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Dauer larva quiescence alters the circuitry of microRNA pathways regulating cell fate progression in *C. elegans*

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SUMMARY

In *C. elegans* larvae, the execution of stage-specific developmental events is controlled by heterochronic genes, which include those encoding a set of transcription factors and the microRNAs that regulate the timing of their expression. Under adverse environmental conditions, developing larvae enter a stress-resistant, quiescent stage called 'dauer'. Dauer larvae are characterized by the arrest of all progenitor cell lineages at a stage equivalent to the end of the second larval stage (L2). If dauer larvae encounter conditions favorable for resumption of reproductive growth, they recover and complete development normally, indicating that post-dauer larvae possess mechanisms to accommodate an indefinite period of interrupted development. For cells to progress to L3 cell fate, the transcription factor Hunchback-like-1 (HBL-1) must be downregulated. Here, we describe a quiescence-induced shift in the repertoire of microRNAs that regulate HBL-1. During continuous development, HBL-1 downregulation (and consequent cell fate progression) relies chiefly on three let-7 family microRNAs, whereas after quiescence, HBL-1 is downregulated primarily by the lin-4 microRNA in combination with an altered set of let-7 family microRNAs. We propose that this shift in microRNA regulation of HBL-1 expression involves an enhancement of the activity of lin-4 and let-7 microRNAs by miRISC modulatory proteins, including NHL-2 and LIN-46. These results illustrate how the employment of alternative genetic regulatory pathways can provide for the robust progression of progenitor cell fates in the face of temporary developmental quiescence.

KEY WORDS: Dauer, Quiescence, Heterochronic, microRNA, *C. elegans*

INTRODUCTION

During animal development, key developmental events, such as cell division and differentiation, occur in precisely timed sequences. The precision of developmental timing is ensured by networks of regulatory genes, such as the heterochronic genes of *C. elegans* that control stage-specific cell fate decisions in the worm (reviewed by Moss, 2007; Resnick et al., 2010; Rougvie, 2001). A potential challenge to the regulation of developmental timing is the interruption of development by cellular quiescence, a reversible non-proliferating state. In mammals, quiescence is an important feature of many adult stem cells. Despite lengthy quiescent periods, stem cells possess the capacity to maintain their tissue specificity and multipotency. Defects in this process can lead to a failure to maintain tissue homeostasis, and are thought to be an important aspect of the aging process (Sharpless and DePinho, 2007).

In *C. elegans*, a form of developmental and cellular quiescence occurs during the dauer larva stage, an optional developmentally arrested form that is adopted in response to unfavorable environmental cues. These cues are transduced through a complex signaling network, including TGFβ, insulin and nuclear hormone receptor pathways (Fielenbach and Antebi, 2008). Some key regulators of dauer quiescence, including the DAF-16/FoxO transcription factor, and the DAF-18/PTEN phosphatase, are also key regulators of quiescence in mammalian adult stem cells. Furthermore, the p21CIP/p27KIP cell-cycle inhibitors are required to maintain cell cycle arrest in both quiescent mammalian adult stem cells and quiescent progenitor cells in *C. elegans* dauer larvae (Hong et al., 1998; Tothova and Gilliland, 2007). Dauer quiescence occurs specifically after the second larval molt, and all cells exit the cell cycle for the duration of the quiescence. The length of dauer quiescence is variable, from hours to potentially months – longer than the entire lifespan of animals that developed continuously (Fielenbach and Antebi, 2008). If favorable environmental conditions are encountered, larvae recover from dauer quiescence and resume development. Remarkably, the pattern and sequence of cell divisions in post-dauer larvae are identical to those of continuously developing larvae (Fig. 1) (Braendle and Félix, 2008; Euling and Ambros, 1996; Liu and Ambros, 1991). Thus, progenitor cells in *C. elegans* larvae possess a capacity to maintain their precise state of cell fate specification during a lengthy developmental sequence.

A clue to the mechanism underlying the ability of *C. elegans* to accommodate dauer quiescence comes from the study of heterochronic genes, which have been studied primarily for their roles in the stem-cell-like 'seam cell' lineage of the hypodermis (reviewed by Moss, 2007; Resnick et al., 2010; Rougvie, 2001). At each larval stage, seam cells express a particular stage-specific cell fate, defined by a specific pattern and sequence of cell divisions (Fig. 1A). At adulthood, seam cells exit the cell cycle, differentiate, and secrete an adult-specific cuticular structure called 'adult alae'. Furthermore, the seam cells and other hypodermal cells express an adult-specific collagen encoded by *col-19* (Ambros and Horvitz, 1984; Liu et al., 1995). Heterochronic genes regulate stage-specific seam cell fates and, accordingly, mutations in these genes can result in either 'precocious' development, wherein events of a particular larval stage are skipped and later events occur precociously, or

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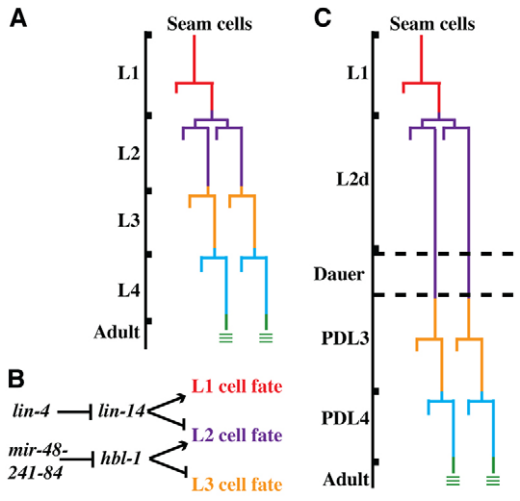

Fig. 1. Heterochronic genes regulate stage-specific cell fates. (**A**) Lineage diagram of seam cells (V1-V4, V6) during continuous development in wild-type *C. elegans* hermaphrodites (Sulston and Horvitz, 1977). Asymmetric seam cell divisions generate an anterior cell that fuses with the surrounding syncytial hyp7 cell, and a posterior replacement seam cell. A symmetric division in the L2 stage doubles the number of seam cells. The cell division pattern appropriate to each stage is color-coded. Adult alae are represented by three horizontal lines. (**B**) The key heterochronic genes that form the early timer during continuous development (Abbott et al., 2005; Rougvie, 2001). (**C**) Lineage diagram of seam cells during dauer-interrupted development. Dashed lines indicate the interruption of development by dauer quiescence for a variable amount of time. PD, post-dauer.

'retarded' development, wherein events of a particular larval stage are reiterated in subsequent larval stages (Ambros and Horvitz, 1984).

The heterochronic gene network can be thought of as a molecular timer that regulates stage-specific cell fate progression from the first larval stage (L1), through subsequent larval stages (L2-L4) to the adult (reviewed by Rougvie, 2001). During continuous development through the four larval stages, transcription factors that specify early cell fates are progressively downregulated by microRNAs (miRNAs), levels of which rise over time. In the first larval stage, the LIN-14 transcription factor is required for L1 cell fate. Expression of the lin-4 miRNA begins soon after the onset of larval development, and lin-4 levels rise during the L1 stage. When lin-4 levels are high enough, lin-4 downregulates *lin-14*, a direct lin-4 target, thereby permitting progression to L2 fate (Fig. 1B) (reviewed by Rougvie, 2001). In a similar manner, the Hunchback-like-1 (HBL-1) transcription factor is expressed in the first part of the L2 stage when it promotes L2 fates and prevents precocious expression of L3 fates. Levels of three redundant members of the let-7 family rise during the L2 stage and downregulate *hbl-1*, permitting progression to L3 cell fates (Fig. 1B) (Abbott et al., 2005). By contrast, the expression of post-dauer L3 cell fates should not rely on the same timer, because development is arrested throughout dauer quiescence, independent of the amount of time that elapses. In support of the expectation that post-dauer timing mechanisms are distinct from the timer that operates during continuous development, certain heterochronic genes that are normally required for stage-specific cell fate specification have been found to be dispensable in situations in which progenitor cells undergo a period of dauer quiescence (Abrahante et al., 2003; Liu and Ambros, 1991; Morita and Han, 2006). These observations indicate that alternative genetic mechanisms regulate stage-specific cell fate progression during dauer-interrupted development compared with continuous development (Liu and Ambros, 1991). However, little is known about the nature of the post-dauer developmental timing mechanisms, or how timing mechanisms shift from the continuous development mode to the post-dauer mode.

In this work, we probe the mechanisms that regulate developmental timing after dauer quiescence, focusing on the role of the three let-7 family miRNAs that downregulate *hbl-1* during continuous development. We find that dauer quiescence shifts the requirement for progression to L3 cell fate from resting primarily on the let-7 family miRNAs to resting on the parallel action of two miRNA families: the lin-4 family and the let-7 family. We propose that there is a potentiation of the activity of both lin-4 and let-7 family miRNAs after dauer quiescence. This potentiation involves *nhl-2*, which encodes a miRISC modulator that enhances let-7 family miRNA activity, and *lin-46*, which encodes a putative scaffolding protein that, in this context, seems to enhance lin-4 miRNA activity.

MATERIALS AND METHODS

Nematode growth conditions

C. elegans Bristol strain was grown on standard nematode growth medium with *Escherichia coli* strain OP50 as a food source. Animals were maintained at 20°C, and all experiments were carried out at 20°C except where indicated. The complete genotype of all strains is listed in supplementary material Table S1. The genotype of all newly derived strains was confirmed by PCR genotyping.

lin-4 overexpression transgenes

Transgenic lines *arEx1311* and *arEx1313* were created by injecting N2 hermaphrodites with 1 ng/μl p915 [*hsp16-2::lin-4*], 1 ng/μl p716 [*myo-3::mCherry*] and 50 ng/μl N2 genomic DNA digested with *Pvu*II. p915 was constructed using by PCR amplifying the *lin-4* precursor from N2 genomic DNA, and cloning it into pPD49.78. The *lin-4* precursor sequence used was the same sequence previously shown to rescue lin-4 phenotypes in vulval development (Li and Greenwald, 2010). Once lines were established by mCherry expression, the arrays were crossed into *maIs105[col-19::GFP]*, *nhl-2(ok818); maIs105*, or *lin-46(ma164) maIs105* backgrounds. To carry out mis-expression experiments, ten gravid adults of these strains were permitted to lay eggs on 60-mm nematode growth media (NGM) plates seeded with OP50 for two hours. The embryos developed ~15 hours at 20°C and then were subject to heat-shock at 33°C for one hour. After heat-shock, the plates were returned to 20°C where development continued until larvae were scored during the L4 stage, ~2 days after heat-shock. mCherry expression was used to determine whether the larvae carried the array and thus mis-expressed *lin-4* upon heat-shock.

Characterization of developmental phenotypes

Retarded phenotypes were determined in young adult hermaphrodites that had either grown continuously or had interrupted their development with dauer quiescence. Young adult animals were scored for adult alae formation and/or *col-19::GFP* expression using a Zeiss Axio Imager D1 with an AxioCam MRm camera, and an X-Cite 120Q light source (EXFO Photonic Solutions). Continuously developing animals were grown with plenty of food and sparse population density. Populations were synchronized by allowing mothers to lay eggs for several hours, or by dissecting embryos out of gravid mothers for strains in which all hermaphrodites are egg-laying defective. Post-dauer populations were synchronized by selecting dauer larvae from a starved and crowded plate using 1% SDS, then adding the larvae to NGM plates seeded with OP50 to stimulate recovery. For certain strains, some animals with otherwise dauer-like morphology nevertheless displayed sensitivity to SDS treatment, perhaps owing to incomplete expression of the dauer differentiation

programs (Liu and Ambros, 1989). In these cases, we collected dauer larvae manually based on their characteristic morphology rather than relying on SDS selection. *hbl-1::GFP[ctIs39, ctIs37]* or *LIN-28::GFP[maIs108]* expression in post-dauer-L3 larvae was ascertained by picking dauer larvae (identified by morphology) from crowded and starved plates and allowing them to recover for ~14 hours at 20°C. At this point, the morphology of the gonad and vulval precursors resembled that of continuously developing L3 staged larvae. We used the presence of dauer alae on the cuticle to verify that these larvae had previously been in dauer quiescence.

Real-time qRT-PCR

To obtain continuously developing L3 staged larvae, embryos isolated by hypochlorite treatment were placed on NGM plates seeded with OP50 and incubated at 20°C until larvae reached the L3 stage, as judged by the extent of gonad development. Post-dauer L3 staged larvae were isolated from crowded and starved plates incubated at 20°C by SDS-selection. SDS-resistant dauer larvae were recovered on fresh NGM plates seeded with OP50 and incubated at 20°C for ~14 hours. RNA from these larvae was extracted using the Trizol reagent (Invitrogen). Levels of 107 microRNAs were quantified using miR-Taqman-based real-time PCR (Applied Biosystems), and data were analyzed as described (Karp et al., 2011). Three biological replicates were assayed for each strain.

RESULTS

lin-4 can substitute for let-7 family miRNAs after dauer quiescence

Three let-7 family miRNAs (miR-48, miR-84, miR-241) are functionally redundant and downregulate *hbl-1* during the L2 stage in order to allow progression to L3 cell fates (Abbott et al., 2005). Mutant larvae that lack all of these miRNAs inappropriately express L2 cell fates during the L3 stage, thereby delaying subsequent larval cell fates. This ultimately causes a failure to produce adult-specific structures when animals reach reproductive maturity, a classic 'retarded' phenotype (Abbott et al., 2005; Ambros and Horvitz, 1984). Specifically, the retarded phenotype of the triple mutant *mir-48(0) mir-241(0); mir-84(0)* [hereafter '*mir-48-241-84(0)*'] includes (1) a reiteration of the L2-specific proliferative division of the seam cells during the L3 stage, (2) a failure to produce adult alae at the L4 molt, and (3) a failure of young adults to express the adult-specific collagen *col-19* (Fig. 2) (Abbott et al., 2005; Liu et al., 1995). By contrast, L3 and subsequent cell fates are executed normally in *mir-48-241-84(0)* mutants that interrupt their development with dauer quiescence (Fig. 2). Therefore, although these let-7 family miRNAs are required for normal progression to L3 cell fate during continuous development, they are not crucial for development following a period of developmental and cellular quiescence.

The regulation of developmental timing following a variable and potentially lengthy quiescent period implies the need to utilize regulatory mechanisms that do not rely on either chronological time or continuous progression through development. Indeed, many heterochronic genes that are required during continuous development are dispensable after dauer quiescence (Abrahante et al., 2003; Liu and Ambros, 1991; Morita and Han, 2006). The question therefore arises: What developmental timing mechanism might operate in post-dauer development, such that the let-7 family miRNAs are not required? A simple hypothesis is that another miRNA might substitute for *mir-48-241-84* after dauer quiescence. One candidate is *lin-4*, as the *hbl-1* 3′UTR contains at least one site complementary to the lin-4 miRNA. Furthermore, previous work has demonstrated that certain mutant strains carrying a *lin-4(0)* mutation can display a post-dauer retarded phenotype, albeit less severe than during continuous development (Liu and Ambros, 1991). Therefore, *lin-4* might participate in a developmental timing mechanism that operates after dauer quiescence. To test this hypothesis, we constructed a strain that lacks *lin-4*, as well as *mir-48-241-84*. This strain contains another mutation, *lin-14(n179)*, which reduces the activity of a lin-4 target that would otherwise block dauer formation (Liu and Ambros, 1991; Liu and Ambros, 1989). Note that the *lin-14(n179)* allele is included in all *lin-4(0)* strains and their controls discussed below, although for simplicity it is not always mentioned.

We find that *lin-4(0); mir-48-241-84(0)* displays a completely penetrant retarded phenotype during both continuous and dauer-interrupted development (Fig. 3); in particular, these animals exhibit a reiteration of L2 cell fates during post-dauer L3 and post-dauer L4 stages (supplementary material Fig. S1). The observation that post-dauer *lin-4(0); mir-48-241-84(0)* animals display a retarded phenotype whereas post-dauer *mir-48-241-84(0)* animals

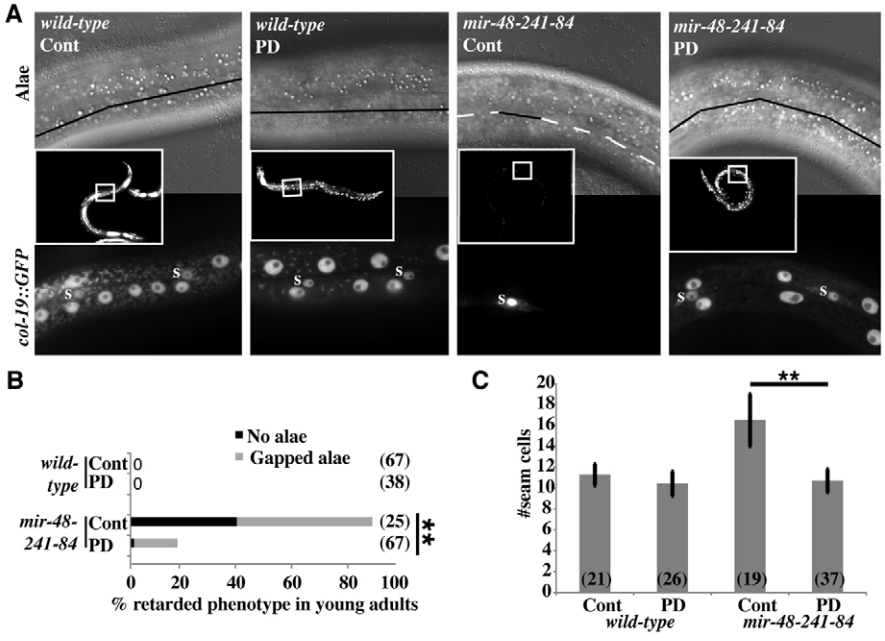

Fig. 2. Post-dauer suppression of *mir-48 mir-241(nDf51); mir-84(n4037)* retarded phenotypes. (**A**) Young adult *C. elegans* hermaphrodites were scored for adult alae formation (top) and *col-19::GFP* expression (inset and bottom). Inset shows GFP fluorescence with 10× objective. Top shows DIC image with 63× objective. Black solid lines indicate alae, white dashed lines indicate no alae. Bottom shows GFP fluorescence with 63× objective. GFP-positive seam cells are marked with 's'. Other GFP+ cells are hyp7 nuclei. (**B**) Percentage of young adult hermaphrodites exhibiting retarded alae defects in N2 (wild type) and VT1066 *mir-48 mir-241(nDf51); mir-84(n4037)*. $n \geq 25$. *$P<0.01$, Fisher's Exact Test. (**C**) Average seam cell number of L3 or post-dauer-L3 larvae of strains containing *wIs51[scm::GFP]*. GFP+ cells were counted between the pharynx and the rectum of each larva. $n>19$. Error bars indicate s.d. **$P<0.01$, two-tailed *t*-test. Cont, continuous development; PD, post-dauer.

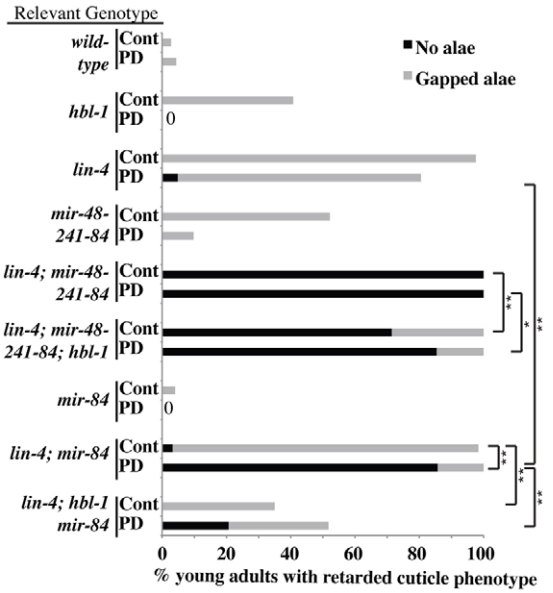

Fig. 3. *lin-4* can substitute for *mir-48-241-84* after dauer quiescence. Percentage of young adult *C. elegans* hermaphrodites exhibiting retarded alae defects. *lin-14(n179)* is included in all strains (except the *hbl-1* single mutant); *lin-14(n179)* moderates the severity of the retarded phenotypes of *lin-4(0)* and *mir-48-241-84(0)* strains during continuous development and, in particular, allows *lin-4(e912)* worms to enter dauer quiescence (Liu and Ambros, 1989) (full genotypes listed in supplementary material Table S1). $n \geq 20$. $^{*}P<0.05$, $^{**}P<0.01$, Fisher's Exact Test.

do not (Figs 2, 3) suggests that *lin-4* is sufficient to promote L3 cell fate during dauer-interrupted development. As *mir-48-241-84(0)* display a retarded phenotype during continuous development even when *lin-4* is present (Fig. 2), *lin-4* is not sufficient to promote L3 cell fate during continuous development. Thus, dauer quiescence produces a shift in which miRNAs are important for L3 cell fate. In addition, post-dauer *lin-4(0); mir-48-241-84(0)* animals are more retarded than post-dauer *lin-4(0)* animals (Fig. 3), indicating that *mir-48-241-84* function together with *lin-4* to control post-dauer stage-specific cell fate progression.

Because *lin-4* can substitute more effectively for *mir-48-241-84* during dauer-interrupted than continuous development (Figs 2, 3), we hypothesized that *lin-4* in combination with the let-7 family miRNAs is more important after dauer quiescence than during continuous development. If this hypothesis is correct, the loss of this combination of miRNAs should produce a stronger phenotype after dauer quiescence than during continuous development. Because *lin-4(0); mir-48-241-84(0)* mutants already display a completely penetrant retarded phenotype during continuous development, we constructed a mutant strain lacking *lin-4* that was also mutant for *mir-84*, one member of the let-7 family. These *lin-4(0); mir-84(0)* animals display a moderately retarded phenotype during continuous development, comparable to that of *lin-4(0)*. By contrast, this same strain displays a dramatic retarded phenotype after dauer quiescence (Fig. 3). Thus, *lin-4* and *mir-84* are more crucial for the proper progression of cell fates during post-dauer development than they are for the same progression of cell fates during continuous development. Note that the severely retarded post-dauer phenotype of *lin-4(0); mir-84(0)* animals is the result of loss of both *lin-4* and *mir-84* because the control strains [*lin-4(0); mir-84(+)* and *lin-4(+); mir-84(0)*] are significantly less retarded than the *lin-4(0); mir-84(0)* double mutant after dauer quiescence (Fig. 3). Loss of *mir-48* or *mir-241* also enhances the retarded phenotype of *lin-4(0)* post-dauer animals, consistent with the functional redundancy of the three let-7 family miRNAs (Table 2, lines 9 vs 17, 10 vs 16, $P \leq 0.015$, Fisher's Exact Test). By contrast, the lin-4 family member *mir-237* does not appear to be important for post-dauer developmental timing (Table 2, lines 5, 6). We interpret the above findings to mean that lin-4 and let-7 family miRNAs act in parallel to ensure cell fate progression after quiescence.

Potentiation of let-7 family miRNA activity by NHL-2 after dauer quiescence

The observations that *lin-4* can substitute for *mir-48-241-84* during dauer-interrupted development, but not during continuous development, and that *lin-4* and *mir-84* are together more important for dauer-interrupted development than in continuous development suggests a shift in the programming of microRNA regulation of L2-to-L3 cell fate progression after dauer quiescence. This shift could involve changes in the levels of lin-4 and let-7 family miRNAs and/or changes in their activities. For example, if lin-4 were more abundant post-dauer than during continuous development, that could explain why *lin-4* can substitute for *mir-48-241-84* only after dauer quiescence. However, wild-type dauer and post-dauer larvae do not express higher levels of mature lin-4 or let-7 family microRNAs than do continuously developing larvae. On the contrary, certain let-7 family miRNAs, particularly miR-48 and miR-241, are reduced in level in dauer and post-dauer L3 stages (Table 1) (Bethke et al., 2009; Hammell et al., 2009a; Karp et al., 2011).

Because our results indicate that lin-4 and let-7 family miRNAs play crucial roles in the expression of stage-specific cell fates after dauer quiescence, yet the levels of these mature miRNAs are either unaffected or reduced after dauer quiescence, we reasoned that protein factors might enhance the activity of the miRNAs after dauer quiescence, perhaps by potentiating the activity of the miRNA-induced silencing complex (miRISC). The miRISC is the miRNA-protein complex that binds to target messenger RNAs and leads to downregulation of the expression of protein from the target mRNA (Krol et al., 2010). A candidate factor that might enhance post-dauer miRNA activity is *nhl-2*, which encodes a miRISC

Table 1. Fold change of mature miRNA in continuously developing L3 larvae compared with post-dauer L3 larvae

	Wild type‡		*mir-48-241-84(0)*§	
miRNA	FC	*P*	FC	*P*
lin-4 family				
lin-4	1.2±0.3	0.19	0.82±0.69	0.68
miR-237	2.3±1.0	0.06	1.25±1.00	0.63
let-7-family				
let-7	1.0±0.6	0.98	0.60±0.48	0.34
miR-48	2.0±0.6*	0.04	UD	UD
miR-84	0.68±0.22	0.13	UD	UD
miR-241	2.5±1.10*	0.03	UD	UD
miR-793	1.1±0.9	0.83	0.76±0.56	0.53
miR-794	UD	UD	UD	UD
miR-795	0.83±0.55	0.64	0.69±2.24	0.75

*$P \leq 0.05$, *t*-test.
‡N2. Data for N2 larvae are from Karp et al., 2011.
§VT1066 *mir-48 mir-241(nDf51); mir-84(n4037).*
FC, fold change microRNA level in continuously developing L3 larvae compared with post-dauer L3 larvae ± modified s.d. (Karp et al., 2011). UD, undetected [Ct≥30 (see Karp et al., 2011)].

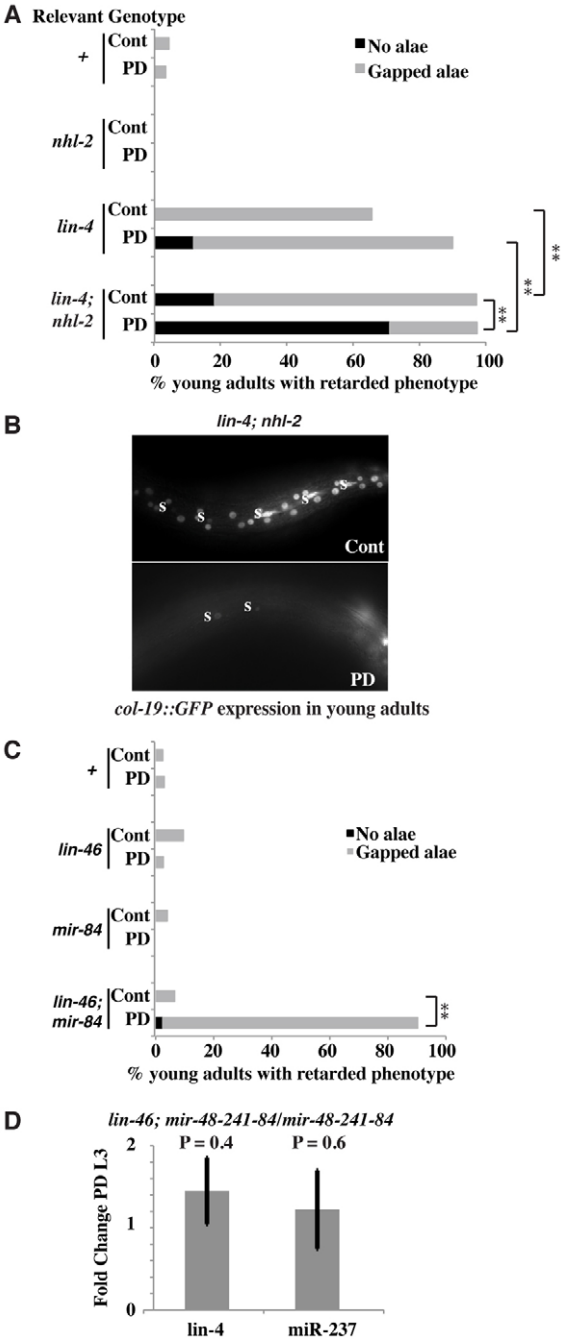

Fig. 4. Compromising both lin-4 and let-7 family pathways simultaneously causes more severe defects after dauer quiescence. (**A**) Percentage of young adult *C. elegans* hermaphrodites exhibiting retarded alae defects for the indicated genotypes. *lin-14(n179)* is included in all strains in order to allow *lin-4(e912)* worms to enter dauer quiescence (Liu and Ambros, 1989) $n \geq 22$. (**B**) GFP fluorescence visualized using a 40× objective in young adult hermaphrodites expressing *col-19::GFP*. GFP-positive seam cells are marked with 's'. Other GFP+ cells are hyp7 nuclei. See supplementary material Fig. S2 for quantification of retarded *col-19::GFP* phenotypes. (**C**) Percentage of young adult hermaphrodites exhibiting retarded alae defects. $n \geq 21$. (**D**) Levels of mature lin-4 family microRNAs in post-dauer L3 larvae are not different between VT1145 *lin-46(ma164) mir-48 mir-241(nDf51); mir-84(n4037)* and VT1066 *mir-48 mir-241(nDf51); mir-84(n4037)*. Error bars represent modified s.d. (Karp et al., 2011). $^{**}P<0.01$, Fisher's Exact Test. Cont, continuous development; PD, post-dauer.

modulator. *nhl-2* has been shown to potentiate the activity of the let-7 family miRNAs during continuous development, without affecting their levels (Hammell et al., 2009b). If *nhl-2* activity also enhances let-7 family miRNA activity after dauer quiescence, then loss of *nhl-2* should have a similar effect as loss of *mir-48-241-84* in combination with *lin-4(0)*. Indeed, *lin-4(0); nhl-2(0)* animals display a dramatic post-dauer retarded phenotype (Fig. 4A,B; supplementary material Fig. S2). Furthermore, the *lin-4(0); nhl-2(0)* retarded phenotype is stronger after dauer quiescence than it is in animals that developed continuously (Fig. 4A,B), similar to the *lin-4(0); mir-84(0)* mutant strain (Fig. 3). These results suggest that *nhl-2* acts in parallel to *lin-4* during post-dauer development, perhaps to potentiate the activity of certain let-7 family miRNAs.

We wondered next whether *nhl-2* might also affect lin-4 activity. During continuous development, *nhl-2* is known to affect activity of several miRNAs in addition to let-7 family members, but its effect on lin-4 is unknown (Hammell et al., 2009b). In order to address this question, we investigated whether *nhl-2* was required for a *lin-4* gain-of-function phenotype. The assay we used is similar to the *mir-48* gain-of-function assay that was used previously to demonstrate that *nhl-2* potentiates let-7 family miRNA activity (Hammell et al., 2009b). In brief, mis-expression of miR-48 causes precocious phenotypes, presumably owing to premature downregulation of *hbl-1* (Abbott et al., 2005; Li et al., 2005). These precocious phenotypes are suppressed by loss of *nhl-2*, implying that *nhl-2* is required for full miR-48 activity (Hammell et al., 2009b). To create an analogous experiment, we mis-expressed lin-4 using the heat-shock promoter. lin-4 mis-expression during continuous development caused precocious phenotypes, as expected if lin-4 targets such as *lin-14* and *lin-28* are downregulated prematurely (supplementary material Fig. S3). However, loss of *nhl-2* has little to no effect on the lin-4 mis-expression phenotype, suggesting that lin-4 activity is not affected by *nhl-2*, at least during continuous development (supplementary material Fig. S3). Because both *lin-14(0)* and *lin-28(0)* precocious phenotypes are completely suppressed by post-dauer development (Liu and Ambros, 1991), we could only perform this experiment for continuously developing animals.

To test whether *nhl-2* can affect the activity of miRNAs in addition to miR-48-241-84 (such as lin-4) after dauer quiescence, we observed the post-dauer phenotype of *nhl-2(0); mir-48-241-84(0)* mutant animals, in comparison to the post-dauer phenotype of *mir-48-241-84(0)*. If *nhl-2* activity is important only to enhance miR-48-241-84 activity after dauer quiescence, then we would not expect to see any effect of removing *nhl-2* when *mir-48-241-84* are absent. However, we do find a moderate enhancement of the post-dauer phenotype of *mir-48-241-84(0)* when *nhl-2* is removed (Table 2, lines 37, 38). This indicates that *nhl-2* might indeed enhance the activity of miRNAs other than miR-48-241-84, such as one or more of the remaining let-7 family miRNAs (let-7, miR-793, miR-794, miR-795) (Ibáñez-Ventoso et al., 2008), and/or lin-4.

The *C. elegans* genome encodes four members of the TRIM-NHL family, NHL-1-3 and LIN-41, plus a related protein, NCL-1; (reviewed by Loedige and Filipowicz, 2009). Quadruple mutants *ncl-1(0); nhl-1(0); nhl-2(0); nhl-3(0)* do not display any post-dauer retarded phenotype, indicating that the TRIM-NHL proteins do not function redundantly for directing stage-specific cell fate during post-dauer development (Table 2, lines 21, 22). This is similar to the lack of redundancy observed in *ncl-1(0); nhl-1(0); nhl-2(0); nhl-3(0)* mutant animals that develop continuously (Hammell et al., 2009b; Hyenne et al., 2008).

Table 2. Adult alae phenotype in post-dauer young adult hermaphrodites

	Strain	Genotype	Adult alae (%)‡ None	Gap	Comp.	*n*
1	N2	wild type	0	0	100	38
2	GS5770	*maIs105[col-19::GFP]*	0	3	97	34
3	VT1874	*hbl-1(ve18)*	0	0	100	23
4	DR441	*lin-14(n179)*	0	4	96	23
5	RF194	*mir-48-241(0)*	0	1	99	120
6	RF195	*mir-48-241(0); mir-237(0)*	0	1	99	88
7	VT1066	*mir-48-241-84(0)*	1	18	81	67
8	VT1178	*mir-48-241-84(0); lin-14(n179)*	0	10	90	41
9	VT573	*lin-4(0); lin-14(n179)*	5	76	20	41
10	VT2283	*lin-4(0); maIs105; lin-14(n179)*	12	78	10	50
11	VT1058	*lin-4(0); mir-48-241(0); lin-14(n179)*	88	12	0	17
12	VT1207	*lin-4(0); mir-48-241-84(0); lin-14(n179)*	100	0	0	39
13	MT13651	*mir-84(0)*	0	0	100	32
14	GS5767	*lin-14(n179) mir-84(0)*	0	0	100	20
15	VT1065	*lin-4(0); lin-14(n179) mir-84(0)*	86	14	0	63
16	VT2308	*lin-4(0); mir-48(0) maIs105; lin-14(n179)*	47	47	7	15
17	VT2307	*lin-4(0); mir-241(0); lin-14(n179)*	47	53	0	19
18	VT1685	*lin-46(0) maIs105*	0	3	97	38
19	VT1685	*lin-46(0) maIs105* 15°C	0	9	91	35
20	VT1313	*maIs105; mir-84(0)*	0	0	100	24
21	VT1273	*nhl-2(0); maIs105*	0	0	100	24
22	VT2080	*nhl-3(0); nhl-2(0) ncl-1(0) nhl-1(0)*	0	0	100	39
23	VT2086	*lin-46(0) maIs105; mir-84(0)*	2	88	10	41
24	VT1314	*nhl-2(0); maIs105; mir-84(0)*	0	13	87	30
25	VT1145	*lin-46(0); mir-48-241-84(0)*	80	20	0	25
26	VT2026	*lin-46(0); mir-48-241-84(0); hbl-1(ve18)*	0	0	100	36
27	VT1102	*lin-28(0); lin-46(0); mir-48-241-84(0)*	90	10	0	21
28	GS5740	*maIs105; lin-14(n179)*	0	4	96	28
29	GS5684	*lin-46(0) maIs105; lin-14(n179)*	0	0	100	20
30	GS5744	*nhl-2(0); maIs105; lin-14(n179)*	0	0	100	27
31	VT2275	*lin-4(0); lin-46(0) maIs105; lin-14(n179)*	20	80	0	30
32	VT2273	*lin-4(0); nhl-2(0); maIs105; lin-14(n179)*	71	26	3	38
33	VT2296	*lin-4(0); maIs105; lin-14(n179) mir-84(0)*	79	21	0	14
34	VT2274	*lin-4(0); lin-46(0) maIs105; lin-14(n179) mir-84(0)*	100	0	0	14
35	VT1573*	*nhl-2(0); lin-46(0)*	0	48	52	27
36	VT1377	*nhl-2(0); lin-46(0); hbl-1(ve18)*	0	0	100	31
37	GS5778	*mir-48-241-84(0); maIs105*	0	1	99	76
38	VT2319*	*nhl-2(0); mir-48-241-84(0); maIs105*	0	38	62	55
39	GS5806	*maIs105; lin-14(n355n679)*	0	7	93	30
40	GS5807	*lin-46(0) maIs105; lin-14(n355n679)*	0	52	48	27
41	VT2325	*maIs105; alg-1(0)*	0	0	100	23
42	GS5217	*alg-1(0)*	0	10	90	20
43	VT1274	*alg-2(0); maIs105*	0	0	100	30
44	VT2008	*maIs105; ain-1(0)*	0	5	95	19
45	VT1278	*nhl-2(0); maIs105; alg-1(gk214)*	0	7	93	27
46	GS6036	*lin-46(0) maIs105; alg-1(0)*	6	77	16	31

Strains were grown at 20°C unless otherwise indicated. Full genotypes are listed in supplementary material Table S1.
*Animals were derived from a parent strain where *nhl-2* was balanced by *hT2*, but maintained homozygous for *nhl-2* for several generations.
‡Adult alae were visualized by DIC microscopy. Hermaphrodites were categorized by whether alae were absent ('none'), indicating all seam cells exhibited larval cell fate, whether there were gaps in the alae ('gap'), indicating some seam cells exhibited larval cell fate, or whether alae were complete ('comp.'), i.e. covered the length of the body from pharynx to rectum, indicating all seam cells exhibited adult cell fate. The number of each category (one side each) over the total number of young adults scored (*n*) was calculated to give the percentage shown here.

lin-46 promotes post-dauer cell fate progression in parallel to let-7 family miRNAs

As *nhl-2* potentiates the activity of let-7 family miRNAs, but does not appear to potentiate lin-4 activity, the question arises as to what factor might enhance lin-4 activity after dauer quiescence, such that *lin-4* can substitute for *mir-48-241-84* during post-dauer development. One candidate is *lin-46*, which promotes the proper timing of L2 and L3 cell fates during continuous development in parallel with *nhl-2* and *mir-48-241-84*. LIN-46 shares sequence homology with the gephyrin scaffolding protein, but the molecular mechanism by which LIN-46 regulates developmental timing is unknown (Abbott et al., 2005; Hammell et al., 2009a; Pepper et al., 2004). We first tested whether *lin-46* could also contribute to stage-specific cell fates after dauer quiescence. *lin-46(0); mir-48-241-84(0)* animals display a severely retarded post-dauer phenotype, indicating that *lin-46* is required for the post-dauer suppression of *mir-48-241-84(0)* retarded phenotypes (Table 2, lines 7, 25). Furthermore, *lin-46(0); mir-84(0)* mutant animals display a retarded defect after dauer quiescence, but not during continuous development, indicating that these two genes (*lin-46* and *mir-84*) are together more important for the regulation of stage-specific cell fates in post-dauer animals than they are in continuously developing animals (Fig. 4C). Finally, *lin-46* also acts in parallel to *nhl-2* after dauer quiescence, because *nhl-2(0); lin-46(0)* animals display a retarded post-dauer phenotype,

whereas the single mutants do not (Table 2, lines 18, 21, 35). Therefore, similar to continuous development, *lin-46* acts after dauer quiescence in parallel to *mir-48-241-84* and *nhl-2* to promote cell fate progression.

The genetic relationship between *lin-46* and let-7 family miRNAs is similar to the relationship between *lin-4* and let-7 family miRNAs. First, *lin-46(0)* and *lin-4(0)* both cause a similar enhancement of the retarded phenotype of *mir-48-241-84(0)* during continuous or post-dauer development (Fig. 3, Table 2) (Abbott et al., 2005). Second, *lin-46(0)* and *lin-4(0)* both cause a retarded post-dauer phenotype when combined with *mir-84(0)* (Fig. 3, Fig. 4C). These observations suggest that *lin-46* might act in the same genetic pathway as *lin-4*. If this were the case, loss of *lin-46* should not enhance the post-dauer retarded phenotypes caused by loss of *lin-4*. With respect to alae formation, this is indeed what we see (Table 2, lines 10, 31, and 33, 34; P=0.2 and P=0.1, respectively, Fisher's Exact Test). However, using the same strains (as for lines 10 and 31 of Table 1) we find that *lin-46(0)* can moderately but statistically significantly (P=0.0007) enhance the post-dauer retarded *col-19::GFP* defects observed: 68% (n=34) of *lin-4(0)* young adults display defects in *col-19::GFP* expression in seam cells, compared with 100% (n=30) of *lin-4(0); lin-46(0)* young adults. This is consistent with additional roles for *lin-46* with respect to the regulation of *col-19* expression. We propose that *lin-46* acts in the same genetic pathway as *lin-4* to regulate the timing of adult alae formation, and also acts downstream of or in parallel with *lin-4* to affect *col-19* expression.

As *lin-46* acts in parallel with *mir-48-241-84* but might act in the same pathway as *lin-4*, one possibility is that *lin-46* might affect lin-4 levels. Specifically, the post-dauer retarded phenotype observed in *lin-46(0); mir-48-241-84(0)* mutant animals could be due to reduced lin-4 expression. To test this hypothesis, levels of mature miRNAs were quantified in *lin-46(0); mir-48-241-84(0)* and *mir-48-241-84(0)* post-dauer L3 staged larvae using Taqman real-time qRT-PCR. No statistically significant difference in the levels of lin-4 or its homolog miR-237 were found between these two mutant strains (Fig. 4D).

We next examined whether *lin-46* could potentiate lin-4 activity in a manner analogous to the potentiation of miR-48-241-84 activity by *nhl-2* (though not necessarily via the same molecular mechanism). We addressed this question using the same *lin-4(gf)* assay that we used to assess whether *nhl-2* could affect lin-4 activity. We found that *lin-46* was required for the complete expression of *lin-4(gf)* phenotypes during continuous development (supplementary material Fig. S3), and that, in this context, *lin-46* appears to potentiate lin-4 activity via the *lin-14* 3′UTR (supplementary material Fig. S4).

LIN-46 and NHL-2 potentiate downregulation of HBL-1 by lin-4 and let-7 family miRNA pathways after dauer quiescence

Downregulation of HBL-1 is required for progression to L3 cell fates during continuous development, and *mir-48-241-84*, *lin-46* and *nhl-2* all contribute to *hbl-1* downregulation (Abbott et al., 2005; Hammell et al., 2009a). HBL-1 is also downregulated during dauer quiescence, via a mechanism involving the *hbl-1* 3′UTR (Karp and Ambros, 2011). To test whether mis-expression of HBL-1 causes the retarded postdauer phenotypes of *lin-4(0); mir-48-241-84(0)* and *lin-4(0); mir-84(0)* animals, *lin-4(0); mir-48-241-84(0); hbl-1(lf)* and *lin-4(0); mir-84(0); hbl-1(lf)* strains were constructed. *hbl-1* null alleles are not available, and RNAi suggests that they would probably be embryonic lethal (Fay et al., 1999). In both cases, reduction of *hbl-1* partially suppressed the retarded phenotypes of both post-dauer and continuously developing animals (Fig. 3). This suggests that the retarded phenotype of *lin-4(0); mir-48-241-84(0)* mutants results from mis-expression of HBL-1. The observed incomplete suppression by *hbl-1(lf)* could be due to mis-expression of additional targets, or to residual *hbl-1* activity from the *hbl-1(lf)* allele. It is noteworthy that the same *hbl-1(lf)* allele very efficiently suppressed the *lin-46(0); mir-48-241-84(0)* post-dauer phenotypes (Table 2, lines 25, 26). This suggests a lesser degree of *hbl-1* mis-expression during post-dauer development in *lin-46(0); mir-48-241-84(0)* animals compared with *lin-4(0); mir-48-241-84(0)* animals. Finally, the retarded post-dauer phenotypes of *nhl-2(0); lin-46(0)* are also suppressed by reduction of *hbl-1* (Table 2, lines 35, 36). This supports the idea that *lin-46* and *nhl-2* might function in the same pathways as lin-4 and let-7 family miRNAs to regulate HBL-1 during post-dauer development.

Consistent with a direct role for lin-4 in downregulation of HBL-1, the *hbl-1* 3′UTR contains a putative lin-4 binding site. However, during continuous development, *lin-4* has not been shown to contribute substantially to the downregulation of HBL-1 between the L2 and L3 stages, although *lin-4* does affect *hbl-1* expression at later stages (Abrahante et al., 2003; Lin et al., 2003). Our results indicate that for proper development after dauer quiescence, lin-4 must act together with let-7 family miRNAs to repress HBL-1 levels; animals multiply deficient for *lin-4* and *mir-48-241-84* exhibit persistent expression of the *hbl-1* reporter during the post-dauer L3 stage (Fig. 5E,F). Loss of *lin-4* alone can also cause persistent expression of the *hbl-1* reporter (Fig. 5I,J), consistent with the retarded phenotypes observed in *lin-4(0)* animals after dauer quiescence (Fig. 3) (Liu and Ambros, 1991).

We also tested two known lin-4 targets, *lin-14* and *lin-28*, for their involvement in post-dauer retarded phenotypes (Lee et al., 1993; Moss et al., 1997; Wightman et al., 1993). The *lin-14* and *lin-28* 3′UTRs contain putative binding sites for let-7 family as well as lin-4 miRNAs. To test *lin-14*, we made use of a complex *lin-14* allele *(n355n679)*, which lacks most of the *lin-14* 3′UTR, rendering it insensitive to miRNAs. In particular, all predicted lin-4 and let-7 family binding sites are deleted (G. Hayes and G. Ruvkun, personal communication) (Reinhart and Ruvkun, 2001). In addition, this allele includes a mis-sense mutation that lowers *lin-14* activity sufficiently to allow dauer formation to occur (Liu and Ambros, 1989; Reinhart and Ruvkun, 2001). If overexpression of *lin-14* is the cause of the post-dauer retarded phenotypes observed when lin-4 and let-7 family miRNAs are deleted, then removing *lin-4* or *mir-84* should not affect post-dauer phenotypes in a *lin-14(n355n679)* background. Instead, loss of *lin-4*, *mir-84* or both miRNAs enhanced the retarded phenotype of *lin-14(n355n679)* during both continuous and post-dauer development (Fig. 5K). Indeed, *lin-4(0); lin-14(n355n679) mir-84(0)* animals display a more retarded phenotype if their development is interrupted by dauer quiescence (Fig. 5K), similar to *lin-4(0); lin-14(n179) mir-84(0)* animals (Fig. 3). Therefore, these retarded phenotypes are caused by target genes other than *lin-14*, probably *hbl-1*. Additionally, *lin-46(0)* enhances the post-dauer retarded phenotype of *lin-14(n355n679)* (Table 2, lines 39, 40; P=0.0002, Fisher's Exact Test). This is consistent with *lin-46* promoting post-dauer cell fate progression through a mechanism that does not involve the *lin-14* 3′UTR, such as through lin-4 regulation of *hbl-1*.

Unfortunately, we could not perform an equivalent experiment to test *lin-28*, because of the lack of availability of an miRNA-independent allele. LIN-28 expression was therefore examined in wild-type and mutant post-dauer animals. A rescuing LIN-28 reporter is mis-expressed in both *lin-4(0)* and *lin-4(0); mir-48-241-*

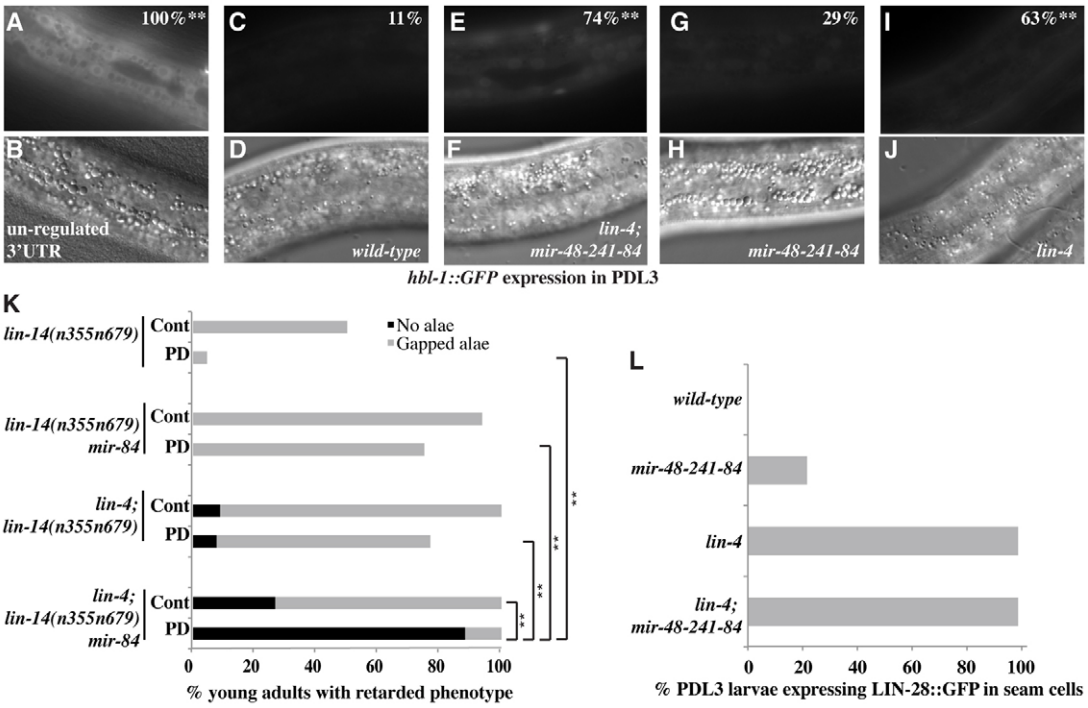

Fig. 5. Expression or activity during post-dauer development of known targets of lin-4 or the let-7 family microRNAs miR-48, miR-241 and miR-84. (**A-J**) *lin-4* and *mir-48*, *mir-241* and *mir-84* contribute to downregulation of *hbl-1::GFP* during post-dauer development. The hypodermis of post-dauer L3-staged larvae was visualized with GFP fluorescence (40 ms) (A,C,E,G,I) or DIC optics (B,D,F,H,J), 63× objective. Anterior is to the left. Note that *ctIs39* (C,E,G,I) and *ctIs37* (A) contain the identical regulatory sequence, apart from the 3′-UTR. *ctIs39* contains the *hbl-1* 3′UTR, whereas *ctIs37* contains the unregulated *unc-54* 3′UTR (Fay et al., 1999). Percentage of larvae that display unambiguous GFP expression in hypodermal nuclei at a 40 ms exposure time, out of the total number examined is shown. ***P*<0.01, Fisher's Exact Test compared with wild type (C). (**K**) Retarded alae phenotype of *lin-4(e912); mir-84(n4037)* and controls when *lin-14* activity is increased by a complex mutation that deletes all predicted lin-4 and miR-84 binding sites from the *lin-14* 3′UTR [*lin-14(n355n679)*]. ***P*<0.01, Fisher's Exact Test. Cont, continuous development; PD, post-dauer. $n \geq 15$. (**L**) Percentage of post-dauer L3 larvae that express a rescuing LIN-28::GFP translational fusion in seam cells (Moss et al., 1997). $n \geq 21$.

84(0) animals during the post-dauer L3 stage (Fig. 5L). Therefore, mis-expression of LIN-28 could contribute to post-dauer retarded phenotypes in strains that lack *lin-4*. However, loss of *lin-28* does not suppress the post-dauer retarded phenotypes of *lin-46(0); mir-48-241-84(0)* (Table 2, lines 25, 27), consistent with the idea that *hbl-1* mis-expression is the major cause of the post-dauer retarded phenotypes in this strain.

Dauer quiescence affects the requirement for core miRISC components

We wondered whether dauer quiescence could affect the requirement for core components of the miRISC. Two core components of the miRISC are Argonaute proteins and GW182 proteins (reviewed by Krol et al., 2010). In *C. elegans*, each of these protein classes is represented by two partially redundant homologs: the Argonautes ALG-1 and ALG-2, and the GW182 proteins AIN-1 and AIN-2. Although simultaneous loss of both *alg-1* and *alg-2* or simultaneous loss of both *ain-1* and *ain-2* causes embryonic lethality, single mutants are viable (Ding et al., 2005; Grishok et al., 2001; Zhang et al., 2007). However, *alg-1(0)* and *ain-1(0)* single mutant animals display various pleiotropic defects, including notably retarded alae defects, consistent with a reduced ability of let-7 family miRNAs to downregulate *hbl-1* in these backgrounds (Brenner et al., 2010; Ding et al., 2005; Grishok et al., 2001; Hammell et al., 2009b; Zhang et al., 2007). Indeed, an *hbl-1* reporter is mis-expressed (albeit at low penetrance) in *ain-1(0)* mutant larvae (Hammell et al., 2009b).

By contrast, the *alg-1(0)* and *ain-1(0)* retarded phenotypes are not evident after dauer quiescence, suggesting that *alg-2* and *ain-2* substitute more effectively for their counterparts after dauer quiescence (Table 2, lines 41, 42, 44). However, *alg-2(0)* mutant animals do not display retarded post-dauer phenotypes (Table 2, line 43), indicating that the lack of *alg-1(0)* post-dauer phenotype cannot be explained by a specificity of *alg-2* for post-dauer development. Interestingly, *lin-46(0); alg-1(0)* double mutants display a penetrant post-dauer retarded phenotype (Table 2, line 46). By contrast, *nhl-2(0); alg-1(0)* double mutants display only a mild post-dauer retarded phenotype, similar to *alg-1(0)* single mutants (Table 2, lines 41, 42, 45). Thus, like *mir-48-241-84*, *alg-1* appears to act in the same pathway as *nhl-2* but in parallel to *lin-46*. One possibility therefore is that *alg-1(0)* retarded phenotypes observed during continuous development could be due primarily to a reduction in the activity of the let-7 family miRNAs.

DISCUSSION

C. elegans larvae develop through one of two alternative life histories depending on environmental conditions: animals with ample resources develop continuously through the four larval stages, whereas in response to certain stresses the progression of developmental events is interrupted between the L2 and L3 stages by dauer larva quiescence, which is of indefinite length (Fielenbach and Antebi, 2008). Strikingly, precisely the same sequence of cell fate events occurs during the L3 and L4 stages of post-dauer

development as during the L3 and L4 stages of continuous development. The interruption of development by dauer between the L2 and L3 would pose a challenge if developmental timing mechanisms controlling L2-to-L3 cell fate transitions were to rely on a strictly time-critical sequence of molecular events, as appears to occur during continuous development (Abbott et al., 2005; Rougvie, 2001). Previous findings had shown that, in fact, alternative mechanisms are employed during continuous vs post-dauer development to control the timing of L3 cell fates (Liu and Ambros, 1991). That previous study was not able to identify the molecular basis of these alternative developmental timing mechanisms. Here, we provide insight into the manner in which heterochronic gene activity is modulated in order to accommodate the interruption of development by dauer quiescence. We provide evidence that the post-dauer suppression of *mir-48-241-84(0)* retarded phenotypes results, at least in part, from a potentiation of lin-4 activity after dauer quiescence. Our data indicate that dauer quiescence causes a shift from reliance primarily on let-7 family miRNAs for the expression of L3 cell fates, to a reliance on parallel activity by both lin-4 and let-7 family miRNAs and, furthermore, that this shift requires the activity of NHL-2 and LIN-46. Indeed, disruption of both of these parallel pathways causes more severe phenotypes after dauer quiescence than in continuously developing animals. As described below, we propose that the quiescence-specific regulation of miRNA expression on the one hand, and activity on the other hand contributes to the robustness with which cell fate transitions occur despite the interruption of development by dauer quiescence.

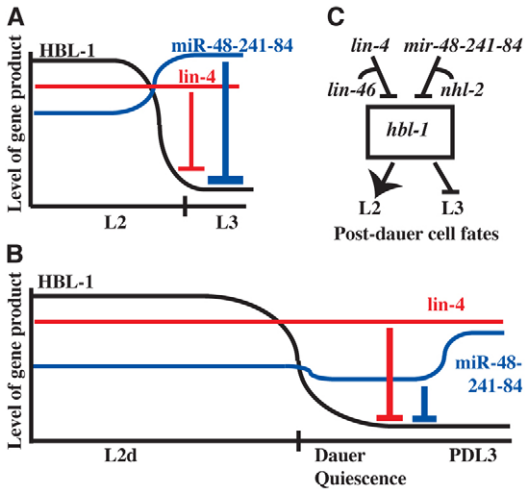

Fig. 6. Model of parallel miRNA pathways after dauer quiescence. (**A**,**B**) Schematic of the changing levels of HBL-1 (as extrapolated from *ctIs39[hbl-1::GFP::hbl-1]* (Fig. 5) (Abbott et al., 2005; Karp and Ambros, 2011), and mature miR-48-241-84 and lin-4 miRNAs (Karp et al., 2011) during the L2 and L3 stage of continuous development (A), or the pre-dauer L2d, dauer quiescence, and post-dauer L3 stages of dauer-interrupted development (B). High HBL-1 levels promote L2 cell fate and oppose L3 cell fate. *mir-48-241-84* downregulate *hbl-1* and promote progression to L3 cell fate during continuous development (Abbott et al., 2005; Abrahante et al., 2003; Lin et al., 2003), and levels of these miRNAs are high during the L3 stage (A) (Abbott et al., 2005; Karp et al., 2011). Although lin-4 is present in continuous development, it plays a minor role in repression of HBL-1 relative to *mir-48*, *mir-241* and *mir-84* (Abbott et al., 2005; Lin et al., 2003). By contrast, *lin-4* plays a major role in repression of HBL-1 during post-dauer L3, perhaps in part to accommodate the lower combined levels of miR-48, miR-241 and miR-84, compared with continuous L3 (B; Table 1). (**C**) A genetic model of the proposed parallel miRNA pathways that promote progression to L3 cell fate after dauer quiescence by downregulation of HBL-1 (and possibly other factors). In this model, *lin-46* acts with *lin-4*, and might potentiate lin-4 microRNA activity, whereas *nhl-2* potentiates the activity of let-7 family miRNAs (see main text).

Previous work has shown that levels of certain mature let-7 family miRNAs are reduced before and during dauer quiescence, owing to the activity of the dauer-promoting form of the DAF-12 nuclear hormone receptor (Bethke et al., 2009; Hammell et al., 2009a; Karp et al., 2011). This reduction of let-7 family levels is important to allow the continued expression of HBL-1 throughout the extended pre-dauer L2d stage, which is >50% longer than the rapid L2 stage of continuous development (Fig. 6A,B) (Bethke et al., 2009; Golden and Riddle, 1984; Hammell et al., 2009a; Karp and Ambros, 2011). However, as larvae recover from dauer quiescence and prepare to express L3 cell fates, levels of miR-48 and miR-241 remain low relative to continuously developing L3-staged larvae (Table 1, Fig. 6B) (Karp et al., 2011). Despite these lower levels of some let-7 family miRNAs, *hbl-1* is efficiently downregulated in post-dauer-L3 staged larvae, in a 3′UTR-dependent manner (Fig. 5). By contrast, lin-4 levels remain constant after the L1 stage, and are not affected by dauer quiescence (Fig. 6) (Karp et al., 2011). Perhaps this stable expression is the reason that reliance on *lin-4* for progression to L3 cell fates increases after dauer quiescence. Finally, we propose that *lin-46* and *nhl-2* potentiate the activity of lin-4 and let-7 family miRNAs, respectively, to regulate the proper progression to L3 cell fates after dauer quiescence (Table 2, Fig. 4). As the expression of rescuing LIN-46 and NHL-2 reporters (Hammell et al., 2009b; Pepper et al., 2004) is not obviously increased during or after dauer quiescence (not shown), it will be interesting to discover at what level these factors are regulated during dauer-interrupted development.

In conclusion, we have identified a mechanism that enables cell fate progression to be robust to the interruption of development by a period of quiescence through alteration of let-7 family and lin-4 microRNA activities. Because lin-4 and let-7 miRNA families are evolutionarily conserved, similar relationships among these microRNAs could be operative in the context of developmental contingencies in other organisms (Ibáñez-Ventoso et al., 2008). let-7 and miR-125 (the lin-4 homolog in other species) family microRNAs are generally, though not exclusively, associated with promoting differentiation in a variety of cell types, analogous to their roles in cell fate progression in *C. elegans* (Mallanna and Rizzino, 2010; Nimmo and Slack, 2009). In flies, certain miRNAs have been shown to provide robustness to environmental stress, and the importance of miRNAs in the response to various types of stress has been proposed in mammals as well (Kosik, 2010; Leung and Sharp, 2010; Li et al., 2009). In general, such stress-buffering roles for microRNAs could reflect the sort of conditional shift in microRNA activity on an mRNA target like that described here. These findings also underscore the importance of protein co-factors that modulate miRNA activity. As potentiation of miRNA activity by the NHL-2 homolog TRIM32 has been described in mammals (Schwamborn et al., 2009), it will be interesting to explore whether TRIM32 and other miRNA co-factors are important for the robustness of cell fate specification in mammalian stem/progenitor cells to lengthy quiescent periods.

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Competing interests statement
The authors declare no competing financial interests.

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