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Caenorhabditis elegans microRNAs of the let-7 family act in innate immune response circuits and confer robust developmental timing against pathogen stress

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Edited by Phillip A. Sharp, MIT, Cambridge, MA, and approved March 26, 2015 (received for review November 30, 2014)

Animals maintain their developmental robustness against natural stresses through numerous regulatory mechanisms, including the posttranscriptional regulation of gene expression by microRNAs (miRNAs). Caenorhabditis elegans miRNAs of the let-7 family (let-7-Fam) function semiredundantly to confer robust stage specificity of cell fates in the hypodermal seam cell lineages. Here, we show reciprocal regulatory interactions between let-7-Fam miRNAs and the innate immune response pathway in C. elegans. Upon infection of C. elegans larvae with the opportunistic human pathogen Pseudomonas aeruginosa, the developmental timing defects of certain let-7-Fam miRNA mutants are enhanced. This enhancement is mediated by the p38 MAPK innate immune pathway acting in opposition to let-7-Fam miRNA activity, possibly via the downstream Activating Transcription Factor-7 (ATF-7). Furthermore, let-7-Fam miRNAs appear to exert negative regulation on the worm’s resistance to P. aeruginosa infection. Our results show that the inhibition of pathogen resistance by let-7 involves downstream heterochronic genes and the p38 MAPK pathway. These findings suggest that let-7-Fam miRNAs are integrated into innate immunity gene regulatory networks, such that this family of miRNAs modulates immune responses while also ensuring robust timing of developmental events under pathogen stress.

let-7 family microRNAs | p38 | Pseudomonas aeruginosa | developmental timing | innate immunity

During development, animals routinely encounter environmental, physiological, and nutritional challenges that threaten to compromise the robust execution of developmental programs. Therefore, the genetic programming of development includes mechanisms to ensure that developmental events occur flawlessly despite stressful conditions (1, 2). Several studies indicate that microRNAs (miRNAs) are used to maintain the robustness of biological processes under stress conditions (3–10). miRNAs are endogenous, noncoding, small RNAs that posttranscriptionally regulate gene expression primarily through binding to the 3′UTR of target miRNAs, which results in translation inhibition and/or mRNA degradation (11). miRNAs with the same seed sequence (nucleotides 2–7 of the mature miRNA sequence), which are predicted potentially to share the same set of targets (12), are grouped into a family.

The miRNA lin-4 and the miRNAs of the let-7 family (let-7-Fam) are central to the regulation of pluripotency and differentiation in many animal systems, including mammals (13–18). Four Caenorhabditis elegans let-7-Fam miRNAs, let-7, mir-48, mir-84, and mir-241, function in concert to repress key heterochronic gene targets, including daf-12, lin-41, and hbl-1, to stage-specifically regulate the timing of the hypodermal seam cell fates (16, 19–22). In C. elegans, the temporal patterns of cell division and cell fate during larval development are exquisitely invariant, even though larvae develop as free-living inhabitants of a changing environment in soil and decaying plant materials.

In the wild, worms encounter a variety of bacteria species as food sources. Several of these species are proven to be pathogenic to C. elegans (23), thus representing environmental stressors. Interestingly, recent studies have shown that let-7-Fam miRNAs seem not only to regulate developmental events but also to regulate the antibacterial and inflammatory response in several animal systems (24–28). At least two of the C. elegans let-7-Fam miRNAs are shown to regulate the worm’s survival to Pseudomonas aeruginosa infection (27). These previous findings suggest that let-7-Fam miRNAs could possibly coordinate developmental timing and innate immune responses so as to contribute to the robustness of development during pathogen infection.

Here, we show that let-7-Fam miRNAs are engaged in reciprocal interactions with innate immune pathways. Using genetically sensitized backgrounds, we find that the developmental timing phenotypes of let-7-Fam miRNA mutants are modified by their bacterial diet, particularly by growth on pathogenic P. aeruginosa. The let-7-Fam miRNA activity is negatively regulated on P. aeruginosaa by the p38 MAPK pathway. Moreover, let-7-Fam miRNAs exhibit negative regulation on pathogen resistance, possibly through several pathways, including p38 MAPK signaling pathway, and also through downstream heterochronic genes, particularly the let-7-Fam miRNA targets lin-41 and hbl-1. Our findings suggest that genetic redundancy among let-7-Fam miRNAs enables these miRNAs to control the expression of developmental cell fates robustly, while also modulating innate immune responses according to the pathogenicity of the worm’s bacterial diet.

Results

Growth on P. aeruginosa Aggravates the Heterochronic Phenotypes of let-7-Fam miRNA Mutants. The let-7-Fam miRNAs act semiredundantly in controlling the developmental timing of certain

Significance

Proper animal development requires the robust execution of cell fates under stressful conditions. In Caenorhabditis elegans, reciprocal interactions between heterochronic genes and the p38 innate immune pathway help to coordinate development with pathogen defense. Importantly, the robustness of developmental cell fate expression during infection depends on functional redundancy among genes encoding microRNAs (miRNAs) of the let-7 family. These findings underscore the importance of miRNA pathways in conferring robustness to developmental programs under stressful conditions and highlight roles for heterochronic genes not only as developmental timers but also as modulators of innate immune responses. The let-7 family miRNAs and p38 innate immune pathway are evolutionarily conserved; therefore, this study presents implications for similar integration of these two pathways in other animal systems.

Author contributions: Z.R. and V.R.A. designed research; Z.R. performed research; Z.R. analyzed data; and Z.R. and V.R.A. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1422858112/-/DCSupplemental.
stage-specific hypodermal seam cell fates in *C. elegans*. Loss of *let-7-Fam* miRNAs results in reiterations of early larval seam cell division patterns at later stages, and many seam cells in these mutants also fail to differentiate adult-specific cuticular structures (called adult alae) properly at the larval stage 4 (L4) molt (15, 16) (Fig. L4). These heterochronic *let-7-Fam* miRNA mutant phenotypes are easily quantified by using microscopy to measure the number of seam cells and to score for the formation of adult alae in young adults. To investigate whether different bacterial food sources could have an impact on the regulation of developmental timing by *let-7-Fam* miRNAs, we used a genetically sensitized *let-7-Fam* miRNA mutant strain [miR-48 mir-241 (nDf51)] that exhibits a partially penetrant heterochronic phenotype. Wild type (WT) animals have an average number of 16 seam cells, and 100% of the animals have complete adult alae at the young adult stage, whereas miR-48 mir-241(nDf51) animals display an average of 18.5 seam cells, and ~60% of the animals exhibit incomplete adult alae.

We scored seam cell numbers and adult alae formation for WT and miR-48 mir-241(nDf51) animals after development on six different bacterial diets, including three strains of *Escherichia coli* that are typically used as laboratory food sources (HB101, OP50, and HT115) and three other bacterial species (*Comamonas sp*. DA1877, *P. aeruginosa* PA14, and *Salmonella enterica* SL1344) that have been shown to have a significant effect on the physiological state of *C. elegans* (23, 29). As expected, WT animals exhibited no evidence of developmental timing abnormalities regardless of the bacterial diet (Fig. 1B and Table 1). However, miR-48 mir-241(nDf51) animals showed a quantitatively different seam cell phenotype, dependent upon the bacterial food source (Fig. 1B). Notably, when grown on *P. aeruginosa*, miR-48 mir-241(nDf51) animals exhibited an enhanced seam cell phenotype compared with growth on HB101 (Fig. 1B). Consistent with this enhanced seam cell phenotype, miR-48 mir-241(nDf51) animals also exhibit an enhanced adult alae phenotype on *P. aeruginosa* (Table 1). These results suggest that bacterial food source can modulate the activity of the heterochronic gene pathway. Because animals with all *let-7-Fam* miRNAs intact showed no heterochronic phenotypes on any of the bacterial diets, these results indicate that *let-7-Fam* miRNAs act redundantly to maintain the robustness of developmental timing under the influence of dietary stress.

The most dramatic effect of diet on the developmental timing phenotypes of miR-48 mir-241(nDf51) animals was from growth on the pathogenic bacterium *P. aeruginosa*. Therefore, we focused our further studies on the effects of *P. aeruginosa*. The heterochronic phenotype enhancement in miR-48 mir-241(nDf51) animals on *P. aeruginosa* suggests that the activity of the remaining family members (chiefly *let-7* and *mir-84*) may be decreased upon exposure to *P. aeruginosa*. To examine whether this decrease of *let-7-Fam* miRNA activity is restricted to certain members of the family, we tested a series of other *let-7-Fam* miRNA mutants for their developmental timing phenotypes upon *P. aeruginosa* treatment. Two other *let-7-Fam* miRNA mutants showed enhancement in their heterochronic phenotypes on *P. aeruginosa*. Specifically, miR-48(n4097); mir-84(n4037) animals showed an enhancement in seam cell phenotype (Fig. 5A), whereas let-7(n2853) animals exhibited an adult alae phenotype enhancement (Table 1). These results suggest that the activity of *mir-241* and *mir-48* could also be decreased by *P. aeruginosa* treatment. In conclusion, the activities of all four *let-7-Fam* miRNAs appear to be decreased upon growth on *P. aeruginosa*.

**Modulation of *let-7-Fam* miRNA Activity by the p38 MAPK Pathway in Response to *P. aeruginosa* Infection.** The observed effects of bacterial diet on the developmental timing phenotypes of *let-7-Fam* miRNA mutants could be caused by various properties of the bacterial food, such as nutritional quality and pathogenic toxicity. This issue is of particular interest in the case of *P. aeruginosa*, which can support *C. elegans* larval development as a sole food source yet is also a pathogen capable of infecting *C. elegans*. To determine if the pathogenicity of *P. aeruginosa* is required for the modulation of heterochronic phenotypes elicited in *let-7-Fam* miRNA mutants, we cultured miR-48 mir-241(nDf51) larvae on the gacA mutant of *P. aeruginosa* PA14. GacA is an important regulator of the cell density-dependent gene expression in *P. aeruginosa*, and it is required for the production of exoenzymes and secondary metabolites (30). The pathogenicity of the PA14 gacA strain is dramatically decreased compared with the WT PA14 (31). We observed that the enhancement of *let-7-Fam* miRNA mutant phenotypes was substantially reduced for larvae grown on PA14 gacA compared with larvae grown on WT PA14.
Table 1. Adult alae phenotypes of WT and let-7-Fam miRNA mutants cultured on different bacterial food sources

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Complete</th>
<th>Gapped</th>
<th>No alae</th>
<th>n</th>
<th>Test^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (VT1367)</td>
<td>E. coli HB101</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>E. coli OP50</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>E. coli HT115</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>N.S.</td>
</tr>
<tr>
<td>Comamonas sp. DA1877</td>
<td>P. aeruginosa PA14</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>N.S.</td>
</tr>
<tr>
<td>P. aeruginosa PA14 gacA</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>S. enterica SL1344</td>
<td>P. aeruginosa PA14 gacA</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

| mir-48 mir-241(nDf51) | E. coli HB101 | 40 | 60 | 0 | 42 |   |
|                       | E. coli OP50   | 28 | 72 | 0 | 39 | N.S.  |
|                       | E. coli HT115  | 60 | 40 | 0 | 40 | N.S.  |
| Comamonas sp. DA1877 | P. aeruginosa PA14 | 40 | 60 | 0 | 15 | N.S.  |
|                       | P. aeruginosa PA14 gacA | 8 | 92 | 0 | 37 | *** |
|                       | S. enterica SL1344 | 30 | 70 | 0 | 21 | N.S.  |

| mir-(n4097); mir-84(n4037) | E. coli HB101 | 100 | 0 | 0 | 31 |   |
|                          | P. aeruginosa PA14 | 93 | 7 | 0 | 30 | N.S.  |

| let-7(n2853) | E. coli HB101 | 0 | 77 | 23 | 30 |   |
|             | P. aeruginosa PA14 | 0 | 42 | 58 | 26 | ** |

^1The χ² test was used for comparison between E. coli HB101 (control) and other bacterial treatment for the same genotype. N.S., not significant. **P < 0.01; ***P < 0.001.

(Fig. 1B and Table 1). This observation suggests that the pathogenicity of P. aeruginosa is crucial for the modulation of let-7-Fam miRNA activity.

Although C. elegans can develop throughout postembryonic development and into the adult stage with P. aeruginosa as a sole food source, adults succumb to P. aeruginosa infection within a few days. A key pathway that enables larvae to survive on P. aeruginosa is the conserved p38 MAPK cascade (TIR-1/NSY-1/SEK-1/PMK-1) that initiates the innate immune response for antibacterial defenses (32, 33). We found that genetic removal of any p38 MAPK pathway component blocks the enhancement of let-7-Fam miRNA activity when animals are grown on P. aeruginosa (Fig. 1B and Table 1). These data suggest that the p38 MAPK pathway negatively regulates let-7-Fam miRNAs and that this regulation is tissue- and stage-specific.

PMK-1 and the p38 MAPK pathway are crucial for the modulation of let-7-Fam miRNA activity. Further more, one of the major downstream transcription factors of the p38 MAPK pathway is ATF-7 (34). We therefore tested whether p38 loss of function could affect the transcriptional activities of let-7-Fam miRNAs. We observed that mir-48 mir-241(nDf51) animals homozygous for a null mutation of ATF-7 [atif-7(qd22q130)] or for a phosphorylation-defective allele of ATF-7 [atif-7(qd22p)] (34) did not exhibit enhanced heterochronic phenotypes on P. aeruginosa (Fig. 2 A and C). Interestingly, even for animals grown on E. coli, loss of ATF-7 led to a significant enhancement of heterochronic phenotypes in mir-48 mir-241(nDf51) animals (Fig. 2B). Therefore, ATF-7 seems to exhibit a positive regulation on let-7-Fam miRNA activity when animals are grown on E. coli, and upon P. aeruginosa infection, this regulation could be altered depending on the phosphorylation potential of ATF-7. Models for the effects of different atf-7 alleles on let-7-Fam miRNA activity are shown in Fig. S2 B–D.

**Effects of P. aeruginosa Infection and p38 Signaling on let-7-Fam miRNA Gene Expression.** To investigate possible mechanisms for the apparent reduction of let-7-Fam miRNA activity upon P. aeruginosa infection, we first measured the mature let-7-Fam miRNA levels in animals exposed to P. aeruginosa throughout larval development to young adults. We observed an approximately twofold decrease in mir-241 but no significant change in the levels of other let-7-Fam miRNAs on P. aeruginosa (Fig. 3). Because we did not observe any reduction of mir-84 or let-7 levels on P. aeruginosa, the enhanced heterochronic phenotypes of mir-48 mir-241(nDf51) animals cannot simply be accounted for by a reduction of let-7-Fam miRNA levels in whole animals. Upon P. aeruginosa infection, it is possible that let-7-Fam miRNAs are regulated through modulation of the potency of their action, without affecting their overall levels. However, because our miRNA quantitation was performed on total RNA from whole worms, we would not necessarily detect tissue-specific changes in let-7-Fam miRNA levels.

The results of our phenotypic analysis indicate that the activities and/or levels of let-7-Fam miRNAs are modulated by the p38 MAPK pathway. Because the p38 MAPK pathway is known to regulate gene expression transcriptionally, we examined whether p38 loss of function could affect the transcriptional activities of let-7-Fam miRNA genes. For these studies, we used transgenic worms carrying transcriptional reporters expressing GFP from let-7-Fam miRNA gene promoters. We observed stage- and tissue-specific increases of GFP expression for Pmir-48::GFP, Pmir-84::GFP, and Plet-7::GFP in the pmk-1 mutant compared with WT animals (Fig. 4). The developmental stages at which we detected differences in reporter activities between WT and pmk-1 mutant animals were L4 and young adult stages for Pmir-48::GFP, day 1 adult for Pmir-84::GFP, and L3, young adult, and day 1 adult for Plet-7::GFP. For mir-48 and mir-84, up-regulation of these reporters by loss of pmk-1 was observed in the hypodermis, suggesting a cell-autonomous effect of pmk-1 signaling on let-7-Fam miRNA transcription (Fig. 4 A, B, D, and E). Also, loss of pmk-1 resulted in detectable Pmir-84::GFP expression in the hyp7 syncytium compared with essentially undetected expression of Pmir-84::GFP in hyp7 for WT animals. For the let-7 transcriptional reporter, an increase of reporter activity in the absence of pmk-1 was apparent in the intestine but not in the hypodermis (Fig. 4 C and F). These data suggest that the p38 MAPK pathway negatively regulates the transcription of let-7-Fam miRNAs and that this regulation is tissue- and stage-specific.
miRNAs. Our results indicate that during larval development, worms actively down-regulate their let-7-Fam miRNA activity upon exposure to *P. aeruginosa*. Additionally, we found that pmk-1 regulates the transcription of let-7 in the intestine, a major tissue for pathogen resistance in *C. elegans* (35). Therefore, we hypothesized that this inhibition of let-7-Fam miRNA activity may reflect a survival response to the presence of pathogen during larval development. Central to this hypothesis is the premise that let-7-Fam miRNAs may negatively regulate pathogen resistance; hence, the down-regulation of their activity would promote survival. Accordingly, we examined the survival capacity of several let-7-Fam miRNA mutants placed on *P. aeruginosa* beginning as L4-stage animals.

First, we measured the survival of animals homozygous for let-7(mg279), a weak loss-of-function allele (with very weak developmental timing phenotypes), and animals homozygous for let-7(n2853), a stronger loss-of-function allele (with developmental timing phenotypes resembling the let-7 null mutant) on *P. aeruginosa*. We observed that let-7(mg279) animals displayed a dramatic improvement in survival against *P. aeruginosa* infection compared with WT animals (Fig. S4A), whereas let-7(n2853) animals displayed the opposite, a decreased survival on *P. aeruginosa* (Fig. S4B). Interestingly, populations of the double-mutant mir-48 mir-241(nDf51) and mir-48(n4097); mir-84(n4037) animals exhibited biphasic survival curves (Fig. S4A). Apparently, for each of these double mutants, a portion of the population dies faster than WT, whereas the remainder of the population survives longer than WT on *P. aeruginosa*.

To confirm that the opposite pathogen survival phenotypes of distinct let-7 mutants are attributable to the reduction of let-7 gene activity, we tested the ability of a single-copy let-7 transgene (nals380), which fully rescues the developmental phenotypes of both let-7 alleles (Fig. S3A and S3B), to also rescue the characteristic survival phenotypes of let-7(mg279) and let-7(n2853) mutants. nals380 rescued the extended survival phenotype of let-7(mg279) animals (Fig. S3C) and the shortened survival phenotype of let-7(n2853) animals on *P. aeruginosa* (Fig. S3D). Interestingly, the let-7 rescuing transgene also rescued the biphasic survival phenotype of mir-48 mir-241(nDf51) and mir-48(n4097); mir-84(n4037) animals (Fig. S3E and F). These results support the conclusions that let-7-Fam miRNAs function redundantly to regulate survival on *P. aeruginosa* and that all of the survival phenotypes of let-7-Fam miRNA mutants on *P. aeruginosa* result from different degrees of let-7-Fam miRNA loss of function.

To determine whether the complex survival phenotypes of let-7-Fam miRNA mutants on *P. aeruginosa* could reflect
The *let-7*-Fam miRNAs regulate a set of specific target gene mRNAs during larval development to control developmental timing. For *let-7*, the most prominent targets are *lin-41* and *hbl-1* (20–22). To determine if *lin-41* and *hbl-1* are downstream of *let-7* in the regulation of pathogen resistance, we tested the survival of *let-7*(*mg279*) animals on *P. aeruginosa* in the presence of *lin-41* or *hbl-1* loss-of-function mutations. Interestingly, animals with partial loss of function for *lin-41* exhibited decreased survival upon *P. aeruginosa* infection, and *lin-41* also suppressed the enhanced survival phenotype of *let-7*(*mg279*) animals (Fig. 6A). These findings suggest that aside from its developmental timing role downstream of *let-7*, *lin-41* is also possibly one of the targets of *let-7* for the regulation of pathogen resistance.

Downstream Heterochronic Genes May Mediate the Enhanced Survival of *let-7*(*mg279*) Animals on *P. aeruginosa*. To investigate further the mechanism by which *let-7*-Fam miRNAs negatively regulate pathogen resistance, we focused on understanding the enhanced survival phenotype of *let-7*(*mg279*) animals. This emphasis was for several reasons. First, *let-7*-Fam miRNAs contribute redundantly to survival on *P. aeruginosa*. Hence, *let-7* can serve as a proxy for the other *let-7*-Fam miRNA genes in our genetic analyses. Additionally, *let-7*(*mg279*) animals exhibit a striking survival phenotype on *P. aeruginosa* that we expected would be more tractable for genetic analysis than the complex survival phenotypes of the other *let-7*-Fam miRNA mutants. Finally, among the *let-7*-Fam mutants tested here, *let-7*(*mg279*) animals exhibited the least reduced lifespan on *E. coli*. Therefore, using *let-7*(*mg279*) animals to explore the mechanism of *let-7*-Fam miRNAs in pathogen resistance could minimize potentially confounding effects of lifespan in our analysis.
hbl-1(mg285) also suppressed the enhanced survival phenotype of let-7(mg279) on *P. aeruginosa*, but unlike lin-41(ma104), the hbl-1(mg285) mutation alone did not cause any obvious survival phenotype (Fig. 6B). Because hbl-1(mg285) is only a partial loss-of-function mutation, epistasis cannot be interpreted unequivocally, but these results are consistent with both hbl-1 and lin-41 functioning downstream of let-7 for pathogen sensitivity. We note that hbl-1 has been shown to regulate the transcription of let-7 negatively (36). Therefore, the suppression of let-7(mg279) enhanced survival phenotype by hbl-1(mg285) could reflect a derepression of let-7 gene transcription.

In addition, we examined the longevity on *E. coli* HB101 of lin-41(ma104) and hbl-1(mg285) animals along with animals mutant for lin-41(ma104) or hbl-1(mg285) in combination with let-7(mg279) (Fig. 6C and D). The results suggest that lin-41 and hbl-1 positively regulate lifespan on *E. coli*. However, because hbl-1(mg285) animals exhibit similar survival on *P. aeruginosa* compared with WT animals (Fig. 6B), the suppression of hbl-1(mg285) on let-7(mg279) animals’ enhanced survival phenotype on *P. aeruginosa* is likely caused by a role of hbl-1 in pathogen resistance, rather than longevity. On the other hand, the suppression of let-7(mg279) animals’ survival phenotype on *P. aeruginosa* by lin-41 could be contributed, in part, by the role of lin-41 in longevity and, in part, by a role in pathogen resistance.

The p38 MAPK Pathway Is Required for the Prolonged Survival Phenotype of let-7(mg279) Animals on *P. aeruginosa*. In addition to targeting lin-41 and hbl-1, let-7 could (directly or indirectly) regulate genes in the innate immune response pathways to regulate pathogen resistance negatively. Interestingly, in the p38 MAPK pathway, tir-1, nsy-1, and sek-1 are all predicted targets of let-7-Fam miRNAs (37). Therefore, we hypothesized that let-7 could function upstream of the p38 MAPK pathway to regulate survival on *P. aeruginosa*. Accordingly, we examined the survival of animals doubly mutant for let-7(mg279) and loss-of-function mutations in p38 MAPK pathway components. We observed that loss of any p38 MAPK pathway component could suppress the enhanced survival phenotype of let-7(mg279) animals (Fig. 6E and Fig. S4). Also, previous studies indicate that the p38 MAPK pathway does not affect longevity in *C. elegans* (32, 38). Therefore, these results indicate that let-7(mg279) animals’ enhanced pathogen resistance phenotype requires the p38 MAPK pathway.

To examine whether let-7 may regulate factors upstream of p38 in the MAPK pathway, we tested for an elevated level of phospho-p38 in protein extracts of let-7(mg279) animals compared with extracts of WT animals. These experiments did not conclusively reveal an impact of let-7 on p38 phosphorylation (Fig. S3). However, because these assays for phosphorylated p38 were performed on extracts of whole animals, it is possible that let-7 may function upstream of the p38 MAPK pathway in a tissue-specific manner. Although, genetically, the p38 MAPK pathway is downstream of let-7 in regulating survival on *P. aeruginosa*, we cannot rule out the possibility that it might function in parallel with lin-41 and hbl-1 for the prolonged survival of let-7(mg279) animals on *P. aeruginosa*.
between WT and let-7(mg279) animals (Fig. S6). However, after 48 h of infection, we observed a significant reduction of outer membrane vesicles (OMVs) in the intestinal lumen of let-7(mg279) animals compared with WT animals (Fig. 7). OMVs are secreted by P. aeruginosa and known to function as a virulence factor and toxin delivery platform (39). These results suggest that the prolonged survival of let-7(mg279) animals on P. aeruginosa reflects an enhanced countervirulence activity in let-7(mg279) animals compared with WT animals.

Discussion

Animals are challenged by diverse physiological and environmental stresses during development but they nevertheless execute temporal and spatial patterns of developmental events with remarkable robustness. The let-7-Fam miRNAs function in the heterochronic gene pathway to regulate the specification and execution of stage-specific cell fates during C. elegans larval development (15, 16). Here, we report evidence that the activity of let-7-Fam miRNAs is regulated by the p38 innate immune response pathway during growth on the bacterial pathogen P. aeruginosa (Fig. 8A). Furthermore, we show that let-7-Fam miRNAs and several other heterochronic genes also function in the modulation of the worm’s resistance against P. aeruginosa infection (Fig. 8B). Our findings uncover fundamental connections between the heterochronic gene pathway and innate immune response pathway, and suggest that these connections could serve to optimize the coordination of temporal cell fate specification and antibacterial responses in the developing larvae.

An important implication of our findings is that the collective function of let-7-Fam miRNAs provides robustness to the specification of temporal fates in WT C. elegans larvae, especially when they are challenged by pathogenic bacteria. This conclusion is based on a few interesting findings in our study. First, growth on pathogen affects temporal seam cell fate phenotypes only in sensitized genetic backgrounds where the full complement of let-7-Fam miRNAs is compromised by mutation. For WT, most of the single
let-7 miRNA activity upon let-7-Fam infection, presumably depending on its phosphor-
mRNA mutant miRNA activity, certain as-
mRNAs func-

P. aeruginosa miRNA geno-

miRNA mutants reveal a regulatory circuit con-
P. aeruginosa

miRNA sensitized genetic background, albeit with less
E

miRNA activity with and without

manifests, and let-7(mg279) mir-84(n4037) animals, developmental
timing phenotypes were not affected by P. aeruginosa infection (Fig. S1A), which indicates that the down-regulation of let-7-Fam
miRNA activity caused by P. aeruginosa is tolerated in these
animals (without compromising temporal cell fate specification)
due to the genetic redundancy of let-7-Fam miRNAs. Moreover,
we also observed an increase in the variation of seam cell number
in let-7-Fam miRNA mutants as the activity of this miRNA family
decreases, and the variation in seam cell number is enhanced by
P. aeruginosa infection in those same let-7-Fam miRNA mutants
where we observed P. aeruginosa-induced heterochronic pheno-
type enhancement (Fig. S1B). This variation of seam cell number
could reflect noisy target gene expression caused by reduction of
let-7-Fam miRNA activity, and could also represent a breakdown in the robustness of seam cell fate determination. These data
(Fig. S1B) further support the idea that let-7-Fam miRNAs func-
tion to protect the robustness in the temporal patterns of the seam
cell program, especially in the face of P. aeruginosa infection.

We observed that certain other bacterial food sources, besides
P. aeruginosa, can also modulate the seam cell phenotype in the
let-7-Fam miRNA sensitized genetic background, albeit with less
potency than P. aeruginosa (Fig. 1B). One surprising result is that
E. coli OP50, a routine food source for most laboratories, also
enhances the seam cell numbers of mir-48 mir-241(nDf51) ani-
mals (although this enhancement is very mild compared with the
effects of P. aeruginosa and there is no change in the adult alae
phenotype). Interestingly, pmk-1(nm25) partially suppresses the
enhanced heterochronic phenotypes of mir-48 mir-241(nDf51)
animals on OP50 (Fig. S1 C–E), consistent with previous findings
that OP50 could be slightly pathogenic to worms (40). Therefore,
the enhanced seam cell phenotype of the let-7-Fam miRNA
mutant on OP50 could be partially due to the slight pathoge-
nicity of the bacteria. However, other properties of OP50 could
contribute to the phenotype as well because pmk-1(nm25) did
not fully suppress all of the enhanced heterochronic phenotype of
mir-48 mir-241(nDf51) animals on OP50 (Fig. S1E). Nevertheless,
it is noteworthy that WT larvae exhibit robustly normal de-
velopmental timing regardless of the bacterial diets tested here,
supporting our conclusion that the full let-7-Fam miRNA geno-
type underlies the robustness of developmental timing to dietary
and/or pathogenic stress.

Our findings that P. aeruginosa elicits phenotypic modulations of
let-7-Fam miRNA mutants reveal a regulatory circuit connect-
ing the p38 MAPK innate immunity pathway and the het-
erochronic gene developmental timing pathway via let-7-Fam
miRNAs. The role of the p38 pathway in this process is sup-
ported by our observation that loss-of-function mutations that
disable the p38 MAPK block the phenotypic enhance-
ment of let-7-Fam miRNA mutants on P. aeruginosa. Also in
support of this conclusion is our result that constitutive activa-
tion of p38 by knocking down of a negative regulator of p38
signaling, vhp-1 (the p38 phosphatase) (41, 42), phenocopied the
P. aeruginosa-induced heterochronic phenotype enhancement in
let-7-Fam miRNA mutants (Fig. S2 F and G).

Although our findings show that the p38 MAPK pathway is
involved in regulation of let-7-Fam miRNA activity, certain as-
pects of this regulatory circuit differ in interesting ways from
canonical p38 innate immune signaling. First, the tir-1(ok1052)
allele that lacks the N-terminal Heat/Armadillo motif of the
protein blocks the enhancement of let-7-Fam miRNA mutant
phenotypes (Fig. S2A), even though this motif has been shown to
be dispensable for pathogen resistance (43, 44). This result sug-
gests that activation of the p38 MAPK pathway is necessary for the
regulation of let-7-Fam miRNA activity upon P. aeruginosa
infection but that p38 signaling alone without the activity of the
Heat/Armadillo motif of TIR-1 is not sufficient. Moreover, we
observed that ATF-7 exerts complex and allele-specific modes of
regulation on let-7-Fam miRNA activity with and without
P. aeruginosa infection, presumably depending on its phosphor-
ylation potential (Fig. S2 B–E). This finding is consistent with pre-
viously published observations that upon phosphorylation by PMK-1
in the face of P. aeruginosa infection, ATF-7 switches its mode of

Fig. 7. let-7(mg279) animals exhibit reduced abundance of bacterial OMVs in their intestinal lumen during P. aeruginosa infection. Transmission electron micrographs of transversal midbody sections of a WT (N2) animal (A) and let-7(mg279) animal (B) infected with P. aeruginosa PA14 for 48 h. b, bacterial cell; mv, microvilli. Arrowheads point to representative OMVs. (Scale bars: 0.5 μm.) (C) Ratio of OMVs to bacteria in electron micrographs. The electron micrographs were randomly sampled with a 1-μm square five times, and the numbers of OMVs and bacteria within the square were counted. Two animals were tested for both the WT and let-7(mg279) groups. Error bars represent SDs. ***P < 0.001; two-tailed t test.

Fig. 8. Model. (A) Model for the regulation of developmental timing by P. aeruginosa and the p38 MAPK pathway in C. elegans. (B) Model for the roles of heterochronic genes in pathogen resistance on P. aeruginosa. *Predicated targets of let-7-Fam miRNAs in the p38 MAPK pathway. X represents other possible pathways that are regulated by let-7 to promote pathogen resistance on P. aeruginosa. The dotted line indicates proposed regulatory interactions not yet tested experimentally.
regulation on pmk-1/p38-mediated gene expression (34). More intriguingly, the let-7-Fam allele enhances the phenotypes of the let-7-Fam miRNA mutant even more than the let-7 null allele does, and the phenotype is suppressed when animals were exposed to *P. aeruginosa* (Fig. S24). This let-7-Fam allele removes the first exon from two let-7 gene isoforms, leaving the remaining isoforms unaffected, suggesting that there is an activation domain in the N-terminal region of ATF-7 protein. Removal of this region in the gk715 allele results in a reversal of function for the protein. Previous findings have suggested that the N-terminal region of mammalian ATF-7 homologs is essential for transcriptional activity (45, 46), a situation that, according to our results, appears to be evolutionally conserved in *C. elegans*. Although further studies are required to understand these noncanonical functions of TIR-1 and ATF-7 on let-7-fam miRNA activity, nevertheless our results provide a basis for uncovering the mechanisms for how the p38 MAPK pathway regulates let-7-fam miRNAs.

Our experiments using let-7-Fam miRNA gene transcriptional reporters in WT and pmk-1 mutant growing on *E. coli* suggest that p38 signaling could inhibit let-7-Fam miRNA activity, at least in part, at the transcriptional level. Thus, upon *P. aeruginosa* infection, activated p38 could further reduce let-7-Fam miRNA gene expression relative to growth on *E. coli*, which correlates with the enhanced heterochronic phenotype of let-7-Fam miRNA mutants on *P. aeruginosa*. However, we were not able to explore the GFP reporter activity of let-7-Fam miRNA genes upon *P. aeruginosa* infection directly due to apparent nonspecific degradation of GFP (possibly caused by cellular autophagy and/or necrosis) when animals were treated with *P. aeruginosa*. Additionally, it should be noted that the transcriptional regulation by p38 of let-7-Fam miRNAs could be more complex than observed here, because the reporter transgenes used in our study may not have necessarily contained all relevant regulatory elements (47).

The negative regulation of let-7-Fam miRNAs upon *P. aeruginosa* infection is presumably beneficial to worms and can enhance their response to the pathogen. This conclusion is supported by our observation that let-7(mg279) animals with mildly reduced activity of let-7 exhibit enhanced survival in the face of *P. aeruginosa* infection. Even though we have shown that the p38 MAPK pathway and the let-7 targets *lin-41* and *hbl-1* are required for this phenotype, it is also possible that additional downstream stress response effectors, such as *skn-1* (27), could mediate the regulation for pathogen resistance by let-7-Fam miRNAs. Predicted targets of let-7-Fam miRNAs include components of several pathways involved in pathogen resistance, including the p38 MAPK pathway, the unfolded protein response pathway, the oxidative stress response pathway, and the autophagy pathway (27, 32, 48, 49). We propose that let-7-Fam miRNAs could function during normal, unstimred development to dampen several stress response pathways, and that under stress conditions, such as *P. aeruginosa* infection, the down-regulation of let-7-Fam miRNA activity reported here would broadly augment the worm’s stress response.

The intestine, hypodermis, and neurons have been shown previously to be involved in the host response to pathogen infection in *C. elegans* (35, 43). Although tissue-specific rescue and knock out experiments will be required to determine the anatomical sites of action and to test for cell autonomy of let-7-Fam miRNAs for their regulation of pathogen resistance, our findings suggest that let-7-Fam miRNAs may have an impact on pathogen resistance by acting in the intestine and possibly in the hypodermis. We did not observe any noticeable difference in the pathogen avoidance behavior between let-7(mg279) animals and WT animals (Fig. S7), arguing against an exclusively neuronal effect. However, our EM results indicate that let-7(mg279) animals actively fight off *P. aeruginosa* infection in the intestine. Finally, based on the transcriptional reporter results, let-7-Fam miRNAs are specifically regulated in the intestine and the hypodermis.

Implications of our findings include intriguing possibilities for evolutionarily conserved roles of let-7-Fam miRNAs in cell fate and innate immune gene regulatory networks. In mammalian cells, let-7-Fam miRNA levels have been shown to be reduced in response to infection by the bacterium *Salmonella* (24), the parasitic protozoan *Cryptosporidium parvum* (25, 26), or an inflammatory response of Src activation (28). In the latter context, let-7-Fam miRNA levels were regulated by lin-28 (28), which also functions in the heterochronic pathway with let-7-Fam miRNAs in *C. elegans* (16, 50). In the context of *Salmonella* infection, Toll-like receptor 4 and the NFκB pathway, and possibly other innate immune signals, appear to mediate the down-regulation of let-7-Fam miRNAs (51). This repression of let-7 by NFκB is analogous to the p38 MAPK pathway’s regulation of let-7-Fam miRNAs in the context of *P. aeruginosa* infection of *C. elegans*, whose genome does not contain an NFκB homolog (52, 53). It is not currently clear whether p38 could contribute to the regulation of let-7-Fam miRNAs in mammalian cells upon innate immune activation.

In conclusion, our study demonstrates that let-7-Fam miRNAs function in a feed-forward loop with the p38 MAPK pathway to promote pathogen resistance upon infection, and that the genetic redundancy among let-7-Fam miRNAs assists animals in maintaining their robust developmental programs under various stress conditions.

**Materials and Methods**

**Nematode and Bacteria Methods.** *C. elegans* was cultured on nematode growth media (NGM) (54) and fed with *E. coli* HB101, unless otherwise noted. All of the *C. elegans* strains used in this study are listed in Table S1. Synchronized populations of developmentally staged worms were obtained by standard methods (55). To test different bacterial foods for effects on heterochronic phenotypes, all of the *E. coli* strains and *Comamonas* sp. were seeded onto NGM plates from saturated cultures. Procedures for culturing *P. aeruginosa* and *S. enterica* for feeding nematodes were carried out as described by Powell and Ausubel (56).

**Heterochronic Phenotype Analysis.** Gravid adult animals raised at 20 °C were placed on control or treatment plates at 20 °C, and their progeny were scored at the young adult stage for adult lateral alae formation and seam cell number. Nomarski Differential Interference Contrast (DIC) microscopy and fluorescence microscopy with the *mais105 [col19]; gfp* transgene to mark lateral hypodermal cell nuclei were used to score alae formation and seam cell number, respectively.

**RNA Extraction.** Animals were collected and flash-frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Invitrogen).

**Firefly miRNA Assay.** Two micrograms of total RNA was used for Firefly miRNA assay ([www.fireflybio.com](http://www.fireflybio.com)). A standard protocol provided by the manufacturer (Firefly BioWorks) was followed. Guava easyCyte BHT (Millipore) was used for analysis. Signals (arbitrary units) were normalized to small nuclear RNA U18 and then to the control group (HB101).

**Confocal Microscopy.** Animals were mounted on glass slides with 2% (wt/vol) agarose pads and anesthetized with 10 mM levamisole. A Leica TCS SPE microscope was used to acquire images. The mean intensities were calculated with the Leica Application Suite Advanced Fluorescence software platform.

**P. aeruginosa** Killing Assays and Lifespan Assays. *P. aeruginosa* PA14 and *E. coli* HB101 were cultured in LB and seeded onto slow-killing plates (56) containing 100 μM 5-fluorodeoxyuridine (Sigma) for *P. aeruginosa* killing assays and lifespan assays, respectively. The seeded plates were incubated at 37 °C for 24 h and then transferred to 25 °C for 24 h before use. Assays were conducted by transferring L4-stage animals raised on *E. coli* HB101 to *P. aeruginosa* killing assay plates and lifespan assay plates at 25 °C. Animals that died prematurely due to developmental abnormalities (leading to bagging and bursting vulva) or that died after crawling off the plate were censored. Data were normalized by adjusting the numbers of live animals at
ACKNOWLEDGMENTS. We thank members of the V.R.A. laboratory for helpful discussions, S. Burke, K. McElhinney, and C. Stirling for comments on the manuscript; and the laboratories of C. Mello, A. Walker, R. Davis, and K. Matsumoto for reagents and technical assistance. Several nematode and bacteria strains used in this study were kindly provided by the Caenorhabditis Genetics Center (which is funded by the NIH Office of Research Infrastructure Programs [Grant P40 OD010440] and the laboratories of F. Ausubel and D. Kim. The EM was performed by the core EM facility at the University of Massachusetts Medical School, supported by Award S10RR027897 from the National Center for Research Resources. Firefly BioWorks developed the firefly miRNA assay for C. elegans-specific miRNAs. This work was funded by NIH Grant R01 GM34028 (to V.R.A.).