Functional Genomic Analysis of *C. elegans* Molting

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Ecdysozoan animals, including nematodes and arthropods, develop through periodic larval stage molts when the exoskeleton is shed and synthesized anew. Although molting is the hallmark of the most abundant and diverse group of animals on the planet, including a wide variety of human pests and pathogens, the endocrine circuits that regulate molting in response to environmental and physiologic cues are not well understood. Moreover, little is known about the molecular mechanisms of release and *de novo* production of the exoskeleton.

Endocrine and neuroendocrine pathways regulate molting in arthropods, and likely operate in nematodes as well. In insects, pulses of the steroid hormone ecdysone trigger molting and metamorphosis. The neuropeptide PTTH (prothoracicotropic hormone) stimulates synthesis of ecdysone in the prothoracic glands. Near the very end of each larval stage, the neuropeptide eclosion hormone (EH), combined with a decline in the titer of ecdysone, prompts release of the peptide ecdysis-triggering hormone (ETH) from glands lining the trachea. ETH promotes behaviors essential for escaping the old exoskeleton, and also stimulates neurons to secrete more EH, creating a positive feedback loop that culminates in a hormonal surge decisive for ecdysis. Environmental cues, including photoperiod, temperature, and humidity, as well as physiologic factors including size, stage, and the nutritional status of the organism, modulate secretion of PTTH in various arthropods, suggesting extensive sensory input to the neuroendocrine secretions that govern molting. However, little is known about circuits that initiate, terminate, or set the pace of the molting cycle in any Ecdysozoan.

Although an endocrine trigger for nematode molting has yet to be identified, several lines of evidence implicate steroid hormones in *C. elegans* molting. Molting of *C. elegans* requires cholesterol, the biosynthetic precursor of all steroid hormones, as well as the LDL receptor-like protein LRP-1, which is thought to endocytose sterols from the growth medium. A sterol-modifying enzyme synthesized in the intestine, LET-767, is also essential for ecdysis, consistent with the production or modification of a hormone derived from steroids. The best evidence of a hormonal cue for molting of *C. elegans* is the requirement for two nuclear hormone receptors, NHR-23 and NHR-25, orthologous, respectively, to the ecdysone-responsive gene products DHR3 and Ftz-F1 of *Drosophila melanogaster*. Ecdysone itself, however, is unlikely to serve as a molting hormone in nematodes because ecdysteroids have not been detected in any free-living nematode, and because orthologs of the ecdysone receptor components EcR and USP (ultraspiracle) have not been identified in the complete genome of *C. elegans*.

The exoskeleton of nematodes, called the cuticle, is a collagenous extracellular matrix secreted by underlying epithelial cells, known as the hypodermis and seam, and by specialized interfacial cells that line openings of the body, including the buccal cavity, pharynx, vulva, and, anus. Molting involves the synthesis and secretion of a new cuticle underneath the old one, separation of the old cuticle from the epidermis (apolysis), and shedding of the pre-molt cuticle (ecdysis). Near the very end of each stage, larvae become inactive for a brief period of time, known as lethargus, coinciding with detachment of the old cuticle from the hypodermis. Stereotypical behaviours then promote ecdysis; larvae flip on their long axis to loosen the body cuticle, regurgitate the anterior half of the pharyngeal cuticle, and then escape the old exoskeleton via forward thrusts. Although elasticity of the cuticle permits growth during each larval stage, particular structures, such as the buccal cavity, grow saltationally at molts. The distinction between collagen in the nematode exoskeleton and chitin in the insect exoskeleton suggests that the enzymatic cascades that mediate release of the exoskeleton in nematodes may be distinct from those that release the exoskeleton in arthropods. Although two collagenases essential for molting have been identified in *C. elegans*, the full

ensemble of membrane signalling proteins and extracellular matrix enzymes required to remodel the exoskeleton has yet to be illuminated.

Human diseases caused by parasitic nematodes affect tropical regions of Africa, Asia, and South America. The World Health Organization estimates that 120 million people endure lymphatic filariasis (elephantiasis), due to infection by the filarial nematodes *Wuchereria bancrofti* or *Brugia malayi*, and that 18 million people endure onchocerciasis (African river blindness), due to infection by *Onchocerca volvulus*. *Ascaris*, hookworms, and whipworms are also important pathogens, infecting approximately 1 billion people. Parasitic nematodes further damage livestock and lay waste to $80 billion of crop plants annually. One promising approach to the discovery of new targets for anti-nematode drugs, vaccines, and pesticides is the identification of nematode-specific gene products essential for the viability of larvae. In the screen we report, a large number of nematode-specific genes essential for molting were identified, and some of these are attractive drug targets.

To identify a full set of endocrine and enzymatic regulators of molting in *C. elegans*, we screened a combined library of 18,578 bacterial clones that each express a double-stranded RNA designed to silence one of the 19,427 predicted worm genes via RNAi. About 25 L1-stage larvae were fed each clone and later examined for molting defects, indicated by the adherence of cuticle from the pre-molt larval stage to the body of the worm (the Mlt phenotype). Gene inactivations observed to prevent molting in the primary library screen were tested again by feeding the bacterial clones to approximately 50 wild-type (N2) and 50 *rrf-3*(*pk1426*) mutant larvae, a genetic background where RNAi is more effective.

Inactivation of 159 genes caused molting defects in both wild-type (N2) and *rrf-3(pk1426)* mutant larvae. 87 of these were assigned a lower priority based on gene annotation or the low penetrance (<10%) of molting defects observed after RNAi in *rrf-3(pk1426*) mutant larvae (Table S4). The blind identification of 9 genes previously described to cause an arrest at a molt, including *lrp-1*, *nhr-23*, *nhr-25*, *skp-1*, *nas-37*, *nas-36*, *rme-8*, *acn-1*, and *bli-4*, verified the efficacy of our genomic, RNAi-based strategy for isolating *bona fide* molting genes. Moreover, the observation of molting defects in larvae with chromosomal deletions or mutations in the genes *qua-1*, *lrp-1*, and *nas-37* verified that RNAi faithfully recapitulates loss-of-function phenotypes in the molting pathway. In addition to the aforementioned 9, 28 more of the 159 gene inactivations described here were independently observed to disrupt molting in broad screens using RNAi. The names *mlt-8*, *mlt-9, and mlt-11*were assigned, respectively, to W08F4.6, F09B12.1, and W01F3.3 after expression data verified a primary function in molting. The genes *fbn-1*, *noah-1*, and *noah-2* were assigned names based on homology to mammalian or insect genes.

Figure 1 shows examples of molting-defective larvae produced by RNAi. Most often, larvae were observed incarcerated in sheaths of old cuticle extending from the anterior end of the worm, as shown for a *mlt-11(RNAi)* larva. The nature of molting defects caused by particular gene inactivations suggested that the corresponding proteins function in a specific anatomical place or stage of ecdysis. For example, *xrn-2(RNAi)* larvae fail to expel or shed cuticle from the anterior pharynx at the end of the L4 stage (results not shown), and thus resemble animals lacking the DNA binding protein PEB-1. *mlt-9(RNAi)* larvae fail to shed cuticle lining the buccal cavity, often causing the lips to invert. Unshed cuticle often forms coronal constrictions on *nas-37(RNAi)* larvae, possibly when animals flip on their long axis to loosen the old cuticle during molting. Inactivation of the collagenase gene *nas-37* also prevents the breakdown of old cuticle at the anterior tip of the worm, thereby blocking escape from the old exoskeleton (results not shown). Although Mlt larvae typically arrest development, some gradually escape from the old cuticle, only to fail again at the next molt, a phenomenon observed often in *qua-1(RNAi)* larvae.

The vast majority of genes identified in our screen are likely essential for all four molts, because their inactivation prevents molting from several larval stages. Moreover, although feeding L1-stage larvae dsRNA for particular genes, such as *mlt-8* or *acn-1*, prevents development beyond the L3 stage, feeding the same

dsRNAs to older larvae also disrupts the final molt. The majority of gene inactivations also disrupt molting from the dauer stage, an alternative L3 stage that is adapted for survival in unfavorable conditions and resembles the infective form of parasitic nematodes.

The majority of genes identified in our screen are conserved in parasitic nematodes responsible for human, animal, and plant diseases. Many genes, including *mlt-8* and *mlt-9*, are conserved only in nematodes. Similar proteins were readily identified among the predicted products of cDNAs or genomic sequences from parasitic and free-living nematodes, but not in the translated genomes of *D. melanogaster* or *H. sapiens*. In contrast, the genes *noah-1* and *noah-2*, which specify putative extracellular matrix components, are conserved in insects and nematodes but not in humans, and thus show the phylogenetic conservation signature expected for molting genes common to Ecdysozoans.

Predicted functions of genes uncovered in the molting screen

Based on experimental evidence of a steroidal pathway, as well as the evolutionary relationship between arthropods and nematodes, we expect that endocrine cues periodically initiate molting in *C. elegans*, stimulating the synthesis and secretion of a new cuticle followed by release of the old cuticle. We expected to isolate many genes essential for ecdysis because we screened for animals arrested at the final stage of the molt. However, we also anticipated the identification of genes required for the production of, or response to, hormonal cues for molting, given that the loss of *nhr-23* in *C. elegans* or the ecdysone receptor in insects results in a terminal failure to ecdyse. Below, we discuss how the annotations of particular genes uncovered by RNAi implicate the corresponding proteins in basic aspects of the molting cycle.

Regulation of gene expression

The identification of several transcription factors in our genomic screen suggests that molting requires extensive changes in gene expression, similar to how transcriptional cascades promote molting and metamorphosis of insects. Particular transcription factors likely alter gene expression in epithelial cells, possibly in response to endocrine cues. Annotated DNA binding proteins and transcription factors required for molting include three zinc-finger proteins, specified by *F10C1.5*, *F25H8.6*, and *lir-1*, that resemble, respectively, *Drosophila* Doublesex, BED subfamily members, and the *C. elegans* transcription factor LIN-26, as well as two nuclear hormone receptors, NHR-23 and NHR-25, that were previously implicated in molting. NHR-23 and NHR-25 are the best candidates for transducing hormonal signals, because the NHRs are expressed in epithelial cells and conserved in insects. In theory, NHRs required for molting might regulate the expression of zinc-finger transcription factors identified in our screen, just as particular NHRs activate zinc-finger proteins in the transcriptional cascades coupled to insect metamorphosis.

Our screen further identified the *xrn-2* gene as essential for molting. The predicted XRN-2 protein is a 5'-3'exoribonuclease that is conserved from yeast to humans. The homologous enzyme, Rat1p, is required for degradation of particular pre-mRNAs as well as the 5' processing of ribosomal and small nuclear RNAs in *S. cerevisiae*. Consistent with a role for XRN-2 in gene regulation during molting, activity of Rat1p contributes to the physiologic regulation of gene expression in yeast.

Intercellular Signaling

The identification of annotated signaling components in our genomic screen suggests that molting requires intercellular communication, possibly to coordinate events in different tissues, or provide feedback on the status of the molt to endocrine regulators. The *qua-1* (quahog-1) gene specifies a protein with a Hint domain at the C-terminus, a hallmark of the hedgehog family of membrane-associated intercellular signaling proteins known to elicit spatially patterned responses in nearby cells during development. Gene products related to the transmembrane transporter-like protein Dispatched are also essential for molting and might export QUA-1 from cells where the protein is synthesized.

Putative intercellular signaling peptides identified in the molting screen include QUA-1, MLT-8, and PAN-1. Features of peptide hormones present in the novel protein MLT-8 include an N-terminal secretory signal sequence, two pairs of basic amino acids suitable for proteolytic processing, and three putative N-linked glycosylation sites. The predicted MLT-8 protein also lacks motifs characteristic of association with membranes. Thus, we expect MLT-8 to be secreted from the cells where it is synthesized, and to move

between cells, rather than localize to the extracellular matrix. We predict that cells secrete PAN-1 based on similar features.

The *acn-1* gene encodes a protein 28% identical to human angiotensin converting enzyme (ACE), the peptide protease that cleaves angiotensin I to angiotensin II. One model for the function of ACN-1 in molting is that worm ACN-1 regulates the production of a peptide molting hormone. However, ACN-1 is unlikely to directly catalyze proteolysis, because the active-site residues that coordinate zinc in human ACE are not conserved. Nevertheless, ACN-1 might bind particular peptides, influencing their maturation or secretion.

Protein Synthesis

The isolation of 25 genes encoding ribosomal proteins or tRNA synthetases confirmed that molting requires a burst of biosynthetic activity, presumably to make components for the new cuticle.

Secretion of the new cuticle

Eighteen components of the general secretion machinery isolated in our screen, including the vesicle coat proteins Sec-23p and B-cop, the small GTPase Sar-1p, and the vesicle fusion factor NSF, are likely essential for synthesis of the new cuticle. Consistent with this view, larvae undergoing RNAi of secretory genes often disintegrate near the time of molting. Alternatively, defects in the secretion of particular proteases or membrane proteins might account for molting defects caused by the loss of particular secretory pathway genes, similar to how the loss of a cytoplasmic adaptor protein, DAB-1, blocks secretion of LRP-1 and also impedes molting.

Remodeling the contractile apparatus

During molting, the basement membrane situated between the muscle and the hypodermis, as well as muscle membrane proteins, must be remodeled such that the contractile apparatus detaches from the old exoskeleton and attaches to the new one. Several genes encoding proteins known to mediate muscle cell adhesion during embryonic development, such as myotactin, were uncovered in the molting screen, implying that the corresponding proteins serve a similar adhesive function in post-embryonic development.

Remodeling of the extracellular matrix

Many genes identified here as essential for molting specify annotated extracellular matrix proteins predicted to directly regulate the production or release of the collagenous cuticle.

Predicted components of the extracellular matrix include FBN-1, a protein that is 30% identical to fibrillin, the microfibril protein defective in Marfan syndrome, a common connective tissue disorder of humans. In addition, the genes *noah-1* and *noah-2* are homologous to NompA, a component of specialized extracellular matrices in the fly. Identification of *fbn-1*, *noah-1*, and *noah-2* as essential for molting suggests that incorporation of the corresponding proteins into macromolecular structures within the new cuticle might be critical for release at the next molt. We further identified three peroxidases likely to modify cuticle components, one of which, BLI-3, is thought to crosslink collagens. Post-secretion enzymatic modifications might therefore be essential for the structural integrity of the new cuticle or release of the cuticle at the next molt.

Two tolloid family metalloproteases independently described as essential for ecdysis, NAS-37 and NAS-36, likely degrade the cuticle of the pre-molt larval stage, or regulate the maturation of other zymogens, as in the blood clotting protease cascade. NAS-37 and NAS-36 might also regulate the assembly of new cuticle, by processing the precursors of particular extracellular matrix proteins, just as tolloid family metalloproteases in vertebrates regulate extracellular matrix formation, in part, by cleaving the C-propeptides of procollagens. The products of *mlt-11* and *bli-5* likely serve as extracellular protease inhibitors, because they contain multiple domains similar to bovine pancreatic trypsin inhibitor (BPTI), and because a related protein from *D. melanogaster*, Papilin, localizes to the extracellular matrix *in vivo* and inhibits ADAMTS metalloproteases *in vitro*. Identification of these probable anti-proteases suggests that proteolysis of the extracellular matrix during molting is highly regulated, either spatially or temporally. In the absence of MLT-11 and BLI-5, extracellular

proteases might be overly active in particular spatial domains, or at inappropriate times, a view consistent with the observation of blisters in the adult cuticle of *bli-5(RNAi)* animals.

The molting RNAi screen further identified proteins that might transduce signals across the plasma membrane of epithelial cells during molting. The presence of MAM domains in the products of *mlt-9* and *ZC13.3* suggests a role in signaling, because the MAM domain is found in numerous transmembrane proteins in the cell-adhesion superfamily, including the receptor-like protein tyrosine phosphatase (PTP) mμ. The *lrp-1* gene, one of the first identified as essential for molting, and also a prominent hit in the genomic screen, encodes an LDL receptor-like protein whose intracellular domain has been proposed to function as a signaling molecule after proteolytic cleavage and release from the plasma membrane of epithelial cells. Signaling via the aforementioned proteins might regulate the spatial or temporal dynamics of ecdysis.

Taken together, the loss-of-function phenotypes and annotations of the aforementioned genes suggest that the corresponding proteins localize to the membrane or extracellular matrix of epithelial cells, where they regulate the formation, condensation, or degradation of the collagenous cuticle during molting. Precise transcriptional or post-transcriptional regulation of the corresponding gene products is likely required for the orderly synthesis and breakdown of cuticle during each molt. In addition to the genes uncovered by RNAi, we identified a single gene, *mlt-10*, as a hypermorphic allele in a forward genetic screen for molting mutants to be described elsewhere. The *mlt-10* gene corresponds to C09E8.3 and represents the founding member of a large family of nematode-specific genes encoding putative extracellular matrix proteins (our unpublished observations).

Temporal and spatial expression patterns of molting genes

We determined the spatial and temporal expression pattern of particular molting genes. We expected some of these genes to act in endocrine cells that trigger molting and some to act in epithelial cells that are remodelled during molting. Further, we anticipated much dynamic regulation during the molting cycle. Because the period between molts is short, only 8 to 10 hours at 25°C, we fused a PEST signal for rapid protein degradation to the C-terminus of GFP, generating a fusion protein with a fluorescent half-life of less than 1 hour *in vivo*. The *gfp-pest* gene was placed under the control of promoters from six genes, *nas-37*, *mlt-11*, *mlt-9*, *acn-1*, *mlt-8*, and *mlt-10*. Fusions with the conventional *gfp* gene revealed the cellular patterns, but not the detailed temporal dynamics, of expression from the promoters of *qua-1* and *xrn-2*. The genes selected for analysis represented the major functional categories from our screen, namely; regulators of gene expression, including the exoribonuclease *xrn-2*; putative signalling pathway components, including the *hedgehog*-like gene *qua-1*, the novel secreted peptide gene *mlt-8*, and the *ACE* homolog *acn-1*; and annotated transmembrane or extracellular matrix proteins, including the MAM domain gene *mlt-9*, the anti-protease gene *mlt-11*, the collagenase gene *nas-37*, and the novel cuticle component gene *mlt-10*. Within functional categories, we selected genes whose inactivation produced highly penetrant molting defects.

Fluorescence from all eight of the *gfp* fusion genes was observed in epithelial cells that synthesize cuticle. The *qua-1*, *nas-37*, *mlt-9*, *mlt-11*, *acn-1*, *mlt-8*, and *mlt-10* fusion genes were each expressed in the hypodermis, including the major body syncytium, hyp7, and hypodermal cells in the head and tail (Fig. 2A-C & E-F, & results not shown). The *nas-37*, *mlt-9*, *acn-1*, and *mlt-11* fusion genes were also expressed in the lateral seam cells, which are essential for molting and morphogenesis of the cuticle. Interestingly, fluorescence from *nas-37p::gfp-pest* in seam cells was observed only before the L4 stage-to-adult molt, when the cells terminally differentiate and fuse, whereas fluorescence from *mlt-9p::gfp-pest* and *mlt-11p::gfp-pest* in seam cells was observed, predominantly, before larval-to-larval molts, when the cells divide. The protease NAS-37 and anti-protease MLT-11 might therefore be required, respectively, to induce or repress fusion of the seam cells. The *gfp* fusion gene for the exoribonuclease *xrn-2* was expressed in specialized myoepithelial cells that secrete the pharyngeal cuticle (Fig. 2D), consistent with the defect of *xrn-2(RNAi)* larvae in shedding cuticle from the pharynx. Particular fusion genes were also expressed in

specialized interfacial cells that secrete cuticle, including the rectal gland, the rectal epithelia, the excretory duct and pore cells, and the vulval epithelium, as well as support cells for head neurons.

A pulse of fluorescence was observed in the hypodermis prior to molting, for each of the six *gfp-pest* fusion genes. Fluorescence from *mlt-8p::gfp-pest* was first detected approximately 3 hours before the L1/L2 molt, or 13 hours after hatchlings synchronized by starvation were fed and incubated at 25°C. The intensity of fluorescence increased until lethargus and then decreased rapidly, such that GFP was barely detectable just 2 hours after molting. When monitoring single transgenic larva over the course of development, fluorescence from *mlt-8p::gfp-pest* was observed starting at 65 ± 2 % and ending at 90 ± 2 % of the way through each larval stage, on average. Expression of *mlt-9p::gfp-pest* and *mlt-10p*::*gfp-pest* in the hypodermis was observed at a similar time, starting, respectively, 64 ± 3% and 63 ± 2% of the way through each larval stage. In contrast, hypodermal expression of GFP from the *mlt-11* promoter was detected earlier, from 51 ± 2% to 72 ± 3% of the duration of each stage, suggesting that the MLT-11 anti-protease, synthesized midway through each larval stage, might repress proteases that are post-translationally activated at ecdysis. Expression of *mlt-9p::gfp-pest* and *mlt-11::gfp-pest* in the seam cells often preceded and lasted longer than expression in hyp7 (results not shown). Expression of *nas-37p::gfp-pest* and *acn-1p::gfp-pest* also cycled in phase with all four molts (results not shown). Likewise, expression of *qua-1p::gfp* in the hypodermis and of *xrn-2p::gfp* in the pharyngeal myoepithelium intensified prior to molting (results not shown). Particular fusion genes were also expressed in the epithelial cells of late embryos that synthesize cuticle for the first larval stage. Moreover, expression of the *gfp* fusion genes was never detected in the hypodermis of gravid adults that no longer molt, whereas *mlt-10p::gfp-pest* and other fusion genes were expressed in adults that undergo a supernumerary molt due to inactivation of the heterochronic gene *lin-29*.

To verify that cycling fluorescence from a *gfp-pest* fusion gene reflects dynamic temporal regulation of gene expression, we examined the level of *mlt-10* messenger RNA by Northern analysis. As predicted by the *mlt-10p::gfp-pest* fusion gene, the abundance of *mlt-10* mRNA in late L4 larvae exceeded that of mid L4 larvae by a factor of 6, and *mlt-10* mRNA was barely detectable in young adults.

Taken together, the spatial and temporal expression patterns of *mlt-8, mlt-9, mlt-10, mlt-11, nas-37, acn-1, qua-1,* and *xrn-2* indicate that the genes are expressed before molting in epithelial cells, such that the corresponding proteins are synthesized in an appropriate time and place to regulate molting. Expression of reporters for *mlt-9, mlt-10, mlt-11,* and *nas-37* in epithelial cells supported our predictions, based on gene annotations, that MLT-9 (MAM domain), MLT-10 (Novel), MLT-11 (anti-protease), and NAS-37 (collagenase) regulate the formation or release of cuticle.

Interestingly, particular fusion genes were expressed in neurons and gland cells that might produce or respond to endocrine signals regulating molting. For example, *xrn-2p::gfp* was expressed in several anterior neurons, including sensory neurons, the PVT neuron that projects along the ventral cord, and the M5 pharyngeal neuron. Expression of *xrn-2* in M5 might be relevant to molting because M5 innervates gland cells whose secretions are thought to expedite release of the pharyngeal cuticle, and because *xrn-2p::gfp* was expressed in M5 only in larvae. The *xrn-2* reporter was also expressed in the intestine, a tissue implicated in the regulation of molting as the site of synthesis of the sterol-modifying enzyme LET-767. However, XRN-2 synthesized in the intestine could serve functions unrelated to molting, because expression of *xrn-2p::gf*p in the intestine persisted in adults that no longer molt. The *mlt-8* reporter was expressed in larvae in a single posterior neuron that remains to be identified. Interestingly, particular fusion genes including *acn-1p::gfp-pest* were expressed in the excretory gland cell of larvae. Although the gland cell is active during ecdysis, ablation of the cell does not prevent molting, indicating that the essential function of *acn-1* is unlikely to stem from expression in this cell. The neurons and other non-epithelial cells that express molting genes represent candidates for foci of endocrine regulation, although the physiologic relevance of molting gene expression in non-epithelial tissues remains to be determined. Analysis of the full expression patterns of these and other molting genes awaits the availability of full-length, functional *gfp* fusion genes that include intronic sequences, or antibodies against the corresponding proteins.

Evidence of an endocrine cue for molting

Observations on the expression of *mlt-10p::gfp-pest* and other reporters lend further support to the hypothesis of an endocrine cue for *C. elegans* molting. In *nas-37(RNAi)* larvae, old cuticle often forms a natural ligature along the longitudinal axis of the worm, typically near the region of the nascent vulva. When *Ex[mlt-10p::gfp-pest] nas-37(RNAi)* larvae with such ligatures were examined late in the L4 stage, 31% (68/216) expressed GFP exclusively in hypodermis on the anterior side of the constriction, whereas no animals were fluorescent on the posterior side alone (Figure S2). 69% (148/216) of ligated larvae expressed GFP on both sides of the ligature, but in many cases fluorescence was barely detectable on the posterior side. Larvae that expressed GFP only in the anterior section stopped expressing GFP as rapidly as control larvae that completed the last molt, and, in some cases, attempted to shed the L4 cuticle from the head, indicating that the animals were not simply delayed at one point in the molting cycle. Moreover, although movement often indicated survival of the tissue on the posterior side of the ligature, the animals failed to express GFP in the posterior section up to 8 hours after the normal time of the L4-to-Adult molt. Together, these observation show that expression of *mlt-10p::gfp-pest*, and, possibly, molting of the posterior hypodermis requires a diffusible cue produced in the anterior of the worm. Similar experiments using man-made ligatures implicated a hormonal cue for molting of the parasitic nematode *Aphelenchus avenae* in 1967. Consistent with the view that a cue produced in the anterior of the worm stimulates molting in *C. elegans*, expression of many of the *gfp* fusion genes typically begins in the anterior hypodermis and then spreads over time to the anterior and then posterior section of the major hypodermal syncytium (hyp7).

Ordering gene expression cascades using *gfp* fusion genes

The molting cycle is a complex temporal program likely to have multiple triggers and checkpoints regulating the expression and activity of transcription factors that control multiple downstream targets. We predicted that particular genes isolated in our RNAi screen would act upstream in the molting pathway, directly or indirectly regulating the expression of genes that promote release of the exoskeleton. The availability of GFP reporters allowed us to visualize the gene regulatory status of the pathway when molting was blocked at various points by the inactivation of particular molting genes.

To order gene expression cascades among the molting genes, we fed larvae expressing either the *mlt-10* or *mlt-8* reporter gene particular dsRNAs of interest. We chose to monitor fluorescence from *mlt-10p::gfp-pest* because all of the transgenic animals grew vigorously and expressed GFP late in each larval stage, and because expression of the *mlt-10* reporter likely signifies the synthesis of components for the new cuticle. The *mlt-8* reporter provided a second marker expressed in the same cells at the same time. We initially examined six gene inactivations representing major functional categories identified in our screen, namely regulators of gene expression (*nhr-23*), putative signaling pathway components (*qua-1, mlt-8,* and *acn-1*), and extracellular matrix proteins (*fbn-1* and *mlt-9*). Transgenic larvae fed the corresponding dsRNAs were monitored for fluorescence and molting over time. Tracking individual worms ensured that expression of GFP was assessed before larval arrest ensued. Analyzing only animals that failed to molt ensured that a defect in expression of GFP would be detected even if a particular RNAi were effective in the minority of animals fed the bacterial clone.

Figure 4A shows that all *nhr-23(RNAi)* animals failed to express GFP from either the *mlt-10* or *mlt-8* promoter prior to their ill-fated molt. Inactivation of *nhr-23* also diminished expression of the reporters for *nas-37, mlt-11, mlt-9, acn-1,* and *qua-1* in the hypodermis, and of the *xrn-2* reporter in the pharyngeal myoepithelium. Thus, the conserved nuclear hormone receptor NHR-23, synthesized in epithelial cells, likely initiates or sustains the pulse of *mlt* gene expression late in each larval stage, perhaps provoking a response to an as-yet unidentified molting hormone. Inactivation of the *acn-1* and *mlt-8* genes likewise abrogated expression of GFP from the *mlt-10* promoter, suggesting that ACN-1 and MLT-8 function downstream of NHR-23 in regulatory cascades.

In contrast, inactivation of the hedgehog-like gene *qua-1*, the *fibrillin* homolog *fbn-1,* or the MAM domain gene *mlt-9* produced larvae that expressed GFP from both the *mlt-10* and *mlt-8* promoters but nevertheless failed to complete ecdysis, indicating that these three genes are dispensable for expression of the *mlt-10* and *mlt-8* reporters, and likely to function downstream of, or in parallel to, the *mlt-10* gene.

To identify additional transcriptional control points, populations of *Ex*[*mlt-10p::gfp-pest*] larvae were fed bacteria expressing dsRNAs corresponding to 76 molting genes, and then monitored for fluorescence late in the L2, L3, and L4 stages. Inactivation of the genes *xrn-2, Y65B4A.6, skp-1, D1054.15, R06A4.9, W09B6.1, M03F8.3, T23F2.1,* and *crs-2* (in addition to *nhr-23, acn-1, and mlt-8*), abrogated expression of GFP during a particular stage and blocked development shortly thereafter, suggesting that the corresponding proteins normally induce or sustain expression of *mlt-10.* The genes identified as putative regulators of the *mlt-10* gene encode, respectively, a 5'-3'exoribonuclease, a DEAD box helicase, a putative co-factor of NHR-23, two WD-beta repeat proteins, the enzyme acetyl-Coenzyme A carboxylase, a homolog of the spliceosome-associated factor CRN1, a glycosyltransferase, and a cysteinyl tRNA synthetase. The *xrn-2* and *Y65B4A.3* genes were verified as positive regulators of *mlt* gene expression by tracking fluorescence from single transgenic larvae fed the corresponding dsRNAs over time (results not shown). We expect the twelve genes upstream of *mlt-10p::gfp-pest* to be required for the initiation or maintenance of synthesis of the new cuticle during molting.

Inactivation of other genes did not significantly ($p>.001$) reduce expression of GFP relative to control larvae of the same stage. In most cases, the formal possibility that the corresponding proteins regulate the *mlt-10* gene cannot be eliminated due to the variable efficacy of RNAi, particularly when inactivation of a gene partly reduced expression of GFP. However, inactivation of particular genes, *bli-3, nas-37, lrp-1, F45G2.5, ZK430.8, unc-52, lev-11, W10G6.3, kin-2, bli-1, gei-16, K04A8.6,* and *F25H8.6,* produced molting-defective larvae that expressed GFP (Fig. 4B and results not shown), demonstrating with certainty that these gene activities are not necessary for induction of *mlt-10p::gfp-pest* and suggesting, instead, that the genes act downstream of, or in parallel to, the *mlt-10* gene. Gene products dispensable for *mlt-10* expression might act very near, or at, the time of ecdysis, consistent with our predictions, based on gene annotations, that the products of *fbn-1, bli-3,* and *ZK430.8* promote assembly or post-secretion modifications of the cuticle. Monitoring expression of the *gfp* fusion genes thus allowed a first sorting of molting genes uncovered by RNAi into pathways.

Discussion

Using functional genomics, we identified a large set of genes essential for molting in *C. elegans*. We have produced a model for the regulation of molting wherein endocrine or neuroendocrine cues generated by as-yet unidentified cells trigger epithelial cells to remodel the exoskeleton at the end of each larval stage. Together, gene annotations as well as spatial and temporal expression studies suggest that many genes uncovered in our screen correspond to extracellular matrix enzymes and structural components as well as transmembrane proteins that are active in epithelial cells at the time of molting, regulating the *de novo* production or release of cuticle in a tightly controlled cycle. Activation of molecules such as the anti-protease MLT-11, the collagenase NAS-37, the MAM domain protein MLT-9, or the LDL-receptor-like protein LRP-1 each represents a potential focus for the spatial and temporal regulation of ecdysis.

Fusions to GFP show that expression of several genes uncovered in our screen cycles in phase with molting, similar to the cyclic expression of particular nuclear hormone receptor and cuticle collagen genes. Analysis of the GFP reporters shows further that the conserved nuclear hormone receptor NHR-23 directly or indirectly activates expression of many genes in epithelial cells, including *mlt-8, mlt-9, mlt-10, mlt-11, nas-37, acn-1,* and *xrn-2* (specifically in the pharynx). NHR-23 is also required for expression of a cuticle collagen gene, *dpy-7,* whose product is incorporated into each larval cuticle. Thus, NHR-23 likely coordinates gene expression in epithelial cells, possibly in response to an endocrine cue for molting. A ligand for NHR-23 has yet to be identified, but the molecule could be synthesized in neuroendocrine cells or in steroidogenic cells coupled to neurons that regulate molting. Production of a ligand for NHR-23 is likely to be exquisitely regulated, similar to how steroidogenesis in the prothoracic gland of insects is both induced by PTTH and repressed by ecdysteroids.

How the multiple genetic pathways uncovered by RNAi converge to regulate *mlt* gene expression remains to be determined. However, one or more of these pathways might couple progression of the molting cycle to physiologic or environmental cues, such as the availability of food.

Our screen further identified the exoribonuclease XRN-2 as a novel regulator of gene expression during molting. One model is that XRN-2 down-regulates the abundance of mRNAs or microRNAs that correspond to negative regulators of molting. Together, the observations that *xrn-2(RNAi)* larvae fail to shed the pharyngeal cuticle and that *xrn-2p::gfp* is expressed in the pharyngeal myoepithelium suggest that XRN-2 promotes ecdysis in the pharynx. However, *xrn-2* is also required for expression of the *mlt-10* reporter in the hypodermis, a tissue where expression of *xrn-2p::gfp* itself has not been detected. One possibility is that XRN-2 activity in the pharynx, intestine, or neurons leads to an intercellular cue that promotes expression of *mlt* genes in hyp7. Alternatively, *xrn-2* might be expressed in the hypodermis but not detectable using this particular *gfp* fusion gene. In theory, the product of *Y65B4A.6,* another gene isolated in our screen, and XRN-2 might work together to regulate gene expression because *Y65B4A.6* encodes a member of the DEAD-box helicase family, enzymes that couple ATP hydrolysis to unwinding dsRNA in a variety of cellular processes, and a DEAD-box helicase functions along with the Xrn1p/Rat1p exoribonuclease in mRNA degradation in yeast. Together, the requirement for *xrn-2* in molting and the recent observation that a closely related gene, *xrn-1*, is essential for epithelial cell movements during embryogenesis establish the XRN family of exoribonucleases as important developmental regulators in *C. elegans*.

Our identification of putative signaling molecules synthesized in epithelial cells, such as QUA-1 and MLT-8, suggests an essential role for intercellular communication in molting of *C. elegans*. One model is that signaling between different epithelial cells, such as the hypodermal syncytium and the lateral seam cells, might coordinate the production or release of cuticle, while signaling between the hypodermis and muscle might coordinate remodeling of the contractile apparatus. Intercellular communication might also coordinate the division or fusion of the seam cells and the endoreduplication of intestinal nuclei with molting. Consistent with this view, transcription factors that regulate the differentiation or fusion of seam cells are also required to shed the cuticle. The hedgehog-like protein QUA-1 is a good candidate for an extracellular signal secreted from the hypodermis. QUA-1 could generate a spatially patterned response in nearby cells, coordinating the molt. We further hypothesize that MLT-8 serves as an autocrine cue that sustains synthesis of the new cuticle, because *mlt-8* promotes expression of the *mlt-10* reporter in the same epithelial cells where MLT-8 itself is synthesized and, presumably, secreted. An alternative view is that cell-autonomous responses to one or a few endocrine cues account for the coordinated activities of different cell types during *C. elegans* molting, similar to how different tissues respond in concert to changes in the titer of 20-hydroxyecdysone during insect metamorphosis.

To set the molting cycle, we further expect secreted signals from epithelial cells to provide feedback on the status of the molt to endocrine or neuroendocrine regulators. The existence of physiologic feedback cues is consistent with the observation that many larvae that fail to ecdyse also arrest development, including those Mlt larvae defective in epithelial functions, like LRP-1. Interference with ecdysone signaling in epidermal tissues similarly triggers a global arrest during *Drosophila* metamorphosis, suggesting the existence of a molting "check-point" in insect development. In theory, any of the signaling components isolated in our screen could function in feedback pathways. Particular peptide hormones might also regulate lethargus or the behaviors characteristic of ecdysis, in much the same way that peptide hormones trigger ecdysis in insects, although none of the putative secreted peptides identified in our screen show obvious sequence similarity to EH or ETH.

With the cycling *GFP* reporters in hand, genes and hormones that function far upstream in the molting pathway of nematodes, possibly in neuroendocrine or endocrine cells, can now be identified as genetic mutants or biochemical activities that trigger precocious or delayed expression of the *mlt* gene reporters. Such master neuroendocrine regulators of molting may well be conserved between nematodes and arthropods. One simple explanation for the abundance of epithelial, as opposed to neuronal, genes uncovered in this particular screen is that RNAi works better in epithelial cells than in neurons.

Identifying genes essential for molting of *C. elegans* enables the development of safe and effective nematicides and insecticides that target gene products conserved only in Ecdysozoans. Current anti-nematode drugs, such as benzimidazoles and avermectins, target, respectively, cytoskeletal components and ion channels that are conserved in mammals, and the drugs therefore can be toxic to humans. Resistance to these compounds is also increasingly common. One potential new drug target is MLT-8, since the

corresponding gene is conserved and highly expressed at the molt in a parasitic nematode, as inferred by the identification of 32 cDNAs matching *Ce mlt-8* (E ¯121) in a library derived from molting *O. volvulus* (Table S7 and results not shown). However, the novel sequence of MLT-8 may pose a serious challenge for drug development. In this regard, molting proteases, like NAS-37, represent more attractive targets for the development of small molecule antagonists, given the success of drug development on protease targets for high blood pressure and HIV. Further, molting genes conserved only in insects and nematodes, such as the extracellular matrix proteins NOAH-1 and NOAH-2, identify potential targets for insecticides expected to only harm Ecdysozoans.