CHAPTER SIX

Developmental Transitions in *C. elegans* Larval Stages

Ann E. Rougvie[*,1], **Eric G. Moss**[†]
[*]Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, Minnesota, USA
[†]Department of Molecular Biology, Rowan University, Stratford, New Jersey, USA
[1]Corresponding author: e-mail address: rougvie@umn.edu

Contents

Abstract

Molecular mechanisms control the timing, sequence, and synchrony of developmental events in multicellular organisms. In *Caenorhabditis elegans*, these mechanisms are revealed through the analysis of mutants with "heterochronic" defects: cell division or differentiation patterns that occur in the correct lineage, but simply at the wrong time. Subsets of cells in these mutants thus express temporal identities normally restricted to a different life stage. A seminal finding arising from studies of the heterochronic genes was the discovery of miRNAs; these tiny miRNAs are now a defining feature of the pathway. A series of sequentially expressed miRNAs guide larval transitions through stage-specific repression of key effector molecules. The wild-type lineage patterns are executed as discrete modules programmed between temporal borders imposed by the molting cycles. How these successive events are synchronized with the oscillatory molting cycle is just beginning to come to light. Progression through larval stages

Current Topics in Developmental Biology, Volume 105
ISSN 0070-2153
http://dx.doi.org/10.1016/B978-0-12-396968-2.00006-3

can be specifically, yet reversibly, halted in response to environmental cues, including nutrient availability. Here too, heterochronic genes and miRNAs play key roles. Remarkably, developmental arrest can, in some cases, either mask or reveal timing defects associated with mutations. In this chapter, we provide an overview of how the *C. elegans* heterochronic gene pathway guides developmental transitions during continuous and interrupted larval development.

1. INTRODUCTION

Developmental transitions can be seen at the level of morphology and widespread cellular changes, as in insect metamorphosis, or by more subtle shifts in cell growth and behavior, as when neural cells produce neurons and then glia. These transitions are fascinating because they imply the existence of molecular mechanisms that coordinate the behaviors of many cells in time. We can expect that such mechanisms are not simple, and consist of many parts with complex interactions and dynamics. To work out how such transitions are regulated and executed, we need experimental systems that permit multiple approaches and provide unusual access to the underlying molecular phenomena.

Two kinds of developmental transitions exemplified by the *Caenorhabditis elegans* larva are the four stage-to-stage molts on the way to adulthood and an optional diapause state that can be initiated midway in that course in response to harsh environmental conditions. The regulation of these two types of transitions intersects when the normal progress of development is interrupted and then resumes where it left off. The molecular mechanisms and regulatory networks underlying these transitions are not simple, but sufficient molecular details have been elucidated that a few principles have emerged. Persistent use of classical genetics, combined with one of the first sequenced genomes, RNA interference, and molecular approaches has revealed a dynamic network of molecular regulators, switches, and oscillators, that ensure that cells do the right thing at the right time.

2. A WORM WELL-SUITED TO TIMING STUDIES

C. elegans is a soil-dwelling nematode, a microscopic worm with an extremely streamlined anatomy. It emerges from eggshell with exactly 558 cells, and having the necessary nervous system and musculature, it is competent to forage and feed (see http://www.wormbook.org/). What it

lacks at the beginning of postembryonic development is the ability to reproduce. Over four larval stages, 53 cells divide and differentiate to ultimately form the gonad and reproductive system, as well as additions and rearrangements to the epidermis, muscles, and nervous system. Each larval stage ends with a molt when the epidermis secretes a collagenous cuticle, and the old cuticle is shed. At the last transition, somatic differentiation is complete and the animal becomes sexually mature: either a self-fertilizing hermaphrodite or a male.

The stage-to-stage transitions during larval development appear relatively subtle. The larva grows roughly continuously in size with little change in overall morphology. Each molt is preceded by a period of lethargus, when the worm seems to sleep while its cuticle changeover takes place. Emerging from the old cuticle, the worm resumes foraging and pumping food into its pharynx, and growth continues. By the fourth stage, about 400 cells have been added to the epidermis, nervous system, gonad, and muscle. The adult hermaphrodite that emerges from the last molt consists of 959 somatic cells and scores of germ cells, and is about 1 mm long. The adult will increase in size, but will only add germ cells, not somatic cells, throughout the rest of its roughly 2–3-week life.

If environmental conditions deteriorate, larval development can undergo a controlled and reversible suspension that is coordinated throughout the animal. The trigger is an increased population density, exacerbated by diminishing food supply and slightly warmer temperatures. Under such conditions, early stage larvae have the opportunity to transition into a morphologically distinct form, called the dauer larva, which is specialized for long-term survival under less than optimal growth conditions. The decision to make this choice occurs during the first larval stage (L1) but the morphological shift is not complete until midway through larval development, at the end of the second stage (L2). Survival as a dauer extends far beyond the normal lifespan of the worm due to numerous physiological adjustments. As the dauer disperses, the crowding dissipates (sensed by the drop in a worm-produced pheromone), and if nutritional conditions improve, the larva morphs back into the feeding/foraging condition and picks up development where it left off. In this instance, the transition into and out of dauer must be coordinated with the transition that normally occurs in continuous development from the L2 to the third stage (L3).

Two features of *C. elegans* make it a particularly powerful experimental system for investigating developmental transitions. First, the position and division of every cell is observable in living specimens due to the worm's

transparency. Because cell behavior is remarkably the same from animal to animal, the lineage of every cell in the animal is traceable through larval development. But what makes *C. elegans* exceptionally informative is the genetics: forward genetic screens have identified a diversity of mutants and these mutants can be combined to tell the tale of how the genes function together.

3. HETEROCHRONIC MUTANTS

Among the many *C. elegans* mutants isolated in genetic screens were a few that eventually were grouped together because they altered the succession of larval developmental events. The gross phenotypes of these mutants are not very different from those of many developmental mutants in the lin or "cell lineage abnormal" class: They are unable to lay eggs, having either a missing or defective egg-laying system (Ferguson & Horvitz, 1985). But cell lineage analysis revealed that this subset displayed features not found in other mutants: the normal order of stage-specific lineage patterns is altered throughout the animal (Ambros & Horvitz, 1984). In one class of mutants, development was precocious, where stage-specific events were skipped, and in another development was retarded, where events were reiterated (Fig. 6.1). The whole group was termed "heterochronic mutants," for their abnormal developmental timing. Initially, the mutations in this collection defined just four genes: *lin-4*, *lin-14*, *lin-28*, and *lin-29*. Many others were to follow.

The mere existence of these mutants allowed two insights: First, the temporal progress of many cell lineages is under direct genetic control, rather than being an emergent property of spatial patterning and tissue-specific regulation. Secondly, the mutants revealed that the stage-specific sublineages were modular, that is, what happens at one stage does not necessarily depend on what happened at a previous stage. One commentator compared the modularity of the *C. elegans* larval stage patterns to parasegments in *Drosophila* embryonic development, with the parasegment boundaries being represented by the molts (Thummel, 2001). Indeed, there is likely a mechanistic connection between temporal patterning and the molting cycle.

The epidermis replaces its cuticle four times, once per larval stage. During the intermolt period, certain epidermal blast cells divide in patterns that are characteristic of the lineage at each stage. For instance, lateral epidermal blast cells V1 to V4 each divide once in the first larval stage producing a daughter that joins the epidermal syncytium and one that remains a blast cell

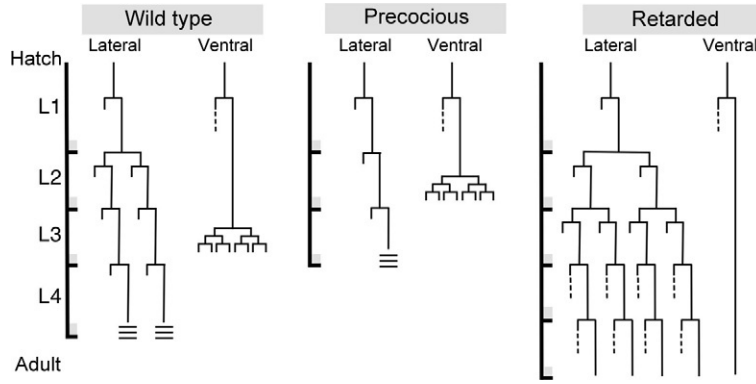

Figure 6.1 Cell lineages of precocious and retarded heterochronic mutants. Post-embryonic development is indicated on the left from hatching to adulthood, encompassing four larval stages. Thick lines represent the molting cycle with gray boxes indicating the lethargus period and the short horizontal lines indicating the molts. Thin lines are lineage diagrams of representative lateral and ventral epidermal cells. Lateral epidermal blast cells terminally differentiate into seam cells of the adult, indicated by three horizontal lines. Ventral cells give rise to the vulva. Dashed lines indicate additional cells not shown. Lineages of representative precocious and retarded mutants are shown. Precocious mutants skip stage-specific lineage events (in this case, those of the L2) and may cease the molting cycle prematurely. Retarded mutants reiterate stage-specific events (e.g., L2), and, as in the example shown, cease molting indeterminantly after five or more cycles.

(Fig. 6.1). This blast cell divides twice in the second larval stage, and once again in the third and fourth stages. At adulthood, these cells fuse to form another syncytium, the lateral seam, which secretes a cuticle structure called adult lateral alae. Tracking of these and other lineages in heterochronic mutants reveals their temporal patterning defects in detail (Ambros & Horvitz, 1984).

lin-4 null mutants reiterate the L1 pattern in subsequent stages and terminal differentiation of the lateral epidermal blast cells never occurs. In the ventral epidermis, the three "vulva precursor cells" (VPCs) normally divide in the L3 to produce the 22 cells of the vulva, but do not do so in *lin-4* mutant animals. A recessive mutation in *lin-14*, on the other hand, causes L1 patterns to be skipped, and all subsequent nongonadal lineages, including the VPCs, advance precociously. In these animals, the lateral epidermal blasts divide twice in the first stage, which they would normally do in the second stage, and differentiation occurs at the end of the L3, one stage early. Thus, *lin-4* and *lin-14* mutants show opposite effects on the L1, resulting in retarded and precocious development, respectively.

lin-28 mutants, like *lin-14* mutants, are precocious, but instead of skipping first-stage events, they skip second-stage patterns (some cells also skip third-stage events). *lin-29* mutants are retarded like *lin-4* animals, but their problems do not begin until the L4 stage. The animal becomes adult, but its epidermis remains larval. Eventually, other heterochronic mutants were identified that showed precocious or retarded development starting at one stage or another. Some heterochronic mutants have stronger null phenotypes than others, and a surprising number affect the L2, and most affect the transition to adulthood, directly or indirectly. The overall theme, however, is that their loss-of-function phenotypes are either precocious or retarded, and the defect begins at a particular stage.

The organization of the heterochronic pathway emerges when individual mutants are combined into multiply-mutant strains (Ambros, 1989). For example, when a null allele of *lin-4*, which reiterates the L1 fates, is put together with a null allele of *lin-14*, which skips the L1 fates, the resulting double-mutant animal resembles the *lin-14* mutant in all respects. By the logic of epistasis analysis, this result suggests that *lin-4* normally negatively regulates *lin-14* in some way. When a *lin-29* mutant is combined with any known precocious mutant, the resulting animal is always retarded for late-stage developmental events, indicating that *lin-29* is the most downstream gene in the pathway.

In this way, the heterochronic pathway has been built up as new genes are added. In most cases, the mutations are strong and the double phenotypes clear. These results give us the core pathway (Fig. 6.2, top). In other cases, however, the gene mutations are weaker (either incompletely penetrant, incompletely expressive, or both), or, the mutations affect a subset of cells or tissues affected by the stronger genes. In these cases, how the genes fit into or onto the core pathway is much less clear. Nevertheless, certain themes of organization have emerged from the genetics. Combined with molecular knowledge gained from cloning and expressing the genes, certain mechanisms that seem particular to developmental timing have emerged.

Among the first mutant animals with heterochronic phenotypes were some that resembled the *lin-4* loss-of-function mutants, but were dominant in their genetic behavior (Ambros & Horvitz, 1987). These are gain-of-function alleles of *lin-14* in which portions of the 3′ untranslated region (UTR) of the gene's mRNA are deleted, leaving the open reading frame intact (Wightman, Burglin, Gatto, Arasu, & Ruvkun, 1991). These alleles, together with analysis of *lin-4*, were key to making the most significant discovery in the pathway: microRNAs.

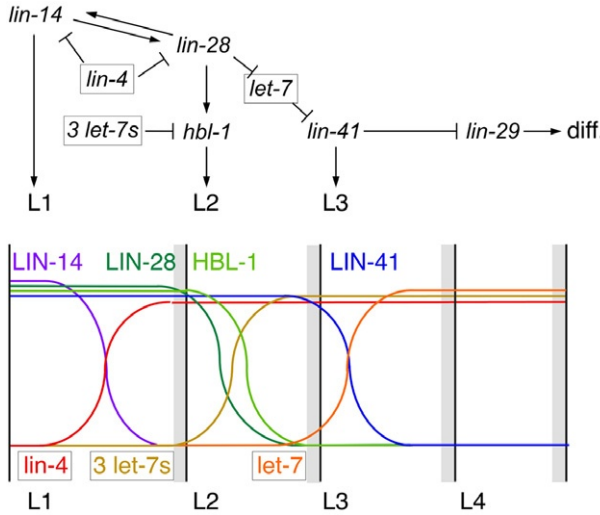

Figure 6.2 Genetic and dynamic models for the heterochronic timing mechanism. The top panel shows one model for the established genetic interactions of core players in the timing mechanism indicating approximately when in developmental time they are active. MicroRNA genes are boxed. Stage-specific fates are indicated by larval stage and terminal differentiation by "diff." The bottom panel represents the rising and falling levels of the mature products of key genes. All microRNAs (boxed) begin development in the off state and rise at specific times, and their targets are repressed accordingly, leading to falling protein levels. Not all factors are shown.

4. microRNA SWITCHES

The hallmark of the developmental timing mechanism is the sequential action of microRNAs, which are 21–25 nucleotides long, the shortest functional RNAs known. These RNAs bind sequences in the 3′UTRs of target mRNAs, slowing, reducing, or blocking the accumulation of the encoded proteins (Ambros, 2011).

lin-4 encodes a microRNA which begins to accumulate from the mid-L1 and is constitutively expressed thereafter (Feinbaum & Ambros, 1999; Lee, Feinbaum, & Ambros, 1993). The LIN-14 protein is normally present early in the L1 and is downregulated as development proceeds (Ruvkun & Giusto, 1989). Sequences complementary to the *lin-4* RNA are present in the *lin-14* mRNA's 3′UTR, and these are missing in the *lin-14* dominant alleles (Wightman et al., 1991; Wightman, Ha, & Ruvkun, 1993). Thus, the loss of *lin-4* or the *lin-14* 3′UTR sequences causes LIN-14 protein to

be expressed throughout larval development resulting in reiteration of L1-specific events. The transition from L1- to L2-specific events is governed by this microRNA switch.

In addition to *lin-4*, several other microRNA genes act in the pathway: *let-7* and its three relatives *mir-48*, *mir-84*, and *mir-241* (Abbott et al., 2005; Reinhart et al., 2000). The *let-7* family of microRNAs share nearly identical 5′ halves of their short 22-nucleotide lengths. Theory predicts that these microRNAs can target the same mRNAs, although whether or not they do *in vivo* is an open question. The three *let-7* relatives are expressed with nearly identical profiles and act redundantly to repress *hbl-1*, which encodes a Hunchback-like transcription factor that controls the transition from L2 to L3 (Abbott et al., 2005; Abrahante et al., 2003; Lin et al., 2003). *let-7* accumulates slightly later and its primary target is *lin-41*, which appears to encode an RNA-regulatory protein or ubiquitin ligase (Loedige, Gaidatzis, Sack, Meister, & Filipowicz, 2012; Reinhart et al., 2000; Slack et al., 2000). There is some controversy about whether it controls the L3 to L4 transition or the L4 to adult transition (Vadla, Kemper, Alaimo, Heine, & Moss, 2012).

In general, the microRNAs show an expression profile that is "off early, on late," and their targets are "on early, off late" (Fig. 6.2, bottom). What distinguishes these switches is the time of accumulation of the microRNA and the consequent time of repression of the target. Although largely still ill-defined, some mechanisms controlling microRNA accumulation have been found.

5. LIN-28: A microRNA REGULATOR AT THE PATHWAY'S CENTER

A second target of the *lin-4* microRNA is *lin-28* (Moss, Lee, & Ambros, 1997). *lin-28* encodes a small RNA-binding protein that is known to bind the precursor of the *let-7* microRNA and block its maturation, thereby supporting *lin-41* against repression (Lehrbach et al., 2009; Moss et al., 1997; Vadla et al., 2012; Van Wynsberghe et al., 2011; Viswanathan, Daley, & Gregory, 2008). It does this by recruiting a poly(U) polymerase (encoded by *pup-2*) that helps to stimulate the degradation of the *let-7* precursor RNA (Heo et al., 2008). Thus, the accumulation of *let-7* is prevented until *lin-28* is repressed by *lin-4*.

However, repressing *let-7* is not all that *lin-28* does. As mentioned above, *lin-28*'s primary role is to promote L2 events. Neither *let-7* nor *lin-41* controls the L2. Instead, *lin-28* appears to act via *hbl-1*, supporting *hbl-1* against

repression (Vadla et al., 2012). The mechanism for this is independent of the *let-7* family members, so something else must be repressing *hbl-1*. What this repressor is and how the LIN-28 protein acts in this *let-7*-independent mechanism remain unclear.

lin-28 also has a supportive role in *lin-14* expression, and visa versa. Because *lin-4* targets each of these genes, when *lin-4* is removed both are constitutively expressed (Arasu, Wightman, & Ruvkun, 1991; Moss et al., 1997). Remove either one and the other is repressed (Arasu et al., 1991; Moss et al., 1997; Seggerson, Tang, & Moss, 2002). Thus, *lin-14* and *lin-28* are tied to each other's expression, but again the molecular mechanisms are unknown. Nevertheless, *lin-28* is a stage-specific positive regulator of three stage-specific effectors in the pathway: *lin-14*, *hbl-1*, and *lin-41*.

6. THE HETEROCHRONIC PATHWAY

There are three distinct phases of the heterochronic pathway, each involving a microRNA switch and a connection to the next phase via a key regulator (Fig. 6.2). *lin-4* and *lin-14* comprise the first phase, which transitions the animal from the L1 to the L2. As mentioned, *lin-4* is off early and accumulates during the L1, but how its rise is controlled is not known. *lin-14*, which appears to encode a transcription factor, seems to be all that is needed to specify L1 fates. What was clear from an early analysis of a variety of *lin-14* mutant alleles is that *lin-14* also has a role in determining what happens in the L2 (Ambros & Horvitz, 1987). It is believed that how it exerts that influence is via its positive regulation of *lin-28* (Pepper et al., 2004). Thus, *lin-14* has a direct role in specifying one stage, and influences a key regulator of the next.

The second phase is governed by *lin-28* and *hbl-1*, which are repressed by *lin-4* and the three *let-7* relatives, respectively. *hbl-1* seems to have the more direct role in specifying L2 fates (Abbott et al., 2005; Abrahante et al., 2003). But *lin-28*—like *lin-14*—has the interesting property of controlling two consecutive stages: it positively regulates *hbl-1* and then positively regulates *lin-41* (by inhibiting *let-7*). The repression of *lin-41* by *let-7*, which leads ultimately to the activation of *lin-29*, is the third phase. Recent data suggest the *let-7*/*lin-41* switch might directly regulate L3 fates (Vadla et al., 2012). It is not yet clear if *lin-41* controls two larval stages; a great deal of investigation needs to be done to fill in how this third phase works, including whether *lin-41* acts alone and how *lin-29* is repressed. It has been suggested that *lin-41*, whose mechanism is unclear, might act directly on *lin-29* post-transcriptionally (Del Rio-Albrechtsen, Kiontke, Chiou, & Fitch, 2006).

lin-29, which is required for terminal differentiation of epidermal cells, encodes a transcription factor that can directly regulate terminal differentiation genes (Rougvie & Ambros, 1995).

The genes directing these three phases form the core heterochronic pathway. In general, these genes have highly penetrant and unambiguous phenotypes when mutated. Other factors governing developmental timing in *C. elegans* are characterized by how they integrate into this core pathway. Still, there is quite a bit missing from this picture, not least of which is how the boundaries between the larval stages fit in.

7. DEVELOPMENTAL TRANSITIONS: THE MOLTS

The heterochronic pathway and molting control must be intimately linked. The very boundaries of the stages are the molts, and the heterochronic pathway confers temporal identity on cells during the intermolt period. Furthermore, precocious mutants like *lin-14* and *lin-28* cease the molting cycle at least one stage too soon, while retarded mutants like *lin-4* and *lin-29* continue molting indefinitely. One expects that the mechanisms coordinating the animal-wide process of molting influence the rise and fall of heterochronic factors that determine the stage-specific cell fates.

Molting is a defining feature of the Ecdysozoa clade of which both *C. elegans* and *Drosophila* are members (Aguinaldo et al., 1997). The mechanisms that guide molting are best understood in flies where pulses of the cholesterol-derived steroid hormone 20-hydroxyecdysone (20-E) impose temporal transitions during the life cycle, timing molting and metamorphosis, by acting through a nuclear receptor heterodimer composed of the ecdysone receptor (EcR) and its partner ultraspiracle (USP) (Koelle et al., 1991; Yao et al., 1993; Yao, Segraves, Oro, McKeown, & Evans, 1992). The signals generated by these periodic interactions induce expression of additional nuclear receptors (DHR3, βFtz-F1, and others) in a precise sequence that temporally patterns the molts and metamorphosis in a coordinated fashion throughout the organism.

C. elegans neither synthesizes nor appears to respond to ecdysteroids, and it lacks EcR and USP orthologs (Chitwood & Feldlaufer, 1990; Sluder, Mathews, Hough, Yin, & Maina, 1999), indicating that molting in this organism is launched by a different trigger. Nevertheless, some aspects of molting control appear to be more universal, with steroid mediated responses providing a unifying theme. The worm lacks cholesterol biosynthesis enzymes and instead depends upon dietary intake of this steroid. When

grown on cholesterol deficient media, *C. elegans* larvae exhibit molting defects, supporting a role for steroid ligands in regulating aspects of this process (Yochem, Tuck, Greenwald, & Han, 1999). In addition, downstream components of the fly ecdysone-activated transcriptional cascade are broadly conserved and several function in molting in the nematode. The best characterized of these are NHR-23 (similar to fly DHR3 and mammalian RORα) and NHR-25 (fly βFTZ-F1; mammalian SF1), nuclear receptors whose expression levels cycle with the molts, peaking in the intermolt (Asahina et al., 2000; Gissendanner, Crossgrove, Kraus, Maina, & Sluder, 2004; Gissendanner & Sluder, 2000; Kostrouchova, Krause, Kostrouch, & Rall, 1998, 2001). Whether these conserved receptors respond to ligand, steroidal or otherwise, has not been established.

The loss of NHR-25 function by mutation or RNAi causes molting defects and an array of additional phenotypes, including embryonic and larval lethality and cuticle structural abnormalities (Asahina et al., 2000; Chen, Eastburn, & Han, 2004 Gissendanner & Sluder, 2000; Silhankova, Jindra, & Asahina, 2005). The molting defect is a manifest as an inability to shed the old cuticle and results from the loss of NHR-25 function in the epidermis (Chen et al., 2004). The loss of *nhr-23* function causes a similar suite of phenotypes, including the inability to shed cuticle at each larval stage (Kostrouchova et al., 1998, 2001). Clarifying the roles of these and potentially other nuclear receptors in molting will require the identification of their direct targets, a process now underway (Kouns et al., 2011), and the identification of their ligands (if any). Genome-wide searches have also revealed genes required to execute molts (Frand, Russel, & Ruvkun, 2005; Kang et al., 2013) and together with more directed studies are beginning to reveal the elements of a molting pathway. Although some signaling molecules are included, many of the genes identified act in the mechanics of molting, encoding proteins such as the collagens that build each cuticle and various peptidases whose functions include detaching the old cuticle from the epidermis. These types of genes are critical, providing the nuts and bolts of the molt. But perhaps more fascinating are the factors that orchestrate molting behaviors with the stage-specific developmental programs. Periodic regulation of molting is required during larval stages and must ultimately be terminated to halt the program when the adult stage is reached. How this regulation is achieved brings us back to the heterochronic gene pathway.

The failure of retarded heterochronic mutants to limit molting to four cycles reveals that a terminal output of the timing pathway is to coordinate the cessation of molting with the transition to adulthood. Here too,

miRNAs play a central role. *let-7* mutants on their own, as well as animals multiply mutant for *let-7* family miRNAs, inappropriately enter lethargus as adults and partly shed the cuticle, indicating these miRNAs engage the mechanism to terminate molting upon reproductive maturity (Abbott et al., 2005; Hayes, Frand, & Ruvkun, 2006; Reinhart et al., 2000). Let-7 family miRNAs appear to terminate the molting cycles through repression of *nhr-23* and *nhr-25* (Hayes et al., 2006); however, only the *nhr-25* 3′UTR contains a predicted *let-7* family binding site, and whether it is a direct target has not been established. Ties among molting, nuclear receptors, miRNAs, and other timing pathway components, are further strengthened by complex genetic interactions between *nhr-25* and various heterochronic genes, and the identification of *apl-1* (amyloid precursor protein-like-1) as a potential *nhr-25* target (Niwa & Hada, 2010; Niwa, Zhou, Li, & Slack, 2008). *apl-1* functions include molting, it acts downstream of *let-7*, and it interacts genetically with other heterochronic pathway members (Hornsten et al., 2007; Niwa et al., 2008). Many details of these gene interactions remain unresolved.

A striking observation is that the extra molt in *let-7 mir-84* double mutants occurs synchronously in the adult population (Hayes et al., 2006), indicating a timing mechanism at work. This finding emphasizes key questions—what paces the molts, and how are the stage-specific programs integrated with the molting timer? Exciting recent work again implicates the heterochronic gene network, this time through *lin-42* (Monsalve, Van Buskirk, & Frand, 2011), a gene first identified by mutations that cause a precocious heterochronic phenotype (Jeon, Gardner, Miller, Deshler, & Rougvie, 1999).

8. LIN-42: A LINK BETWEEN MOLTING AND DEVELOPMENTAL DECISIONS

LIN-42 provides a remarkable link to biological timing mechanisms in flies and mammals: it is the nematode homolog of the Period family of circadian rhythm proteins. *lin-42* and Per genes exhibit an intriguing parallel in regulation: their mRNA and protein levels oscillate over biologically pertinent time scales. LIN-42 levels fluctuate with the molting cycle (~8–10 h at 25 °C) while PER levels are synchronized with the 24-h clock (Hardin, Hall, & Rosbash, 1990; Jeon et al., 1999; Shearman, Zylka, Weaver, Kolakowski, & Reppert, 1997; Sun et al., 1997; Tei et al., 1997; Tennessen, Gardner, Volk, & Rougvie, 2006). As a result of its phenotypes and expression patterns, *lin-42* function has been most studied in a

developmental context; however, one report links *lin-42* activity to a circadian rhythm (Simonetta, Migliori, Romanowski, & Golombek, 2009). For additional consideration of circadian rhythms in *C. elegans*, the reader is directed to the recent literature (Hasegawa, Saigusa, & Tamai, 2005; Migliori, Simonetta, Romanowski, & Golombek, 2011; Olmedo et al., 2012; Temmerman et al., 2011; van der Linden et al., 2010).

The cyclic developmental profile of LIN-42 accumulation dramatically contrasts with the patterns of LIN-14, LIN-28, and HBL-1. Levels of these proteins are initially high in the L1 epidermis, then stage-specifically decrease during early larval stages and remain absent (Fig. 6.2), yet their loss-of-function also causes precocious phenotypes (Abrahante et al., 2003; Lin et al., 2003; Moss et al., 1997; Ruvkun & Giusto, 1989). Thus, *lin-42* appears qualitatively different, with an expression pattern suggestive of multiple or reiterated roles during development. Such a repeated role is not reflected in the early epidermal lineage patterns of *lin-42* mutants which appear normal until the L3 molt when an adult-type cuticle appears one stage early (Jeon et al., 1999). However, certain double mutant combinations do reveal epidermal lineage defects prior to the L3 stage, implying earlier redundant role(s) for *lin-42* in controlling these divisions (Abrahante, Miller, & Rougvie, 1998; Ren & Zhang, 2010). But, it is the conspicuous reiterative pattern of LIN-42 levels, peaking once per larval stage, that is most evocative, suggesting a possible link to the molting cycle.

Curiously, the first described *lin-42* alleles were only observed to have defects in the final molt; the first three molts appeared normal despite the cyclical pattern of *lin-42* expression (Jeon et al., 1999). We now know that early molting defects were hidden by complexities of the *lin-42* locus. LIN-42 and Period proteins share multiple regions of homology, notably a PAS domain that mediates protein–protein interactions and smaller SYQ and LT domains that reside within a C-terminal regulatory region of Per (Chang & Reppert, 2003; Tennessen et al., 2006). *lin-42* encodes four isoforms, two of which do not overlap. One of these nonoverlapping isoforms contains the PAS domain (LIN-42c), while the other contains the SYQ and LT domains (LIN-42a); the remaining two isoforms contain all of these homology domains (Fig. 6.3). The first alleles of *lin-42* left the SYQ/LT isoform intact, but an important recent study of an allele (*ok2385*) that completely deletes this isoform (and truncates the long isoforms) reveals reiterative function(s) for *lin-42* in the molting cycle (Monsalve et al., 2011), and implies that there is some division of labor among the isoforms.

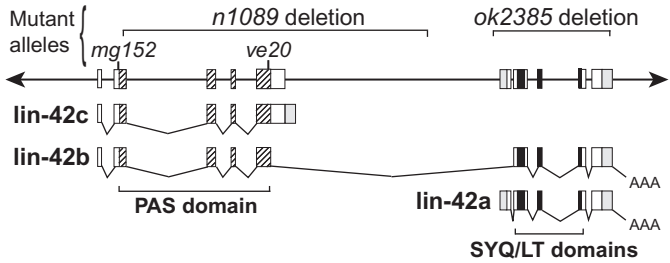

Figure 6.3 Schematic of the *lin-42* locus. The top line indicates the *lin-42* genomic locus with boxes representing exons. The regions encoding the PAS and SYQ/LT domains conserved in fly and mammalian PER proteins are indicated by hatching and black boxes, respectively. Untranslated portions of exons are gray. Positions of lesions in mutant alleles that affect subsets of isoforms are shown above the locus. The first described alleles (e.g., *mg152, n1089, ve20*) did not alter the *lin-42a* transcription unit, which plays a key role in molting regulation and is deleted in *ok2853*. A fourth transcription unit that lacks the last two exons of lin-42b has been omitted for simplicity.

lin-42a mutants differ dramatically from wild-type animals during larval development, exhibiting defects in epidermal cell dynamics and molting at each larval stage, along with the precocious phenotype in the L3 (Monsalve et al., 2011). Rather than developing in near unison as do populations of wild-type animals hatched at the same time, *lin-42a* mutants exhibit severe developmental delays, feed for variable amounts of time prior to an extended lethargus, and molt asynchronously. Animals at each larval stage can become stuck during the molt, failing to complete ecdysis, revealing that LIN-42a is repeatedly required for this process. Moreover, the epidermal seam appears to be the key site of LIN-42 action for regulating these functions. These observations indicate that LIN-42a is required for the rapid synchronous development of wild-type animals and proper execution of molts, leading to the proposal that the rise and fall of the *lin-42a* isoform directs the cyclical entry and exit from each molt, thereby pacing this process. That forced temporal mis-expression of *lin-42a* can induce a lethargus-like state and molting (albeit abnormal) supports this model. This role of *lin-42* in specifying a periodic behavior harkens back again to circadian rhythms, where oscillations in levels of a similar protein in other organisms link behaviors to the 24-h clock.

lin-42a appears to be the elusive factor that integrates successive stage-specific cell lineage programs and epidermal dynamics with the oscillatory molting behaviors, pacing this aspect of postembryonic development. If levels of this LIN-42 isoform are key to the execution and pacing of molts,

then several questions need answers. How are its expression dynamics controlled? What are the targets of LIN-42a activity and do they include *let-7* family miRNAs? And what are contributions of the other isoforms? These issues are presently being intensely studied. Transcription factors required for cyclical *lin-42* expression have not been reported, but nuclear receptors, of which there are many in *C. elegans*, are good candidates and could allow for endocrine control of the process. There is also potential for regulatory interactions among the LIN-42 isoforms. Over-expression of *lin-42a* can rescue the heterochronic defects of all tested mutations in the locus, suggesting that its high dosage can compensate for lack of the other isoforms (Tennessen et al., 2006), perhaps overcoming a missing regulatory or intracellular targeting function. Also curious is the observation that over-expression of the *lin-42c* PAS-domain isoform can antagonize the *lin-42a* mutant phenotype (Monsalve et al., 2011). Although the biochemical roles of each isoform await identification, analogy to Per, together with its nuclear localization, suggests LIN-42 is likely to regulate transcription of downstream genes that control epidermal cell behaviors and integrate them temporally with the molts.

In addition to programming molts, *lin-42* exerts another important influence as it charts an efficient path to reproductive maturity—it mediates organismal responses to environmental conditions. In this capacity, *lin-42* participates in the decision of whether to interrupt the molting cycles of continuous development to enable dauer formation. Here, LIN-42 acts to antagonize the ligand-free form of the nuclear receptor DAF-12 (Tennessen, Opperman, & Rougvie, 2010).

9. REVERSIBLE INTERRUPTION OF DEVELOPMENTAL PROGRESSION: DAF-12

The DAF-12 nuclear receptor plays a unique role in nematode life history regulation by controlling the choice between developing rapidly through each larval stage to reach reproductive maturity versus executing a contingency plan, dauer larva formation, in response to unfavorable growth conditions (Antebi, Culotti, & Hedgecock, 1998; Antebi, Yeh, Tait, Hedgecock, & Riddle, 2000). The ability to interrupt reproductive development for dauer formation intertwines many regulatory pathways. Environmental signals are received and conveyed throughout the animal to interrupt the molting cycle and halt one set of stage-specific programs in order to implement another, while maintaining the ability to coordinately

reverse the process and restart reproductive development from the point where it left off.

DAF-12 is distinctive among worm nuclear receptors in that its ligands, the dafachronics acids (DA) have been identified (Held et al., 2006; Motola et al., 2006) (see also Chapter 7). These cholesterol-derived steroidal ligands bind to DAF-12 during favorable growth conditions and promote reproductive development. In adverse conditions of elevated temperature and/or limited food supply, DA levels are low and ligand-free DAF-12 binds the DIN-1 corepressor and promotes dauer formation (Ludewig et al., 2004). Consequently, DAF-12 acts as a switch to modulate nematode life history. Animals lacking *daf-12* activity altogether are dauer defective and undergo reproductive development regardless of conditions. Developmental timing programs in these animals are largely normal, with only a weakly penetrant reiteration of L2 programs at the L3 stage. However, this retarded phenotype predominates in mutants where DAF-12 is truncated in the ligand binding domain, and it is likely to arise from ligand-free DAF-12 acting with DIN-1 as a constitutive repressor (Antebi et al., 1998, 2000). DAF-12 is thus absolutely required for dauer formation at the L2 molt, but plays a more modulatory role in the heterochronic pathway by helping to control the L2 to L3 transition.

LIN-42 inhibits dauer formation by antagonizing ligand-free DAF-12, thereby promoting continuous development under conditions of mild stress and adding a level of robustness to the dauer decision (Tennessen et al., 2010). At elevated temperatures, *lin-42* mutants constitutively enter the dauer pathway in a DIN-1-dependent manner. Consistent with a role in preventing dauer formation, LIN-42 levels are dramatically reduced in larvae destined to be dauers, and forced expression of LIN-42 in this stage can bypass dauer formation. *lin-42* and *daf-12* also act in opposition during continuous development; *lin-42*; *daf-12* double mutants exhibit mutual suppression of epidermal phenotypes (Tennessen et al., 2006). Together, these genes indirectly sense external stimuli and make the choice between reproduction and an extended juvenile phase.

The mechanism by which DAF-12 coordinates postembryonic developmental progression is coming into focus through the identification of its direct targets. DAF-12 targets appear diverse, as might be expected given its global roles in development and aging, but conspicuously include core heterochronic pathway components, such as *hbl-1*, *lin-28*, *lin-41*, and *lin-42*, as well as the *din-1*, *daf-12*'s partner in dauer formation (Hochbaum et al., 2011). Directed studies are now required to tease apart

the relative contributions of these interactions to DAF-12 functions. Also of key relevance are miRNA targets of DAF-12, notably the *let*-7 family miRNA genes. Ligand bound DAF-12 moderately activates *let*-7 family miRNAs during continuous development, and, for at least *mir-84* and *mir-241*, this regulation appears direct, resulting in promotion of L3 fates through repression of HBL-1 levels (Bethke, Fielenbach, Wang, Mangelsdorf, & Antebi, 2009; Hammell, Karp, & Ambros, 2009). Ligand-free DAF-12, in contrast, is a potent repressor of these miRNAs in dauers, suspending their ability to program stage transitions upon dauer entry. Not only are these miRNA genes targets of DAF-12, but repression of DAF-12 levels during the L3 stage is achieved through feedback targeting of *daf-12* mRNAs by the *let*-7 family miRs, regulation that is necessary for proper developmental progression.

10. INTERRUPTED DEVELOPMENT: REPROGRAMMING AND RESYNCHRONIZATION

An unexpected phenomenon was revealed when heterochronic mutants progressed through the dauer larval stage. Certain heterochronic mutants come out of the dauer stage appearing developmentally normal and are able to lay eggs and mate (Abrahante et al., 2003; Euling & Ambros, 1996; Liu & Ambros, 1991). However, only those mutants whose defects begin early, in the L1 or L2, are subject to this postdauer suppression. Mutants whose developmental defects appear late in development, such as *lin-29* mutants, are not suppressed by going through dauer. But regardless of whether the mutant would have ceased molting precociously after the third stage, or whether it would have continued molting after the fourth stage, all of these suppressed postdauer mutants undergo exactly two molts after emerging from the dauer (Fig. 6.4). This phenomenon implies the existence of a molecular mechanism for setting developmental time that is independent of the early-acting heterochronic genes.

This reprogramming of cell fates upon passage through the dauer is seen more vividly in the hermaphrodite vulva (Euling & Ambros, 1996). Because dauer larvae arrest midway through larval development, precocious vulva development sometimes gets caught in process when the dauer arrest occurs (Fig. 6.4). Remarkably, when the animal emerges from the dauer it develops a perfectly normal vulva, but not in the way that one would expect: it does not simply pick up where it left off. Normally, three of six VPCs divide multiple times to generate the 22 cells of the vulva. In precocious mutants that

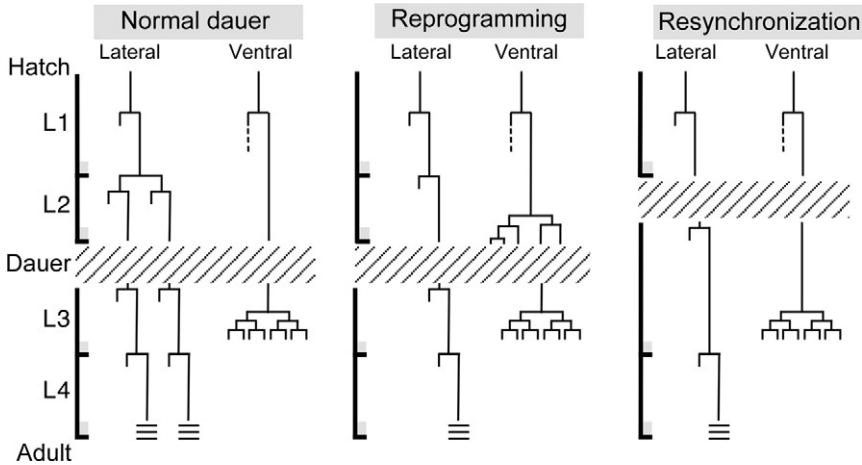

Figure 6.4 Schematic of cell lineage behaviors following dauer development. Examples of lateral and ventral epidermal lineages are shown for wild-type animals and heterochronic mutants that undergo reprogramming or resynchronization. Lineage diagrams are as in Fig. 6.1, with the variable amount of time spent in dauer arrest indicated by hatching. The vulva arises from a subset of the ventral epidermal cells, the vulva precursor cells (VPCs). Vulval reprogramming occurs in precocious mutants where VPCs have divided prior to dauer entry; three VPC progeny (only one is shown for simplicity) will be reprogrammed to divide postdauer, producing the wild-type vulva pattern. Animals that enter dauer too early, at the L1 molt (third panel), undergo a lengthened larval stage upon dauer recovery and tissues resynchronize.

pass through dauer, regardless of how many cells were generated prior to dauer arrest, vulva development is reinitiated, and only three cells divide further to generate a proper vulva. Passage through the dauer stage reprograms the cells so that they revert to their ancestral fates. The only exception is if a VPC divided three times prior to dauer arrest; such cells are incapable of being reprogrammed.

The mechanistic explanation for this reprogramming is incomplete; however, it is known that the expression of certain vulva fate markers and signaling pathways is repressed during the dauer stage, indicating that any commitment prior to arrest is therefore reversible (Karp & Greenwald, 2013). In addition, the important developmental regulator DAF-16/FOXO has a critical role in the VPCs themselves for the maintenance of multipotency (Karp & Greenwald, 2013).

One further variation on this phenomenon is that if certain mutants enter the dauer stage at the wrong time, then they can still emerge from dauer with

"corrected" development. Mutants that alter *lin-14* expression can form dauer larvae either early, at the L1 stage, or late, at the L3 stage (Liu & Ambros, 1989). When these animals emerge from the dauer, they still undergo two additional larval stages; however, despite having undergone a total of three or five larval stages, they can be fully functional, egg-laying competent animals. This is something they would not have achieved if they had developed without arrest. A curiosity is that the postdauer animal seems to synchronize its somatic development, which has undergone one too few or one too many larval stages already, with its gonad development, whose progress is unaffected by the heterochronic genes. Thus, the first postdauer larval stage is sped-up or slowed-down, presumably to get in sync with the gonad. The mechanism of this resynchronization is entirely unknown.

11. LAUNCHING LARVAL DEVELOPMENT

By definition, the postembryonic phase of the worm life cycle begins when the worm hatches from the egg. With this embryo to larva transition, life for the worm changes dramatically; direct exposure to the environment brings with it the ability to forage, feed, and grow. But what launches the larval developmental programs? A growing body of work indicates that postembryonic developmental programs are genetically repressed until appropriate signals are received from the local milieu.

The key trigger is nutritional. Larval progression initiates only in the presence of a food source, typically provided by a lawn of *E. coli* in the lab. When *E. coli* is present, development begins and proceeds apace, in a stereotypic fashion with high temporal synchrony between individuals. Without food, the L1 larva arrests both somatic and germline development, entering a state known as L1 arrest or L1 diapause, and it can survive more than 2 weeks. If a food source becomes available, development resumes synchronously throughout the animal with stage-specific programs appropriately scheduled relative to each other and to the molts, suggesting endocrine involvement. As for postdauer development, what is particularly impressive here is that the same cell division pattern and sequence occurs regardless of the interruption.

Diverse developmental programs are suspended during L1 arrest, and the heterochronic gene pathway is not activated. The *lin-4* miRNA fails to accumulate, and consequently, LIN-14 levels remain high (Arasu et al., 1991; Feinbaum & Ambros, 1999). The *lin-4* response appears transcriptional because this expression pattern is recapitulated by assays of promoter

fusions; the promoter remains inactive unless food is present (Baugh & Sternberg, 2006). Important for understanding how temporal development initiates then is to link nutritional cue(s) supplied by *E. coli* to signaling pathways in the worm that ultimately activate *lin-4* setting off the pathway. Some clues to the mechanism of L1 arrest are beginning to emerge. Notably, components of the insulin/IGF signaling pathway are required for the maintenance of L1 diapause (Baugh & Sternberg, 2006; Fukuyama, Rougvie, & Rothman, 2006; Gems et al., 1998). Animals mutant for DAF-18/PTEN or DAF-16/FOXO initiate postembryonic programs involving cell division, migration, and cell fusion events even under starvation conditions and have reduced survival rates during L1 diapause.

miRNAs help couple nutrient availability to the activation of larval programs. miRNAs have been identified that are required for the maintenance of L1 diapause, survival during diapause, or whose levels correlate with L1 diapause entry and/or exit (Karp, Hammell, Ow, & Ambros, 2011; Kasuga, Fukuyama, Kitazawa, Kontani, & Katada, 2013; Zhang, Zabinsky, Teng, Cui, & Han, 2011). Perhaps most intriguing among these are miR-235 and miR-71. Similar to insulin pathway mutants, miR-235 is required for the arrest of developmental programs during L1 diapause; in its absence, the activation of *lin-4* and *lin-42* is observed, blast cells divide and molting cycles initiate, even when food is lacking (Kasuga et al., 2013). Although much remains to be learned about how activity of this miRNA feeds into heterochronic gene regulation, an insulin pathway mediated reduction in miR-235 levels appears to be an important step in launching larval development.

mir-71 links L1 diapause with postembryonic developmental timing in a surprisingly different way. The loss of *mir-71* has little phenotypic consequence during continuous larval development (Miska et al., 2007) but, unexpectedly, *mir-71* mutants develop with retarded heterochronic defects in the vulva if they have experienced L1 diapause (Zhang et al., 2011). This result suggests that *mir-71* plays a role in ensuring developmental robustness when the worm enters L1 arrest. Further, it predicts that *mir-71* targets mRNAs of proteins whose over-expression would delay the resumption of vulval programs upon feeding; that is, perhaps *mir-71* interfaces with heterochronic gene regulation. Indeed, miR-71 binding sites are predicted in the 3′UTRs of two temporal regulators of vulva development, *lin-42* and *hbl-1*, and these UTRs are *mir-71*-repsonsive during L1 diapause. Given that the loss of *lin-42* or *hbl-1* activity causes precocious defects in vulva formation during continuous development (Abrahante et al., 2003; Tennessen et al., 2006), their

inappropriate elevation could delay vulval cell divisions during recovery from L1 arrest. The influence of nutritional status on *mir-71*, *hbl-1*, and *lin-42* expression in the vulval lineages warrants further investigation.

12. A HETEROCHRONIC PATHWAY FOR LARGER ANIMALS?

A question from the very beginning of the heterochronic gene studies has been: "Is this pathway peculiar to nematodes, or does it recur in other animals?" Of the core pathway components, a few have clear orthologs in animals, including mammals. Others have homologs, but it is too early to say whether those are likely to be performing similar roles.

Mammalian LIN28 has an established role in pluripotency, proliferation, development, and cancer (Shyh-Chang & Daley, 2013). It is expressed in a variety of developing tissues, consistent with a role in timing development on a tissue-by-tissue basis (Yang & Moss, 2003). Its role at the cellular level was explored in neural development where it was shown to have a function remarkably like that in *C. elegans* (Balzer, Heine, Jiang, Lee, & Moss, 2010). Continuous expression of LIN28 caused a kind of retarded development where early fates (neurons) were reiterated at the expense of later fates (glia). It remains to be seen whether all of mammalian LIN28's functions can be traced to defects like those in *C. elegans*, or whether it has diversified its role over evolutionary time.

LIN41 has also been shown to control development in the central nervous system, and mouse LIN41 knockouts display a kind of precocious development in this tissue (Chen, Lai, & Niswander, 2012). LIN41 is also widely expressed and in many of the same tissues as LIN28 (Kanamoto, Terada, Yoshikawa, & Furukawa, 2006; Schulman, Esquela-Kerscher, & Slack, 2005; Yang & Moss, 2003). It is intriguing that these two genes might represent a bit of the heterochronic pathway that has been conserved in mammals. A great deal of work remains to be done to see whether they work together to regulate developmental transitions.

13. CONCLUSIONS AND FUTURE DIRECTIONS

In the interval since Ambros and Horvitz first realized that a few *C. elegans* mutants could be classified as displaying developmental timing defects, much progress has been made toward understanding how temporal

patterning is achieved. It is perhaps fitting that the first two miRNAs were discovered here; miRNAs play pivotal roles in the heterochronic gene pathway, acting as a sequential switches that guide transitions from one larval stage to the next. Indeed, miRNAs now provide a common thread among diverse timing pathways in both plant and animal systems. The key miRNA-target gene associations that guide stage transitions have been identified in the worm, but much remains to be learned about the functions of the protein coding targets. The molecular activities of some remain unresolved and the identities of their direct contacts are largely unknown. In the case of the transcription factors (e.g., LIN-14, HBL-1, DAF-12), direct targets have begun to be identified, but how they time and organize cell division cycles and other events remains unclear. Similarly, with LIN-28 we have a glimpse of one way in which miRNA levels can be timed, but the complete picture of how miRNA levels are controlled in the pathway promises to be more complex with additional transcriptional and posttranscriptional inputs.

The first inkling of how molting cycles are paced and aligned with stage-specific programs has been revealed through the studies of LIN-42; this protein participates in both functions, controlling molt time and execution while also acting to specify epidermal lineage patterns. How oscillatory levels of its various isoforms are programmed and combined to achieve these regulatory outputs remains to be learned, and the identification of LIN-42's activity, targets, and interaction partners are prime goals for future studies.

Layered on top of the core heterochronic gene pathway that times larval development and paces the molts are external inputs that launch and can alter life history paths. How environmental conditions are transduced to coordinately initiate, and sometimes interrupt and subsequently restart, developmental programs are great problems to pursue. Linking these factors to environmental cues are key next steps. How does feeding control *mir-235* levels and how do they ultimately modulate the heterochronic pathway? How does the external environment modulate DAF-12 ligand levels to allow interruption of larval progression? Ultimately, these combined avenues of study will advance our understanding of the intimate connections between environmental cues and the mechanisms that temporally pattern orchestrate developmental transitions.

ACKNOWLEDGMENTS

The authors thank Kate McCulloch for helpful comments on the manuscript. We apologize to our colleagues whose work could not be cited due to space constraints.

REFERENCES

Abbott, A. L., Alvarez-Saavedra, E., Miska, E. A., Lau, N. C., Bartel, D. P., & Horvitz, H. R. (2005). The *let-7* microRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. *Developmental Cell*, *9*(3), 403–414.

Abrahante, J. E., Daul, A. L., Li, M., Volk, M. L., Tennessen, J. M., & Miller, E. A. (2003). The *Caenorhabditis elegans hunchback*-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. *Developmental Cell*, *4*(5), 625–637.

Abrahante, J. E., Miller, E. A., & Rougvie, A. E. (1998). Identification of heterochronic mutants in *Caenorhabditis elegans*: Temporal misexpression of a collagen::green fluorescent protein fusion gene. *Genetics*, *149*(3), 1335–1351.

Aguinaldo, A. M., Turbeville, J. M., Linford, L. S., Rivera, M. C., Garey, J. R., & Raff, R. A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature*, *387*(6632), 489–493. http://dx.doi.org/10.1038/387489a0.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in *C. elegans*. *Cell*, *57*(1), 49–57.

Ambros, V. (2011). MicroRNAs and developmental timing. *Current Opinion in Genetics and Development*, *21*(4), 511–517. http://dx.doi.org/10.1016/j.gde.2011.04.003.

Ambros, V., & Horvitz, H. R. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science*, *226*(4673), 409–416.

Ambros, V., & Horvitz, H. R. (1987). The *lin-14* locus of *Caenorhabditis elegans* controls the time of expression of specific postembryonic developmental events. *Genes & Development*, *1*(4), 398–414.

Antebi, A., Culotti, J. G., & Hedgecock, E. M. (1998). *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. *Development*, *125*(7), 1191–1205.

Antebi, A., Yeh, W. H., Tait, D., Hedgecock, E. M., & Riddle, D. L. (2000). *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes & Development*, *14*(12), 1512–1527.

Arasu, P. A., Wightman, B., & Ruvkun, G. (1991). Temporal regulation of *lin-14* by the antagonistic action of two other heterochronic genes, *lin-4* and *lin-28*. *Genes & Development*, *5*, 1825–1833.

Asahina, M., Ishihara, T., Jindra, M., Kohara, Y., Katsura, I., & Hirose, S. (2000). The conserved nuclear receptor Ftz-F1 is required for embryogenesis, moulting and reproduction in *Caenorhabditis elegans*. *Genes to Cells*, *5*(9), 711–723.

Balzer, E., Heine, C., Jiang, Q., Lee, V. M., & Moss, E. G. (2010). LIN28 alters cell fate succession and acts independently of the let-7 microRNA during neurogliogenesis in vitro. *Development*, *137*(6), 891–900. http://dx.doi.org/10.1242/dev.042895.

Baugh, L. R., & Sternberg, P. W. (2006). DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of lin-4 during *C. elegans* L1 arrest. *Current Biology*, *16*(8), 780–785.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D. J., & Antebi, A. (2009). Nuclear hormone receptor regulation of microRNAs controls developmental progression. *Science*, *324*(5923), 95–98. http://dx.doi.org/10.1126/science.1164899.

Chang, D. C., & Reppert, S. M. (2003). A novel C-terminal domain of *Drosophila* PERIOD inhibits dCLOCK:CYCLE-mediated transcription. *Current Biology*, *13*(9), 758–762.

Chen, Z., Eastburn, D. J., & Han, M. (2004). The *Caenorhabditis elegans* nuclear receptor gene nhr-25 regulates epidermal cell development. *Molecular and Cellular Biology*, *24*(17), 7345–7358. http://dx.doi.org/10.1128/MCB.24.17.7345-7358.2004.

Chen, J., Lai, F., & Niswander, L. (2012). The ubiquitin ligase mLin41 temporally promotes neural progenitor cell maintenance through FGF signaling. *Genes & Development*, *26*(8), 803–815. http://dx.doi.org/10.1101/gad.187641.112.

Chitwood, D. J., & Feldlaufer, M. F. (1990). Ecdysteroids in axenically propagated *Caenorhabditis elegans* and culture medium. *Journal of Nematology*, *22*(4), 598–607.

Del Rio-Albrechtsen, T., Kiontke, K., Chiou, S. Y., & Fitch, D. H. (2006). Novel gain-of-function alleles demonstrate a role for the heterochronic gene lin-41 in *C. elegans* male tail tip morphogenesis. *Developmental Biology*, *297*(1), 74–86.

Euling, S., & Ambros, V. (1996). Reversal of cell fate determination in *Caenorhabditis elegans* vulval development. *Development*, *122*, 2507–2515.

Feinbaum, R., & Ambros, V. (1999). The timing of *lin-4* RNA accumulation controls the timing of postembryonic developmental events in *Caenorhabditis elegans*. *Developmental Biology*, *210*(1), 87–95.

Ferguson, E. L., & Horvitz, H. R. (1985). Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode *Caenorhabditis elegans*. *Genetics*, *110*(1), 17–72.

Frand, A. R., Russel, S., & Ruvkun, G. (2005). Functional genomic analysis of *C. elegans* molting. *PLoS Biology*, *3*(10), e312.

Fukuyama, M., Rougvie, A. E., & Rothman, J. H. (2006). *C. elegans* DAF-18/PTEN mediates nutrient-dependent arrest of cell cycle and growth in the germline. *Current Biology*, *16*(8), 773–779.

Gems, D., Sutton, A. J., Sundermeyer, M. L., Albert, P. S., King, K. V., & Edgley, M. L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. *Genetics*, *150*(1), 129–155.

Gissendanner, C. R., Crossgrove, K., Kraus, K. A., Maina, C. V., & Sluder, A. E. (2004). Expression and function of conserved nuclear receptor genes in *Caenorhabditis elegans*. *Developmental Biology*, *266*(2), 399–416.

Gissendanner, C. R., & Sluder, A. E. (2000). nhr-25, the *Caenorhabditis elegans* ortholog of ftz-f1, is required for epidermal and somatic gonad development. *Developmental Biology*, *221*(1), 259–272. http://dx.doi.org/10.1006/dbio.2000.9679.

Hammell, C. M., Karp, X., & Ambros, V. (2009). A feedback circuit involving let-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18668–18673. http://dx.doi.org/10.1073/pnas.0908131106.

Hardin, P. E., Hall, J. C., & Rosbash, M. (1990). Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. *Nature*, *343*, 536–540.

Hasegawa, K., Saigusa, T., & Tamai, Y. (2005). *Caenorhabditis elegans* opens up new insights into circadian clock mechanisms. *Chronobiology International*, *22*(1), 1–19.

Hayes, G. D., Frand, A. R., & Ruvkun, G. (2006). The mir-84 and let-7 paralogous microRNA genes of *Caenorhabditis elegans* direct the cessation of molting via the conserved nuclear hormone receptors NHR-23 and NHR-25. *Development*, *133*(23), 4631–4641. http://dx.doi.org/10.1242/dev.02655.

Held, J. M., White, M. P., Fisher, A. L., Gibson, B. W., Lithgow, G. J., & Gill, M. S. (2006). DAF-12-dependent rescue of dauer formation in *Caenorhabditis elegans* by (25S)-cholestenoic acid. *Aging Cell*, *5*(4), 283–291.

Heo, I., Joo, C., Cho, J., Ha, M., Han, J., & Kim, V. N. (2008). Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Molecular Cell*, *32*(2), 276–284. http://dx.doi.org/10.1016/j.molcel.2008.09.014.

Hochbaum, D., Zhang, Y., Stuckenholz, C., Labhart, P., Alexiadis, V., & Martin, R. (2011). DAF-12 regulates a connected network of genes to ensure robust developmental decisions. *PLoS Genetics*, 7(7), e1002179. http://dx.doi.org/10.1371/journal.pgen.1002179.

Hornsten, A., Lieberthal, J., Fadia, S., Malins, R., Ha, L., & Xu, X. (2007). APL-1, a *Caenorhabditis elegans* protein related to the human beta-amyloid precursor protein, is essential for viability. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(6), 1971–1976. http://dx.doi.org/10.1073/pnas.0603997104.

Jeon, M., Gardner, H. F., Miller, E. A., Deshler, J., & Rougvie, A. E. (1999). Similarity of the *C. elegans* developmental timing protein LIN-42 to circadian rhythm proteins. *Science*, *286*(5442), 1141–1146.

Kanamoto, T., Terada, K., Yoshikawa, H., & Furukawa, T. (2006). Cloning and regulation of the vertebrate homologue of lin-41 that functions as a heterochronic gene in *Caenorhabditis elegans*. *Developmental Dynamics*, *235*(4), 1142–1149. http://dx.doi.org/10.1002/dvdy.20712.

Kang, Y. L., Yochem, J., Bell, L., Sorensen, E. B., Chen, L., & Conner, S. D. (2013). *Caenorhabditis elegans* reveals a FxNPxY-independent low-density lipoprotein receptor internalization mechanism mediated by epsin1. *Molecular Biology of the Cell*, *24*(3), 308–318. http://dx.doi.org/10.1091/mbc.E12-02-0163.

Karp, X., & Greenwald, I. (2013). Control of cell-fate plasticity and maintenance of multipotency by DAF-16/FoxO in quiescent *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(6), 2181–2186. http://dx.doi.org/10.1073/pnas.1222377110.

Karp, X., Hammell, M., Ow, M. C., & Ambros, V. (2011). Effect of life history on microRNA expression during *C. elegans* development. *RNA*, *17*(4), 639–651. http://dx.doi.org/10.1261/rna.2310111.

Kasuga, H., Fukuyama, M., Kitazawa, A., Kontani, K., & Katada, T. (2013). The microRNA miR-235 couples blast-cell quiescence to the nutritional state. *Nature*, *497*, 503–506. http://dx.doi.org/10.1038/nature12117.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P., & Hogness, D. S. (1991). The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell*, *67*(1), 59–77. http://dx.doi.org/10.1016/0092-8674(91)90572-G.

Kostrouchova, M., Krause, M., Kostrouch, Z., & Rall, J. E. (1998). CHR3: A *Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. *Development*, *125*(9), 1617–1626.

Kostrouchova, M., Krause, M., Kostrouch, Z., & Rall, J. E. (2001). Nuclear hormone receptor CHR3 is a critical regulator of all four larval molts of the nematode *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(13), 7360–7365. http://dx.doi.org/10.1073/pnas.131171898.

Kouns, N. A., Nakielna, J., Behensky, F., Krause, M. W., Kostrouch, Z., & Kostrouchova, M. (2011). NHR-23 dependent collagen and hedgehog-related genes required for molting. *Biochemical and Biophysical Research Communications*, *413*(4), 515–520. http://dx.doi.org/10.1016/j.bbrc.2011.08.124.

Lee, R. C., Feinbaum, R. L., & Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, *75*, 843–854.

Lehrbach, N. J., Armisen, J., Lightfoot, H. L., Murfitt, K. J., Bugaut, A., & Balasubramanian, S. (2009). LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in *Caenorhabditis elegans*. *Nature Structural and Molecular Biology*, *16*(10), 1016–1020. http://dx.doi.org/10.1038/nsmb.1675.

Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., & Gamberi, C. (2003). The *C. elegans hunchback* homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. *Developmental Cell*, *4*(5), 639–650.

Liu, Z., & Ambros, V. (1989). Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in *Caenorhabditis elegans*. *Genes & Development*, *3*(12B), 2039–2049.

Liu, Z., & Ambros, V. (1991). Alternative temporal control systems for hypodermal cell differentiation in *Caenorhabditis elegans*. *Nature*, *350*, 162–165.

Loedige, I., Gaidatzis, D., Sack, R., Meister, G., & Filipowicz, W. (2012). The mammalian TRIM-NHL protein TRIM71/LIN-41 is a repressor of mRNA function. *Nucleic Acids Research*, *41*(1), 518–532. http://dx.doi.org/10.1093/nar/gks1032.

Ludewig, A. H., Kober-Eisermann, C., Weitzel, C., Bethke, A., Neubert, K., & Gerisch, B. (2004). A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging. *Genes & Development*, *18*(17), 2120–2133.

Migliori, M. L., Simonetta, S. H., Romanowski, A., & Golombek, D. A. (2011). Circadian rhythms in metabolic variables in *Caenorhabditis elegans*. *Physiology and Behavior*, *103*(3–4), 315–320. http://dx.doi.org/10.1016/j.physbeh.2011.01.026.

Miska, E. A., Alvarez-Saavedra, E., Abbott, A. L., Lau, N. C., Hellman, A. B., & McGonagle, S. M. (2007). Most *Caenorhabditis elegans* microRNAs are individually not essential for development or viability. *PLoS Genetics*, *3*(12), e215. http://dx.doi.org/10.1371/journal.pgen.0030215.

Monsalve, G. C., Van Buskirk, C., & Frand, A. R. (2011). LIN-42/PERIOD controls cyclical and developmental progression of *C. elegans* molts. *Current Biology*, *21*(24), 2033–2045. http://dx.doi.org/10.1016/j.cub.2011.10.054.

Moss, E. G., Lee, R. C., & Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in *C. elegans* and is regulated by the *lin-4* RNA. *Cell*, *88*, 637–646.

Motola, D. L., Cummins, C. L., Rottiers, V., Sharma, K. K., Li, T., & Li, Y. (2006). Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. *Cell*, *124*(6), 1209–1223.

Niwa, R., & Hada, K. (2010). Identification of a spatio-temporal enhancer element for the Alzheimer's amyloid precursor protein-like-1 gene in the nematode *Caenorhabditis elegans*. *Bioscience, Biotechnology, and Biochemistry*, *74*(12), 2497–2500. http://dx.doi.org/10.1271/bbb.100450.

Niwa, R., Zhou, F., Li, C., & Slack, F. J. (2008). The expression of the Alzheimer's amyloid precursor protein-like gene is regulated by developmental timing microRNAs and their targets in *Caenorhabditis elegans*. *Developmental Biology*, *315*(2), 418–425. http://dx.doi.org/10.1016/j.ydbio.2007.12.044.

Olmedo, M., O'Neill, J. S., Edgar, R. S., Valekunja, U. K., Reddy, A. B., & Merrow, M. (2012). Circadian regulation of olfaction and an evolutionarily conserved, nontranscriptional marker in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(50), 20479–20484. http://dx.doi.org/10.1073/pnas.1211705109.

Pepper, A. S., McCane, J. E., Kemper, K., Yeung, D. A., Lee, R. C., & Ambros, V. (2004). The *C. elegans* heterochronic gene *lin-46* affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin. *Development*, *131*(9), 2049–2059.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., & Rougvie, A. E. (2000). The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*, *403*(6772), 901–906.

Ren, H., & Zhang, H. (2010). Wnt signaling controls temporal identities of seam cells in *Caenorhabditis elegans*. *Developmental Biology*, *345*(2), 144–155. http://dx.doi.org/10.1016/j.ydbio.2010.07.002.

Rougvie, A. E., & Ambros, V. (1995). The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *C. elegans*. *Development*, *121*, 2491–2500.

Ruvkun, G., & Giusto, J. (1989). The *Caenorhabditis elegans* heterochronic gene *lin-14* encodes a nuclear protein that forms a temporal developmental switch. *Nature*, *338*(6213), 313–319.

Schulman, B. R., Esquela-Kerscher, A., & Slack, F. J. (2005). Reciprocal expression of lin-41 and the microRNAs let-7 and mir-125 during mouse embryogenesis. *Developmental Dynamics*, *234*(4), 1046–1054. http://dx.doi.org/10.1002/dvdy.20599.

Seggerson, K., Tang, L., & Moss, E. G. (2002). Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene *lin-28* after translation initiation. *Developmental Biology*, *243*(2), 215–225.

Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, L. F., Jr., & Reppert, S. M. (1997). Two period homologs: Circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron*, *19*(6), 1261–1269.

Shyh-Chang, N., & Daley, G. Q. (2013). Lin28: Primal regulator of growth and metabolism in stem cells. *Cell Stem Cell*, *12*(4), 395–406. http://dx.doi.org/10.1016/j.stem.2013.03.005.

Silhankova, M., Jindra, M., & Asahina, M. (2005). Nuclear receptor NHR-25 is required for cell-shape dynamics during epidermal differentiation in *Caenorhabditis elegans*. *Journal of Cell Science*, *118*(Pt 1), 223–232. http://dx.doi.org/10.1242/jcs.01609.

Simonetta, S. H., Migliori, M. L., Romanowski, A., & Golombek, D. A. (2009). Timing of locomotor activity circadian rhythms in *Caenorhabditis elegans*. *PLoS One*, *4*(10), e7571. http://dx.doi.org/10.1371/journal.pone.0007571.

Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R., & Ruvkun, G. (2000). The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. *Molecular Cell*, *5*(4), 659–669.

Sluder, A. E., Mathews, S. W., Hough, D., Yin, V. P., & Maina, C. V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. *Genome Research*, *9*(2), 103–120.

Sun, Z. S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., & Lee, C. C. (1997). *RIGUI*, a putative mammalian ortholog of the *Drosophila period* gene. *Cell*, *90*, 1003–1011.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., & Hirose, M. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila period* gene. *Nature*, *389*, 512–516.

Temmerman, L., Meelkop, E., Janssen, T., Bogaerts, A., Lindemans, M., & Husson, S. J. (2011). *C. elegans* homologs of insect clock proteins: A tale of many stories. *Annals of the New York Academy of Sciences*, *1220*, 137–148. http://dx.doi.org/10.1111/j.1749-6632.2010.05927.x.

Tennessen, J. M., Gardner, H. F., Volk, M. L., & Rougvie, A. E. (2006). Novel heterochronic functions of the *Caenorhabditis elegans* period-related protein LIN-42. *Developmental Biology*, *289*(1), 30–43.

Tennessen, J. M., Opperman, K. J., & Rougvie, A. E. (2010). The *C. elegans* developmental timing protein LIN-42 regulates diapause in response to environmental cues. *Development*, *137*(20), 3501–3511. http://dx.doi.org/10.1242/dev.048850.

Thummel, C. S. (2001). Molecular mechanisms of developmental timing in *C. elegans* and *Drosophila*. *Developmental Cell*, *1*(4), 453–465.

Vadla, B., Kemper, K., Alaimo, J., Heine, C., & Moss, E. G. (2012). lin-28 controls the succession of cell fate choices via two distinct activities. *PLoS Genetics*, *8*(3), e1002588. http://dx.doi.org/10.1371/journal.pgen.1002588.

van der Linden, A. M., Beverly, M., Kadener, S., Rodriguez, J., Wasserman, S., & Rosbash, M. (2010). Genome-wide analysis of light- and temperature-entrained circadian transcripts in *Caenorhabditis elegans*. *PLoS Biology*, *8*(10), e1000503. http://dx.doi.org/10.1371/journal.pbio.1000503.

Van Wynsberghe, P. M., Kai, Z. S., Massirer, K. B., Burton, V. H., Yeo, G. W., & Pasquinelli, A. E. (2011). LIN-28 co-transcriptionally binds primary let-7 to regulate miRNA maturation in *Caenorhabditis elegans*. *Nature Structural and Molecular Biology*, *18*, 302–308. http://dx.doi.org/10.1038/nsmb.1986.

Viswanathan, S. R., Daley, G. Q., & Gregory, R. I. (2008). Selective blockade of microRNA processing by Lin28. *Science*, *320*(5872), 97–100. http://dx.doi.org/10.1126/science.1154040.

Wightman, B., Burglin, T. R., Gatto, J., Arasu, P., & Ruvkun, G. (1991). Negative regulatory sequences in the *lin-14* 3′-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes & Development*, *5*, 1813–1824.

Wightman, B., Ha, I., & Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*, *75*, 855–862.

Yang, D. H., & Moss, E. G. (2003). Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. *Gene Expression Patterns*, *3*(6), 719–726.

Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., & McKeown, M. (1993). Functional ecdysone receptor is the product of EcR and Ultraspiracle genes. *Nature*, *366*(6454), 476–479. http://dx.doi.org/10.1038/366476a0.

Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M., & Evans, R. M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell*, *71*(1), 63–72. http://dx.doi.org/10.1016/0092-8674(92)90266-F.

Yochem, J., Tuck, S., Greenwald, I., & Han, M. (1999). A gp330/megalin-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. *Development*, *126*(3), 597–606.

Zhang, X., Zabinsky, R., Teng, Y., Cui, M., & Han, M. (2011). microRNAs play critical roles in the survival and recovery of *Caenorhabditis elegans* from starvation-induced L1 diapause. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(44), 17997–18002. http://dx.doi.org/10.1073/pnas.1105982108.