, lin-28*, and *lin-41*, and
**Hormonal control of molting**

In insects, the steroid hormone ecdysone triggers molting at each developmental phase. Likewise, steroid hormone signaling is thought to regulate molting cycles in *C. elegans*. However, with the exception of one steroid hormone that regulates entry into the dauer stage, the specific hormones that regulate normal molting cycles have not been identified. Evidence in support of a role for steroid-hormone signaling in *C. elegans* molting includes the finding that several nuclear hormone receptors are required for molting. In addition, worms that are deprived of cholesterol undergo larval arrest, with a subset displaying defects in molting. Notably, *C. elegans* cannot synthesize cholesterol de novo, and cholesterol serves as a precursor for steroid hormone biosynthesis. Below we discuss the roles of putative components of steroid signaling pathways in *C. elegans* molting (Table 1).

Several genes encoding nuclear hormone receptors have been implicated in *C. elegans* molting, including *nhr-23*, *nhr-25*, and *nhr-67*. Additionally, three other NHRs, *nhr-41*, *nhr-85*, and *daf-12*, control entry into the dauer stage, which is achieved through an alternative molt following the L2 stage. *nhr-23* and *nhr-25* mRNA levels oscillate with the molting cycles according to RT-PCR measurements. *nhr-23* is an ortholog of *Hr3* in *Drosophila melanogaster*, which functions in fly molting. Overall levels of *nhr-23* mRNA are higher during intermolts, suggesting that this receptor is activated before each molt and that NHR-23 initiates a molting cascade. Accordingly, NHR-23 is an important regulator in the expression of several collagens as well as hedgehog-related proteins, which are also important for normal molting. However, it is not known whether the levels of each of the six annotated *nhr-23* isoforms oscillate with the molting cycles.

NHR-25 expression oscillates in a different manner than NHR-23. Full-length *nhr-25* mRNA reaches its highest expression levels during the first three molting periods and also shows a slight increase during the final molt to adulthood. A truncated natural isoform of *nhr-25*, which lacks the DNA-binding domain of the full-length form, has the opposite expression profile, based on RT-PCR, and exhibits its highest levels between molts. *nhr-25* is orthologous to *ftz-f1* in *D. melanogaster*, which is downstream from *Hr3* in the fruit fly molting cascade. However, *nhr-25* transcriptional and translational levels are not affected by *nhr-23*(RNAi), suggesting that this activation cascade is not conserved in *C. elegans* or that NHR-25 expression is redundantly regulated by some other factor.
The cytochrome P450–encoding gene daf-9 acts genetically upstream of daf-12 and may be important for the biosynthesis of dafachronic acid. DAF-9 and its potential upstream activator, SDF-9, a putative protein tyrosine phosphatase, are both expressed in postembryonic neuroendocrine XXX cells, which may be the source of dafachronic acid. This is supported by the finding that ablation of XXX cells, as well as mutations in both daf-9 and sdf-9, leads to constitutive dauer formation under conditions normally conducive for reproductive growth. Taken together, evidence supports an important role for steroid hormone signaling in the control of molting and life cycle progression in C. elegans, including the involvement of genes that are orthologous to well-known ecdysone-triggered molting cascades in D. melanogaster. However, these pathways have diverged considerably, and many gaps remain in our understanding of steroid-hormone signaling pathways in nematodes.

**Cell trafficking and molting**

Intracellular trafficking within the epidermis, including the coordinated processes of exocytosis and endocytosis, are considered to be critical for molting (Table 1). In the case of exocytosis, the secretion of ECM proteins and other components of the cuticle by the epidermis is essential for synthesis of the new cuticle. Consistent with this, inhibition of sec-23, which encodes a component of the COPII complex involved in transport from the endoplasmic reticulum to the Golgi, reduces protein export.
and leads to defects in molting. Conversely, endocytosis may facilitate the recycling of old cuticle components and is important for the internalization of sterols from the environment. In support of this, inhibition of CHC-1, a component of clathrin-coated pits; DAB-1, an adaptor protein; and HGRS-1, an ortholog of yeast Vps27, all lead to molting defects.

More recently, two NIMA family kinases, NEKL-2/NEK8 and NEKL-3/NEK6/7, together with three associated ankyrin domain–rich proteins (MLT-2/ANKS6, MLT-3/ANKS3, and MLT-4/INVS), were shown to function specifically within epidermal syncytia to control each molting cycle in C. elegans. Both the NEKLs and MLTs are expressed in overlapping subsets of epidermal puncta and form at least two distinct complexes consisting of NEKL-2–MLT-2–MLT-4 and NEKL-3–MLT-3. Downregulation of components within the NEKL–MLT network causes changes in the expression pattern of several endosomal markers, including CHC-1, implicating these proteins in vesicular trafficking and possible sterol uptake (discussed below).

Sterol uptake by the epidermis is thought to depend primarily on LRP-1, a conserved low-density lipoprotein receptor related to human megalin. LRP-1 is expressed in an apical punctate pattern in the main epidermal syncytium, hyp7. Interestingly, mosaic analysis did not reveal a requirement for LRP-1 in the intestine, suggesting that the epidermis, and not the intestine, is critical for the absorption of sterols by LRP-1. Depletion of LRP-1 leads to molting defects, which can occur at any of the four larval molts, and similar effects are observed after depletion of the LRP-1 endocytic adaptor protein DAB-1. Furthermore, other endocytosis-related genes, including chc-1, epn-1, nekl-2, aps-2, dpy-23, abp-1, and dyn-1, are important for normal LRP-1 internalization.

Evidence also suggests that the intestine may play a role in sterol uptake. For example, the intestinal steroid dehydrogenase LET-767 is important for molting and other aspects of C. elegans development. In addition, the analysis of fluorescent dehydroergosterol distribution identified the intestine and several other tissues, but not the epidermis, as the primary accumulation point for this cholesterol analog. Similarly, intestinal adsorption of 3β-hydroxy sterols has been demonstrated by filipin staining. However, several aspects of the C. elegans lifestyle might suggest that epidermal sterol uptake could be more important than oral uptake. For example, C. elegans eat bacteria, which do not generally contain sterols, as very few bacterial species are able to produce these organic components. The epidermal intake of sterols might explain why C. elegans in the wild inhabit sterol-rich environments, such as the decomposing tissues of other organisms. Furthermore, the parasitic nematode Ascaris suum primarily absorbs sterols through its body surface, as radiolabeled cholesterol intake is not impaired after occlusion of the digestive tract.

Several other proteins required for the endocytosis of sterols have been implicated in dauer development. These include ncr-1npc-1 and ncr-2npc-2, which encode orthologs of mammalian proteins involved in the transport of sterols. Mutations in mammalian NPC1 lead to neurodegeneration as a result of lysosomal storage defects. ncr-1; ncr-2 double mutants exhibit a constitutive-dauer phenotype, which can be suppressed by increasing the concentration of cholesterol in the growth medium. Furthermore, mutations in ncr-1 cause hypersensitivity to sterol depletion, leading to larval arrest, although the phenotype of these arrested larvae was not well characterized. Genetic analysis indicates that ncr-1 and ncr-2 function upstream of daf-9 and daf-12. ncr-1 is expressed in multiple tissue types, including the intestine and epidermis, whereas ncr-2 is expressed together with daf-9 in XXX cells, which are thought to secrete dafachronic acid. A role for ncr-1 and ncr-2 during normal molting cycles has not yet been demonstrated.

**Hedgehog-like signaling pathways in molting**

Although signaling pathways have been implicated in C. elegans molting, they have not been well characterized. Most notable are several genes with sequence similarity to components of the hedgehog–patched signaling pathway, suggesting that hedgehog-type signaling might be important for molting (Table 1). The hedgehog signaling pathway in C. elegans has, however, diverged from that of the well-characterized hedgehog pathways in fruit flies and vertebrates. Whereas C. elegans has three putative patched orthologs (ptc-1, ptc-2, and ptc-3), 24 patched-related genes, and two dispatched orthologs (che-14 and ptd-2), there is no...
obvious homolog of the hedgehog ligand or the downstream effector smootherned in this nematode species.\textsuperscript{133,135} Computational analysis has, however, revealed 61 and 49 hedgehog-related open reading frames in the \textit{C. elegans} and \textit{C. briggsae} genomes, respectively,\textsuperscript{136} suggesting that these may encode ligands in a hedgehog-like signaling pathway in nematodes.

Relevant to this discussion, patched orthologs, along with several patched-related genes, are important for proper molting, as RNAi of these genes causes molting defects and developmental arrest.\textsuperscript{133} Notably, patched proteins in worms and other species contain sterol-sensing domains,\textsuperscript{135} as do several other components of the hedgehog signaling pathway. Furthermore, in mammalian neuroepithelial cells, ligand binding to patched is assisted by LRP2/megalin,\textsuperscript{137} a homolog of \textit{C. elegans} LRP-1.\textsuperscript{93} LRP2 also controls internalization and trafficking of both hedgehog and patched during forebrain development in mice,\textsuperscript{137} suggesting a potential similar function for LRP-1 in \textit{C. elegans}.

Of the two dispatched orthologs in \textit{C. elegans}, che-14/ptd-1 has been implicated in molting.\textsuperscript{138,139} CHE-14 is expressed in epidermal tissues that are covered by cuticle and is associated with the apical membrane. CHE-14 has a sterol-sensing domain, which might serve in the recognition of a sterol-modified (active) hedgehog-like ligand. Mutations in che-14 cause apical accumulation of vesicles and amorphous material, suggesting a role in epidermal exocytosis.\textsuperscript{139} Notably, dispatched in \textit{D. melanogaster} has a role in the secretion of hedgehog molecules from signaling cells,\textsuperscript{138} suggesting that CHE-14 could be required for the secretion of hedgehog-like molecules in \textit{C. elegans}.

Members of the hedgehog-related group of proteins typically contain a C-terminal autoproteolytic hint/hog domain and one of several types of N-terminal domains, which function in signaling and distinguish the four hedgehog subfamilies: warthog, groundhog, ground-like, and quahog.\textsuperscript{133} Self-cleavage of the C-terminal domain leads to the activation of hedgehog, which subsequently binds a sterol molecule. Although many of the \textit{C. elegans} hedgehog-related proteins are missing a hint/hog domain, they contain one of the conserved N-terminal domains.\textsuperscript{136} Notably, truncated hedgehog orthologs containing only the N-terminal domain are active but cannot be sterol-modified, leading to a change in their hydrophobicity and regulation.\textsuperscript{138,140,141}

Thus, some \textit{C. elegans} hedgehog-related proteins may be regulated in a different manner than their counterparts in flies and vertebrates.

\textit{C. elegans} hedgehog-related genes are expressed in the epidermis and other epithelial cells and are secreted into extracellular matrices, including the internal and external cuticles.\textsuperscript{136} One of the \textit{C. elegans} hedgehog-related genes, \textit{qua-1}, is conserved in free-living and pathogenic nematodes, as well as in higher eukaryotes.\textsuperscript{134} \textit{QUA-1} is cyclically expressed in the main epidermal syncytia at each molt and is secreted into the cuticle. Loss-of-function mutations of \textit{qua-1} lead to strong molting defects and developmental arrest.\textsuperscript{134} Another \textit{C. elegans} hedgehog-related gene implicated in molting is \textit{wrt-5}, a member of the warthog family of ligands.\textsuperscript{142} Prior to and during each molt, WRT-5 is secreted by pharyngeal cells, along with other cell types, and is excreted together with the old cuticle.\textsuperscript{142}

Taken together, orthologs of several components of the hedgehog signaling pathway have been implicated in molting in \textit{C. elegans}. A number of these proteins may be modified by sterols, which are important for molting, and could interact with the sterol-binding receptor LRP-1. These observations provide an alternative, and non-hormone-related, explanation for the role of epidermal sterol absorption in nematode molting, although it is likely that both hedgehog and nuclear hormone receptor signaling pathways have important roles in this process.

\textbf{Conclusions}

Molting is a complex developmental process that is carried out in all nematodes, as well as in many other invertebrate species. Molting allows for the replacement of the apical ECM, known as the cuticle, thereby enabling growth or developmental specialization. As described above, a large and diverse collection of genes have been implicated in molting control in \textit{C. elegans} (Table 1). However, a clear and integrated description of the molting cascade is still missing within nematode species. Further studies will undoubtedly lead to a better understanding of this important developmental process.

Beyond an interest in understanding molting as a developmental process, molting studies may have other significant and practical applications. Notably, molting is a potential focus for antihelminthic drug
development, both for human and veterinary medicine.\textsuperscript{143-145} Diseases caused by nematodes are still widely distributed in the human population and affect hundreds of millions of people, according to the World Health Organization.\textsuperscript{146} Nematode infections can lead to a number of long-term consequences, including death, and can further complicate other diseases. Many of these diseases are zoonoses, meaning that they are naturally transmissible from vertebrate animals to humans. Correspondingly, nematode infections of animals, as well as plants, have significant economic consequences.\textsuperscript{147,148} As such, agricultural industries use extensive prophylactic measures to decrease the prevalence of parasitic nematodoses, which cause material damage and financial loss.

Although molting in nematodes might seem to constitute a specialized biological process within invertebrates, understanding the molecular basis for ECM remodeling and other fundamental events associated with molting has broad relevance for biology and our basic knowledge of disease states. Furthermore, because the \textit{C. elegans} cuticle is largely collagen based, it is potentially useful for understanding dermal physiology and wound healing in higher eukaryotes.\textsuperscript{149} In addition, primary tumor invasion through the ECM is an important process during tumor metastasis and shares many conserved features with ECM remodeling.\textsuperscript{150-152} Notably, many genes implicated in \textit{C. elegans} molting have orthologs in higher eukaryotes, indicating that this biological system represents a powerful tool for exploring the functions and molecular mechanisms underlying a wide range of conserved cellular and developmental processes.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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