

A feedback circuit involving let-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*

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Animal development is remarkably robust; cell fates are specified with spatial and temporal precision despite physiological and environmental contingencies. Favorable conditions cause *Caenorhabditis elegans* to develop rapidly through four larval stages (L1–L4) to the reproductive adult. In unfavorable conditions, L2 larvae can enter the developmentally quiescent, stress-resistant dauer larva stage, enabling them to survive for prolonged periods before completing development. A specific progression of cell division and differentiation events occurs with fidelity during the larval stages, regardless of whether an animal undergoes continuous or dauer-interrupted development. The temporal patterning of developmental events is controlled by the heterochronic genes, whose products include microRNAs (miRNAs) and regulatory proteins. One of these proteins, the DAF-12 nuclear hormone receptor, modulates the transcription of certain let-7-family miRNAs, and also mediates the choice between the continuous vs. dauer-interrupted life history. Here, we report a complex feedback loop between DAF-12 and the let-7-family miRNAs involving both the repression of DAF-12 by let-7-family miRNAs and the ligand-modulated transcriptional activation and repression of the let-7-Fam miRNAs by DAF-12. We propose that this feedback loop functions to ensure robustness of cell fate decisions and to coordinate cell fate with developmental arrest.

gene regulation | microRNA | nuclear hormone receptor

The complexity of animal development requires the coordination of temporal, spatial, and physiological cues. Successful development is necessary to achieve optimal fitness, and natural selection has favored developmental programs that robustly produce a desired outcome in a range of physiological and environmental conditions. Forward genetics has revealed much of the internal programming of several developmental processes in standardized laboratory conditions. A fundamental question emerging from these studies is how these genetic circuits are affected by conditions that are more similar to natural, variable environments. *Caenorhabditis elegans* has been an excellent system to uncover developmental mechanisms because of its well-defined cell lineage and tractability to genetic analysis. *C. elegans* development is robust in a wide range of environmental conditions, providing an excellent opportunity to determine how genetic pathways are modulated in these diverse environments (1).

There are two distinct life histories a *C. elegans* larva may follow: a rapid, continuous life history that occurs in favorable environments and an extended, interrupted life history (2) that occurs in response to increased population density, decreasing food supplies, and elevated temperature (Fig. 1) (3). Within the continuous life history, animals progress through four larval stages (L1–L4) to a reproductively competent adult within ≈ 40 –50 h after hatching (4). By contrast, unfavorable environments sensed during L1 stage cause larvae to enter the predauer L2d stage, which is $\approx 50\%$ longer than the normal L2 (Fig. 1) (2). L2d stage larvae assess their environment while delaying the expression of L3 cell fates. By the end of the L2d stage, the larva commits to one of two developmental programs: (i) expressing L3 cell fates and resuming contin-

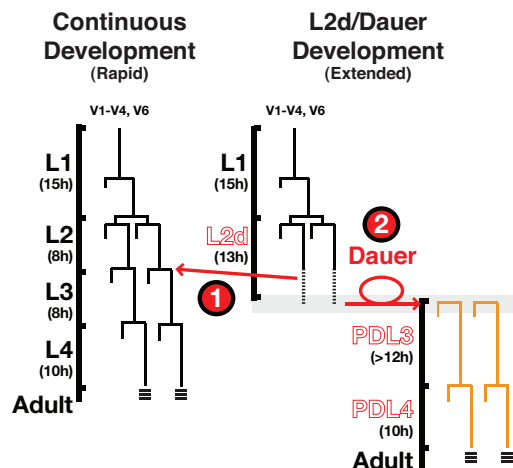


Fig. 1. Temporal development can be modulated by environmental conditions. Wild-type seam cell lineage diagrams for both continuous (Left) and dauer (Right) life histories. In harsh environments, wild-type animals extend the chronological interval between the L1 and L2 molts (L2d). During the L2d, animals enter a critical period in which one of two life histories must be chosen to (1) resume continuous development or (2) arrest as a dauer larva. Once committed to dauer program, wild-type animals can remain in this stress-resistant form for hours to months (2). When food supplies return to favorable conditions, dauer animals will continue development to a reproductively competent adult.

uous development or (ii) entering the developmentally arrested, stress-resistant dauer larva diapause enabling animals to survive for extended periods (5).

Regardless of whether *C. elegans* larvae develop continuously or interrupt development with the dauer diapause, they execute the same specific temporal pattern of cell fates, as exemplified by the lateral hypodermal stem cell (seam cell) lineages (Fig. 1) (6). Each seam cell executes a single asymmetric self-renewing division at each larval stage, with one additional, symmetric division inserted specifically in the beginning of the L2 (or L2d) stage (4). The heterochronic genes regulate the temporal patterning of these seam cell division programs. Mutations in heterochronic genes that specify the L2 cell fates cause a precocious phenotype, where the L2-specific symmetric seam cell division is skipped. By contrast, mutations in genes that promote L3 cell fates cause a retarded phenotype, where the L2-specific symmetric division is reiterated at later larval stages (7). Key to the proper specification of L2 and L3

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seam cell fates is a progressive developmental down regulation of the transcription factor Hunchback-Like-1 (HBL-1) (7). *hbl-1* gene expression is modulated by several regulatory inputs, including the let-7-family miRNAs miR-48, miR-241, and miR-84 (“let-7-Fam miRNAs”). These miRNAs are transcriptionally activated in L2 larvae and are redundantly required to down-regulate *hbl-1* via its 3' UTR (8).

The fact that the specification of L2 and L3 seam cell fates occurs properly regardless of whether the L3 follows rapidly after the L2 (in continuous development) or whether the L3 is delayed indefinitely (by dauer arrest), implies that the regulation of heterochronic genes must be integrated with the genetic programming of dauer larva formation. First, L3 seam cell fates must be postponed during the extended L2d stage. Second, at the end of the L2d stage, seam cells must commit to one of two distinct choices: either expression of L3 fates and the rapid completion of larval development or indefinite postponement of L3 fates in the dauer larva (Fig. 1). This suggests that L2 larvae undergo a complex decision-making process that incorporates an assessment of environmental conditions into the programming of cell fates. Little is currently understood about how genes that regulate dauer formation are integrated with heterochronic gene activity to coordinate this complex decision.

The decision to enter dauer diapause or to develop continuously centers on the activity of the DAF-12 nuclear hormone receptor (9). Direct binding of an endogenous steroid ligand, dafachronic acid (DA), modulates the activity of DAF-12 protein (10). Synthesis of DA ligand is coupled to environmental conditions (5), and DA regulates DAF-12 activity by binding and displacing a corepressor DIN-1S (10, 11). In favorable culture conditions, DA ligand is abundant, and liganded DAF-12 activates transcription. In harsh environments, DA production is dramatically reduced, so most DAF-12 is unliganded and hence free to bind the corepressor DIN-1S (10, 11). The DAF-12:DIN-1 repressive complex is required for dauer formation.

Recently an additional role for DAF-12 has been described whereby DAF-12 directly affects the transcription of several let-7-Fam miRNAs, either positively (as liganded DAF-12) or negatively (as unliganded DAF-12:DIN-1) (12). Importantly, this activity of DAF-12 in let-7-Fam transcriptional regulation appears to be modulatory; null mutations of *daf-12* (*daf-12(0)*) do not display overt heterochronic phenotypes (9, 12, 13), so the temporal activation of these miRNAs occurs independently of DAF-12. However, certain *daf-12* mutations that affect ligand binding have severe heterochronic phenotypes, indicating that DAF-12 has a strong potential activity in the heterochronic pathway that is normally held in check by the DA ligand (9, 13). These observations suggest a complex role in developmental timing for hormone modulated transcriptional regulation by DAF-12.

In this article, we provide evidence for a gene regulatory circuit linking hypodermal seam cell fate specification and environmental signals controlling dauer larva arrest. The essence of this circuit is a complex feedback relationship between DAF-12 and the let-7-Fam miRNAs, wherein DAF-12 regulates let-7-Fam miRNA expression and the let-7-Fam miRNAs temporally down-regulate DAF-12. We show that the DAF-12 component of the pathway modulates the normal temporal transcriptional up-regulation of the let-7-Fam of miRNAs. Specifically, liganded DAF-12 accelerates let-7-Fam miRNA accumulation when environmental conditions favor developmental progression. In contrast, unliganded DAF-12 inhibits let-7-Fam-triggered cell fate transitions when environmental conditions favor developmental arrest. Moreover, we show that let-7-Fam miRNAs feed back and regulate DAF-12 expression, so that the accumulation of let-7-Fam miRNAs governs the sensitivity of the animal to the environmental cues that control DAF-12-mediated developmental arrest.

Results

Unliganded DAF-12 Exerts *din-1*-Dependent Inhibition of let-7-Family miRNA Expression. DAF-12 can directly regulate the transcription of let-7-Fam miRNAs either positively or negatively, depending on the presence of DA ligand (12). However, *daf-12(0)* mutants develop almost normally (9), suggesting that the role for DAF-12 in developmental timing is modulatory rather than essential. We reasoned that the *daf-12(rh61)* mutation, encoding a DAF-12 protein lacking the ligand-binding domain (11, 13), would result in DAF-12 acting as a constitutive repressor of let-7-Fam miRNA expression. Indeed *daf-12(rh61)* animals exhibit developmental timing defects similar to those of *let-7-Fam* compound mutants (8, 9). To test the hypothesis that the activity of *rh61* mutant DAF-12 results in repression of let-7-Fam miRNAs, we assayed the expression of 107 *C. elegans* miRNAs by using real-time-PCR-based TaqMan (ABI) assays on RNA samples extracted from synchronized populations of wild-type or *daf-12(rh61)* mutant larvae at the L2-molt/L3 stage. We found that *daf-12(rh61)* mutant larvae display a dramatic reduction in several let-7-Fam miRNAs (12- to 30-fold reduction in miR-795, miR-48, miR-241, and let-7, $P < 0.01$) (Fig. S1 and Table 1). miR-84 levels were reduced in only one biological replicate and did not qualify as statistically significant in our analysis (see *SI Text*). This is consistent with the more complex regulation of *mir-84* by DAF-12 previously described (12). Of the remaining two let-7-Fam miRNAs, miR-794 was not detectably expressed in any of our samples, and miR-793 did not appear to be affected by *daf-12(rh61)* (Fig. S1, Table 1).

Levels of the *lin-4* family and most other miRNAs were unchanged in mutant extracts compared with wild type. Indeed, levels of only two miRNAs that are not part of the *let-7-Fam* were significantly changed in mutant larvae, suggesting that *daf-12(rh61)* specifically affects the abundance of certain let-7-Fam miRNAs (Table 1, Fig. S1). We conclude that unliganded DAF-12 represses the expression of a specific subset of *C. elegans* miRNAs, including four of the seven let-7-Fam miRNAs, plus two additional, non-let-7-Fam miRNAs.

Although the mutant DAF-12(*rh61*) protein lacks the capacity to bind DA, it retains its ability to bind its corepressor DIN-1S (10), and hence is apparently “locked” in a repressive form. If this constitutively repressive form of DAF-12 is responsible for the strong repression of let-7-Fam miRNAs in *daf-12(rh61)* larvae, relieving this repression should increase the abundance of let-7-Fam miRNAs. We therefore compared the relative expression levels of miRNAs in *daf-12(rh61)* animals to those in animals carrying *daf-12(rh61)* with suppressor mutations that alleviate the severe heterochronic defects of *daf-12(rh61)* (9, 11). First, removal of the DAF-12 corepressor DIN-1S [*din-1(dh181)*; *daf-12(rh61)* animals] partially restored expression of most let-7-Fam miRNAs (Table 1). Furthermore, eliminating the expression of *daf-12(rh61)* via an intragenic null mutation [*daf-12(rh411rh61)*] also resulted in the derepression of the let-7-Fam miRNAs (Table 1) to an extent very similar to that observed in *din-1(dh181)*; *daf-12(rh61)* mutants. The observation that levels of certain let-7-Fam miRNAs are moderately decreased in these suppressor strains indicates that DAF-12 can also positively regulate the expression of let-7-Fam miRNAs, consistent with the previously described role for liganded DAF-12 in activating let-7-Fam miRNA transcription (12). These findings demonstrate that unliganded DAF-12 acts together with its corepressor DIN-1 to inhibit the expression of certain let-7-Fam miRNAs. This suggests that in wild-type development under harsh environmental conditions (low DA concentrations), DAF-12 would attenuate the accumulation of let-7-Fam miRNAs and, hence, retard the down-regulation of let-7-Fam targets, such as *hbl-1*.

DAF-12 Affects Developmental Timing Upstream of *hbl-1* Expression. Three let-7-Fam miRNAs (miR-48, miR-241, and miR-84) redundantly down regulate *hbl-1* to allow progression from L2 to L3 cell

Table 1. Levels of let-7-Fam miRNAs are specifically reduced in *daf-12* mutants or in crowded environments

miRNA	Fold change wild type versus mutant			Sparse vs. crowded
	<i>daf-12(rh61)</i>	<i>daf-12(rh61rh411)</i>	<i>din-1; daf-12(rh61)</i>	Wild type
<i>let-7</i> family				
<i>mir-48</i>	-16.0 ± 4.2	-3.2 ± 0.5	-3.5 ± 0.8	-3.2 ± 1.3
<i>mir-241</i>	-17.8 ± 4.7	-4.3 ± 0.7	-4.5 ± 1.1	-9.4 ± 3.8
<i>mir-84</i>	-2.7 ± 0.7 [†]	-2.8 ± 0.4 [†]	-1.7 ± 0.6	-2.6 ± 0.7
<i>let-7</i>	-12.2 ± 5.5	-12.3 ± 2.5	-6.0 ± 1.6	off*
<i>mir-793</i>	+0.3 ± 0.2	-0.1 ± 0.3	+8.5 ± 4.3 [†]	+0.2 ± 0.3
<i>mir-794</i>	Off*	Off*	Off*	Off*
<i>mir-795</i>	-31.0 ± 17.8	-5.0 ± 1.2[†]	-3.8 ± 1.0[†]	-11.7 ± 5.2
<i>lin-4</i> family				
<i>lin-4</i>	+0.7 ± 0.2	+0.1 ± 0.1	-0.03 ± 0.3	+0.3 ± 0.2
<i>mir-237</i>	-1.2 ± 0.4	-0.1 ± 0.2	-0.75 ± 0.2	-0.7 ± 0.4
Other miRNAs that were significantly reduced [‡]				
<i>mir-230</i>	-3.4 ± 1.5	-1.3 ± 0.2	-1.1 ± 0.2	-0.1 ± 0.2
<i>mir-85</i>	-18.7 ± 5.9	-2.1 ± 0.6	-11.0 ± 2.7	-1.3 ± 0.7
<i>mir-788</i>	-1.7 ± 0.5	-1.7 ± 0.3	-0.3 ± 0.2	2.4 ± 0.7

Values in boldface indicate miRNA significantly changed, $P < 0.01$. Negative values indicate miRNA decreased in *daf-12* relative to wild type, or in crowded relative to sparse conditions. Values close to 0 indicate miRNA was unchanged. Data are displayed as (mean fold-change) ± (mean estimated noise). For details see *Materials and Methods* in *SI Text*.

*Levels were beneath the limit for detection.

[†]Levels changed in only one biological replicate (see *SI Text*).

[‡]No miRNAs were significantly increased in *daf-12* mutants relative to wild type. However, crowded wild-type larvae showed a significant increase (2–24 times) of *mir-242*, *mir-243*, *mir-34*, *mir-71* and *mir-792*.

fates (8). This redundancy suggests that absolute levels of let-7-Fam miRNAs are important for regulation of *hbl-1* and is consistent with the hypothesis that quantitative changes in the levels of let-7-Fam miRNAs are responsible for the heterochronic phenotypes of certain *daf-12* mutants.

If DAF-12 ultimately affects HBL-1 expression by modulating let-7-Fam miRNA expression, we would expect that ligand-independent DAF-12 activity [*daf-12(rh61)*] or reduction of DAF-12 [e.g., *daf-12(RNAi)*] should affect HBL-1 expression in a fashion consistent with the effects on let-7-Fam expression. In particular, because the constitutively repressive form of DAF-12 [*daf-12(rh61)*] causes a greater reduction in let-7-Fam miRNAs than does *daf-12(0)* (see above), we predicted that *daf-12(rh61)* animals should exhibit a greater elevation in HBL-1 expression than *daf-12(RNAi)* animals. Accordingly, we used strains carrying an *hbl-1::GFP::hbl-1* reporter that is normally down-regulated in the hypodermal cells of L3 larvae by let-7-Fam miRNAs (8, 14–16). We find that in *daf-12(rh61)* mutant larvae the *hbl-1* reporter fails to be down regulated in the L3 stage (Fig. 2) (8, 12). We confirmed that this misexpression of *hbl-1* in *daf-12(rh61)* animals depends on DIN-1S, because *din-1s(RNAi)* suppressed both the heterochronic and *hbl-1* misexpression phenotypes of *daf-12(rh61)* animals (Fig. 2). Importantly, reduction of *daf-12* activity by mutation or RNAi does not affect down-regulation of the *hbl-1* reporter (Fig. 2). Thus, consistent with both the severe heterochronic phenotypes and the strong and specific repression of let-7-Fam miRNAs in *daf-12(rh61)* mutant larvae, *daf-12(rh61)* prevents the normal down-regulation of the *hbl-1* reporter more strongly than does a simple reduction of DAF-12 protein. Together, these data suggest that *daf-12* is normally not essential for the transcription of the let-7-Fam miRNAs but functions to modulate their expression in response to environmental conditions and impacts stage-specific gene expression by tuning HBL-1 levels.

DAF-12 Regulates HBL-1 Expression Independently of *lin-28*. HBL-1 expression is tightly controlled by several regulatory pathways in addition to let-7-Fam miRNAs (8). Two well-defined components that regulate HBL-1 in parallel to let-7-Fam miRNAs are *lin-28*, encoding an RNA-binding protein that promotes *hbl-1* translation, and *lin-46*, encoding a scaffolding protein that negatively regulates

hbl-1 (17, 18). Because previous reports have suggested that *lin-28* is downstream of *daf-12* (9), we asked whether *daf-12* regulates stage-specific seam cell fate entirely via modulation of let-7-Fam miRNAs or whether *daf-12* might also act via *lin-28*. We observed LIN-28::GFP expression in wild type, *daf-12(lf)*, and *daf-12(rh61)* mutant backgrounds. Inconsistent with *daf-12* functioning to regulate *lin-28* expression, the LIN-28::GFP transgene is down-regulated normally during the second larval stage in *daf-12* mutants (Fig. S2) (17). This suggests that *daf-12(rh61)* does not require *lin-28* overexpression to promote L2-specific seam cell fates and is consistent with *daf-12* functioning entirely through regulation of let-7-Fam miRNAs. Additional genetic interactions between *daf-12* alleles and *lin-28*, *lin-46*, and miRISC components are also consistent with *daf-12* functioning in the same pathway as the let-7-Fam miRNAs (Table S1, Fig. S3). These data indicate that *daf-12* is one of several modulators of HBL-1 expression and that the effect of *daf-12* mutations on HBL-1 expression and stage-specific cell fates can be entirely explained by the regulation of let-7-Fam miRNA expression by DAF-12.

let-7-Family miRNAs (miR-48, miR-241, and miR-84) Regulate the Expression of DAF-12. Previous studies have demonstrated that the *daf-12* 3' UTR contains let-7 complementary sites that mediate the regulation of *daf-12* 3' UTR reporters during late larval development (19). Because DAF-12 and the let-7-Fam miRNAs (miR-48, miR-241, and miR-84) also function during the L2-to-L3 transition, we investigated whether DAF-12 is regulated by the let-7-Fam miRNAs at this stage. Accordingly, we observed the expression of a functional DAF-12::GFP reporter in *let-7-Fam* mutant larvae. Previous characterization of the DAF-12::GFP reporter in wild-type larvae indicated that DAF-12 is normally down-regulated in hypodermal seam cells (and to a lesser extent in hyp7 cells) after the L2 molt (13). We find that the normal down-regulation of DAF-12::GFP is not observed in L3 stage *mir-48(0)* *mir-241(0)*; *mir-84(0)* mutant larvae (Fig. 3). This indicates that these miRNAs contribute to the down-regulation of DAF-12 between the L2 and L3 stages.

Feedback Between let-7-Family miRNAs and DAF-12 Modulates *daf-12*-Dependent Developmental Events. The data reported above indicate a reciprocal regulatory relationship, whereby DAF-12 reg-

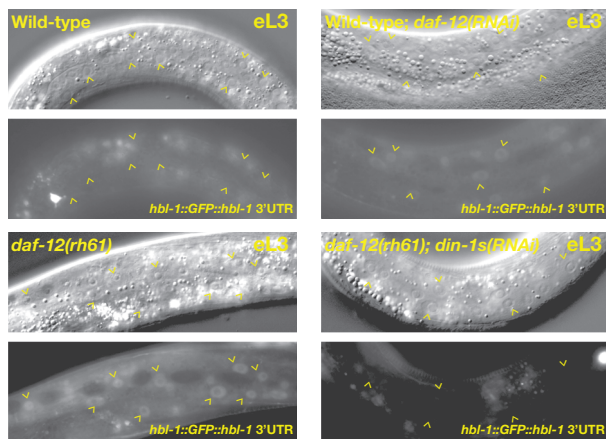


Fig. 2. *hbl-1* is misexpressed in ligand-binding-defective mutants of *daf-12*. The *hbl-1* 3' UTR reporter (*ctls39*) is down-regulated in wild-type and *daf-12(RNAi)* larvae (eL3), but not in *daf-12(rh61)* eL3 larvae. Persistence of *hbl-1* expression in *daf-12(rh61)* larvae depends on the DAF-12 corepressor DIN-15 because RNAi of *din-1s* suppresses the heterochronic phenotypes and corrects the *hbl-1* misexpression of *daf-12(rh61)* animals.

ulates the abundance of let-7-family miRNAs, and the let-7-family miRNAs regulate the abundance of DAF-12. Because let-7-Fam miRNAs affect DAF-12 levels, we tested whether let-7-Fam miRNAs could also affect *daf-12*-dependent developmental programs, including gonad migration and dauer formation.

During larval development, the distal tip cells of the somatic gonad guide gonadal growth and morphogenesis. During the L2 and L3 stages, the gonad arms migrate along the ventral surface of the animal. At the L3 molt, the distal tip cells of both gonad arms migrate dorsally and then reflex back toward the middle of the animal (20). The gonad arms of animals expressing mutant DAF-12 proteins that are unable to bind DA fail to reliably execute the L3 molt gonadal turning (9). Similarly, *daf-9(0)* mutants (which do not make endogenous DA) cultured in the presence of limiting amounts of exogenous DA, exhibit defects in gonadal turning (10). We find that the frequency of gonadal turning defects (the "Mig" phenotype) in *daf-9(0)* mutants is inversely related to DA concentration consistent with an inhibition of L3 gonadal turning by unliganded DAF-12 (Table 2). This implies that at limiting levels of DA, DAF-12 must be down-regulated by the end of the L3 stage, and/or DA levels must rise, to permit gonadal turning.

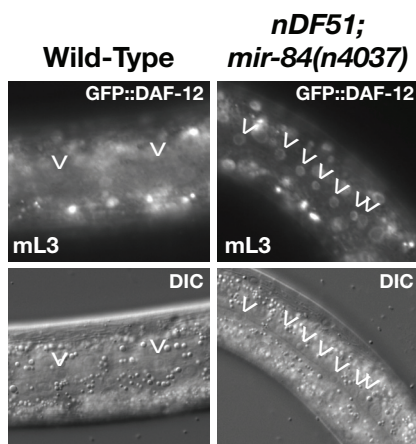


Fig. 3. DAF-12 levels are regulated by let-7-family miRNAs. A translational reporter of DAF-12, containing the *daf-12* 3' UTR, is misexpressed in hypodermal seam cells of *mir-48(0) mir-241(0); mir-84(0)* mutant L3 larvae.

Table 2. Effects on *daf-12*-dependent activity by reduction of let-7-Fam miRNA levels

nM DA ligand	<i>daf-9</i>	<i>mir-241</i> ; <i>daf-9 mir-84</i>	<i>mir-48 mir-241</i> ; <i>daf-9 mir-84</i>
		Mig	
25	83 ± 15 (63)	81 ± 15 (24)	ND
50	35 ± 17 (88)	82 ± 9 (61)	78 ± 16 (52)
100	7 ± 4 (150)	34 ± 21 (127)	ND
		dauer	
0	100 ± 0 (46)	100 ± 0 (33)	ND
25	25 ± 13 (138)	39 ± 4 (95)	ND
50	5 ± 2 (108)	3 ± 1 (79)	19 ± 8 (95)
100	0 ± 0 (150)	0 ± 0 (127)	ND

Data are presented as percentage of animals exhibiting the relevant phenotype, plus or minus the standard error of the mean for three independent trials. Total number of animals scored is in parentheses. ND, not determined.

Because we see that let-7-Fam miRNAs contribute to a down-regulation of DAF-12 between the L2 and L3 stages (see above), we predicted that at constant, limiting concentrations of DA, loss of let-7-Fam miRNAs should result in an increase in the fraction of unliganded DAF-12 and an increase in the Mig phenotype. To eliminate effects on *daf-12* activity attributable to variation in endogenous DA levels, we used a *daf-9(0)* mutation to eliminate endogenous DA, and supplied various levels of synthetic DA exogenously in the culture medium. We find that on plates containing either 50 nM or 100 nM DA, *daf-9(0)* strains that are also reduced in let-7-Fam miRNAs (because of loss of *mir-241* and *mir-84*) indeed display a more penetrant Mig phenotype than *daf-9(0)* controls (Table 2). *daf-9(0)* animals lacking all three let-7-Fam miRNAs [*mir-48(0) mir-241(0); mir-84(0)*] also display an increased Mig phenotype (Table 2). This observation is consistent with the hypothesis that let-7-Fam miRNAs down-regulate DAF-12 as part of a feedback loop.

Another possible consequence of a feedback loop between *daf-12* and the let-7-Fam is that let-7-Fam miRNAs would contribute to the regulation of dauer formation (via the regulation of DAF-12 levels). The ratio of unliganded DAF-12 to liganded DAF-12 during the L2 stage is a critical factor in the decision between dauer vs. reproductive development (for review, see ref. 5) (10). *daf-9(0)* larvae, which lack DA, constitutively form dauer larvae in a *daf-12*- and *din-1*-dependent manner (10, 21). This dauer-constitutive phenotype of *daf-9(0)* animals can be rescued by addition of synthetic DA in a dosage-dependent manner (Table 2) (10). We reasoned that at a constant, limiting concentration of DA loss of let-7-Fam miRNAs should result in an increase in the fraction of unliganded DAF-12 and hence an increase in the frequency of dauer formation. We found that at a DA concentration of 50 nM, 5% of *daf-9(0)* mutants formed dauer larvae, whereas 19% of *mir-48(0) mir-241(0); daf-9(0) mir-84(0)* mutant larvae formed dauer larvae (Table 2). These observations suggest that let-7-family miRNAs affect the dauer formation decision by modulating DAF-12 protein levels and imply that, during wild-type development, the temporal up-regulation of let-7-Fam miRNA levels during the L2 stage could affect the timing and/or frequency of dauer larva arrest.

Note that animals triply mutant for let-7-Fam miRNAs [*mir-48(0) mir-241(0); daf-9(0) mir-84(0)*] display a stronger dauer formation phenotype than do doubly mutant, *mir-48(0) mir-241(0); daf-9(0)* animals (Table 2). This indicates a functional redundancy among let-7-family members with respect to regulation of *daf-12* and dauer formation, analogous to the redundancy among let-7-Fam miRNAs for the regulation of *hbl-1* (8).

Dauer-Promoting Environmental Conditions Affect let-7-Fam Expression in Wild-Type Animals. One prediction of the proposed feedback loop wherein DAF-12 modulates let-7-Fam miRNA gene expres-

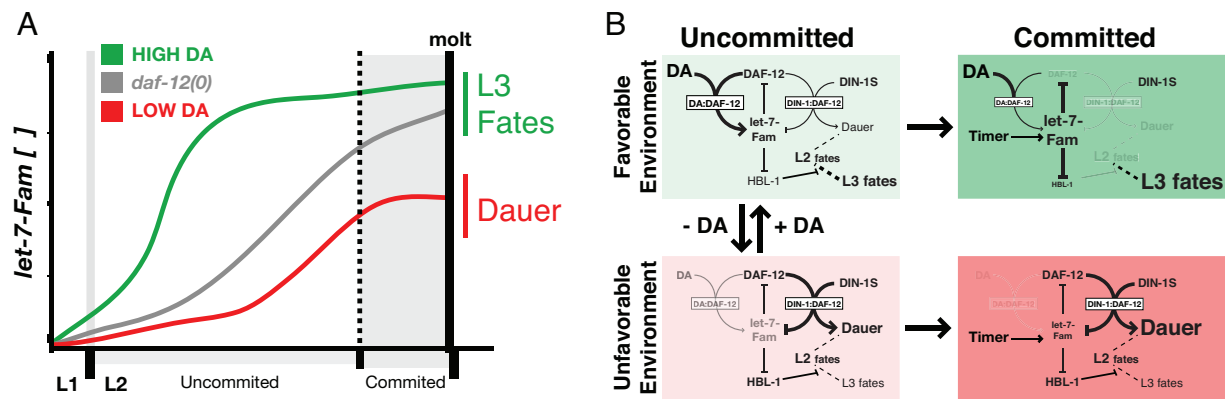


Fig. 4. Model for a feedback circuit that integrates environmental and temporal cues for robust cell fate decisions in different environments. (A) *let-7-Fam* miRNAs are transcriptionally up-regulated as development progresses independently of *daf-12*. However, DAF-12 activity modulates the rate at which the *let-7-Fam* miRNAs are expressed. Liganded DAF-12 (DA:DAF-12) accelerates their up-regulation, whereas unliganded DAF-12 (DAF-12:DIN-1) impedes *let-7-Fam* up-regulation. (B) The feedback circuit between DAF-12 and the *let-7-Fam* miRNAs (see *Discussion*).

sion is that environmental conditions that lead to changes in levels of DA ligand could either accelerate or impede the intrinsic developmental up-regulation of *let-7-Fam* miRNAs during the L1–L3 stages. If the feedback loop were important for integrating stage-specific cell fate decisions with life-history choice, the critical period for this integration would be the second larval stage. We therefore assessed miRNA levels during the second larval stage of wild-type larvae in two environmental extremes: (i) favorable culture conditions (plentiful food supply and sparse population density) and (ii) unfavorable dauer-inducing conditions (simulated high population density induced by addition of dauer pheromone to the growth medium). Exogenous pheromone in these conditions modulates downstream DAF-12 activity via multiple signaling pathways that ultimately regulate *daf-9* expression (21). Larvae on the pheromone-containing plates entered the extended, pre-dauer L2d stage, whereas larvae on the plates without exogenous pheromone developed through the rapid L2 stage. We found that the expression of four of the *let-7*-family members (miR-48, miR-241, miR-84, and miR-795) was significantly reduced in high-pheromone conditions (≈ 3 - to 12-fold; $P < 0.01$, Table 1). As was the case for the effect of *daf-12* mutations on miRNA expression, the effect of environment was relatively specific for the *let-7*-family miRNAs; in this case, only one additional miRNA showed moderate but statistically significant ($P < 0.01$) down-regulation, and five miRNAs showed significant ($P < 0.01$) up-regulation in crowded conditions. Levels of *let-7* and miR-794 were below the limit of detection in some assays, whereas miR-793 was not affected by environmental conditions, consistent with the lack of effect of *daf-12* mutations on miR-793 expression (Table 1). These results indicate that environmental conditions, likely mediated by DAF-12, can significantly impact the expression of *let-7-Fam* miRNAs.

Discussion

The significance of the role for DAF-12 in regulating a choice between continuous vs. dauer-interrupted life history choice in response to environmental conditions has become increasingly clear in recent years, particularly with the identification of DA as a DAF-12 ligand (10). However, the precise role for DAF-12 in influencing developmental timing has remained mysterious. For example, the *daf-12(0)* allele has a profound defect in dauer formation, but a very minor impact on stage-specific cell fates (9). Yet, because certain nonnull alleles of *daf-12* exhibit dramatic developmental timing defects, it was clear that *daf-12* could affect stage-specific cell fate decisions in some fashion (9). This work, together with another recent paper (12), establishes that DAF-12 affects developmental timing by modulating the expression of

let-7-Fam miRNAs. The mechanism for the modulation of *let-7-Fam* miRNAs by DAF-12 is similar to that for regulation of dauer formation. Specifically, in both contexts, liganded DAF-12 functions as a gene activator, and unliganded DAF-12 functions as part of a repressor complex.

Although unliganded DAF-12 is absolutely required for dauer formation (9), we demonstrate that the role for DAF-12 in regulating *let-7-Fam* miRNAs is modulatory rather than essential (Table 1 and Table S1). Specifically, we show that levels of *let-7-Fam* miRNAs are only mildly reduced at the end of L2 in *daf-12(0)* larvae (Table 1). Furthermore, this mild reduction is not sufficient to affect HBL-1 down-regulation or stage-specific cell fate decisions (Table S1 and Fig. 2). However, although DAF-12 exerts a weak positive modulation of *let-7-Fam* expression, molecular and genetic analysis of *daf-12* mutants that cannot respond to environmental conditions because they do not bind DA indicate that DAF-12 possesses a strong negative regulatory potential that is normally held in check by DA. In this scenario, DAF-12 is locked in its repressive form and can override the developmental timer by dramatically inhibiting the temporal up-regulation of *let-7-Fam* miRNAs (Fig. 4). In wild-type animals subjected to unfavorable environmental conditions, DA levels are low, and the repressive DAF-12-DIN-1 activity would dampen the accumulation of *let-7-Fam* miRNAs (Fig. 4A), resulting in a persistence of DAF-12 (and hence promotion of dauer arrest) and HBL-1 (and hence inhibition of L3 cell fates) (Fig. 4B).

A key finding in this work is a reciprocal regulatory relationship wherein DAF-12 and the *let-7-Fam* miRNAs modulate each other's expression. We demonstrate that the expression of *let-7-Fam* miRNAs is regulated by DAF-12 and also that *let-7-Fam* miRNAs down-regulate DAF-12 during larval development (Table 1, Fig. 3). We find that loss of *let-7-Fam* miRNAs can affect DAF-12-dependent events, including stage-specific gonad turning and dauer larva formation (Table 2). We propose that DAF-12 and *let-7-Fam* miRNAs comprise a complex feedback loop that is important both to respond to environmental changes during the uncommitted L2 stage and to reinforce the commitment to one choice that incorporates both life history and stage-specific gene expression. A model for this feedback loop is shown in Fig. 4 and discussed below.

In favorable environments where DA concentrations are high, second-stage larvae progress through the rapid L2 stage that leads to L3 and continuous development (2). Levels of *let-7-Fam* miRNAs rise during this stage in response to temporal cues (Fig. 4A) (8). We interpret the function of the feedback loop in this situation to be to accelerate the up-regulation of *let-7-Fam* miRNAs. Higher levels of these miRNAs will in turn accelerate

the down-regulation of HBL-1, allowing progression to L3 seam cell fates (Fig. 4). Once committed to continuous development and L3 fate choice, the *let-7-Fam* levels will be high because of the combined effect of temporal cues and DAF-12. Therefore, the levels both HBL-1 and DAF-12 will be reduced, precluding both L2 cell fates and dauer formation, irrespective of new changes in environmental conditions.

By contrast, in poor or marginal environments where DA concentration is low, second-stage larvae progress through an extended L2 stage (L2d) (2). For a period during the L2d, larvae remain uncommitted to either the dauer or continuous life histories while they monitor changes in environmental conditions. This uncommitted status of L2d animals is also accompanied by a postponement of L3 cell fates, compared with the rapid L2 stage. We propose that the feedback circuit contributes to both of these processes—the postponement of L3 fates and the eventual selection of reproductive vs. dauer development.

An important component of our model (Fig. 4) is that in dauer-promoting conditions, the DAF-12-DIN-1-repressive complex would dampened the up-regulation of *let-7-Fam* miRNAs, delaying the down-regulation of HBL-1 and DAF-12 and hence postponing the expression of L3 seam cell fates (Fig. 4B). In this model, a delayed down-regulation of DAF-12 serves to retain the developmental options of reproductive vs. dauer development during the extended L2d. If the environment remains poor, the larva will eventually commit to dauer arrest, and in our model, the feedback circuit forms part of the commitment mechanism, whereby DAF-12-DIN-1 complex positively autoregulates by repressing *let-7-Fam* miRNA expression.

If, on the other hand, an uncommitted L2d larva suddenly experiences favorable environmental conditions (and hence a rise in DA level), the direction of the DAF-12-dependent loop can shift, such that reduced DAF-12-DIN-1 repressor complex and increased DA-DAF-12 activator complex would permit accelerated up-regulation of *let-7-Fam* miRNAs. This environmentally triggered rise in *let-7-Fam* levels would promote commitment to reproductive development (by repression of DAF-12) and L3 fates (by repression of HBL-1). In either case, once a life history has been chosen, we propose that the feedback loop reinforces the decision and helps to make the two options mutually exclusive.

A reciprocal relationship between miRNAs and transcription factors that regulate the expression of those miRNAs may be a

common theme elaborated in a variety of developmental contexts. Previously described miRNA-transcription factor feedback loops have been proposed to “tune” the expression of the transcription factors to optimal levels (22–25), consistent with a role for miRNA-transcription factor feedback contributing to the robustness of developmental decisions. Where the transcription factor is a steroid receptor, as in the DAF-12-*let-7-Fam* circuit described here, the characteristics of the circuit can be radically affected by ligand, permitting complex responses to environmental and physiological signals. Depending on the abundance of DA, DAF-12 can either accelerate or retard the up-regulation of *let-7-Fam* miRNAs, enabling ligand-dependent shifts between positive and negative autoregulation. Moreover, DA is a diffusible ligand, enabling the output of the DAF-12 circuit to be coordinated at the organismal level, further ensuring that the distinction between continuous and dauer development is robustly drawn throughout the animal.

Materials and Methods

Nematode Methods. *C. elegans* strains were grown under standard conditions (26). Transformation of animals and RNAi were performed as previously described (8, 27).

Microscopy and Phenotypic Analysis. Lineage analysis and scoring of adult alae phenotypes were performed by picking staged animals of the indicated genotypes and monitoring seam cells derived from the V lineage as described by Sulston and Horvitz (4).

DA Assays. DA acid experiments were performed as in Motola et al. (10). Genotypes of animals assayed in this manuscript and further experimental descriptions can be found in *SI Text*.

TaqMan Data Analysis. Analysis for the TaqMan real-time PCR data were performed similarly to the identification of invariant probes method (28). A detailed description of experimental procedures, calculations and statistical analysis can be found in *SI Text*.

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