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Ren Z, Ambros VR. (2015). Caenorhabditis elegans microRNAs of the let-7 family act in innate immune response circuits and confer robust developmental timing against pathogen stress. GSBS Student Publications. <https://doi.org/10.1073/pnas.1422858112>. Retrieved from https://escholarship.umassmed.edu/gsbs_sp/1884

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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 mRNAs, 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 predicated 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. aeruginosa* 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.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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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. 1A). 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 physiology 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. S1A), 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.

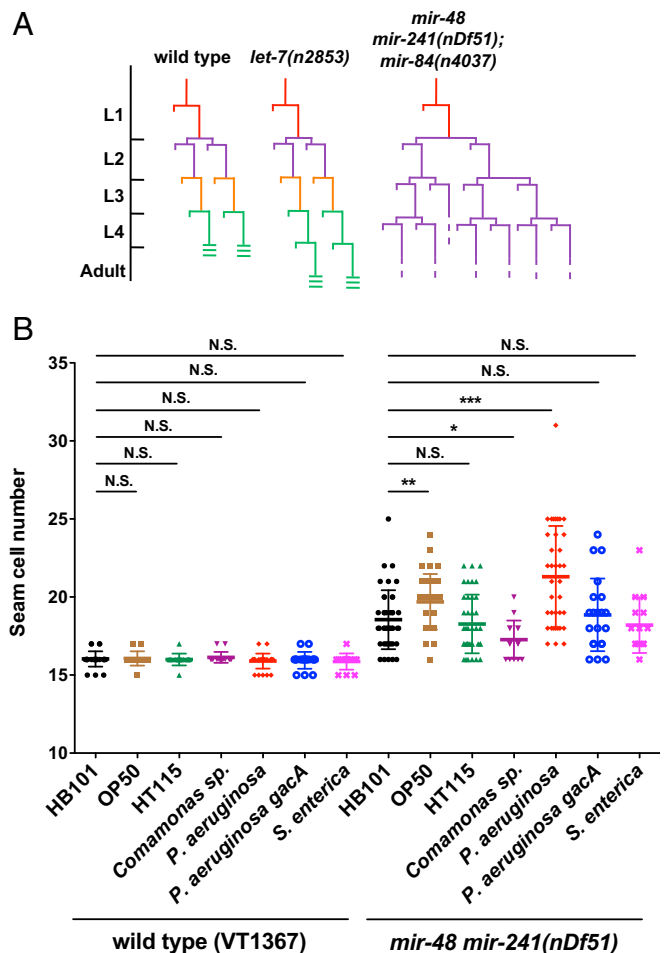


Fig. 1. Effects of bacterial food on the heterochronic phenotype of a *let-7-Fam* partial loss-of-function mutant. (A) Diagrams of seam cell V lineage in WT (N2), *let-7(n2853)*, and *mir-48 mir-241(nDf51); mir-84(n4037)* animals. L1 to L4 are the four larval stages in *C. elegans* postembryonic development. (B) Scatter plot of seam cell number for WT (VT1367) and *mir-48 mir-241(nDf51)* animals raised on seven different bacteria: *E. coli* (HB101, OP50, and HT115), *Comamonas* sp. (DA1877), *P. aeruginosa* (PA14), *P. aeruginosa* (PA14 *gacA*), and *S. enterica* (SL1344). WT animals in this experiment are the strain of VT1367 that carries an integrated *col-19* (an adult-specific collagen) transcriptional reporter (*mals105*) to assist in the quantification of seam cell numbers. The *mals105* is also present in the genetic background of all of the other stains used in heterochronic phenotype analysis. *E. coli* HB101 was used as a control. In this and all subsequent scatter plots, the line in the middle is the mean value and error bars represent SDs. N.S., not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed *t* test ($n \geq 15$).

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

Genotype	Treatment	Percentage of animals with different adult alae phenotypes			<i>n</i>	Test ¹
		Complete	Gapped	No alae		
WT (VT1367)	<i>E. coli</i> HB101 (control)	100	0	0	30	
	<i>E. coli</i> OP50	100	0	0	15	N.S.
	<i>E. coli</i> HT115	100	0	0	15	N.S.
	<i>Comamonas</i> sp. DA1877	100	0	0	15	N.S.
	<i>P. aeruginosa</i> PA14	100	0	0	30	N.S.
	<i>P. aeruginosa</i> PA14 <i>gacA</i>	100	0	0	15	N.S.
	<i>S. enterica</i> SL1344	100	0	0	15	N.S.
<i>mir-48 mir-241(nDf51)</i>	<i>E. coli</i> HB101	40	60	0	42	
	<i>E. coli</i> OP50	28	72	0	39	N.S.
	<i>E. coli</i> HT115	60	40	0	40	N.S.
	<i>Comamonas</i> sp. DA1877	40	60	0	15	N.S.
	<i>P. aeruginosa</i> PA14	8	92	0	37	***
	<i>P. aeruginosa</i> PA14 <i>gacA</i>	30	70	0	21	N.S.
	<i>S. enterica</i> SL1344	27	73	0	15	N.S.
<i>mir-48(n4097); mir-84(n4037)</i>	<i>E. coli</i> HB101	100	0	0	31	
	<i>P. aeruginosa</i> PA14	93	7	0	30	N.S.
<i>let-7(n2853)</i>	<i>E. coli</i> HB101	0	77	23	30	
	<i>P. aeruginosa</i> PA14	0	42	58	26	**

¹The χ^2 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 the *let-7-Fam* miRNA mutant phenotypes on *P. aeruginosa* (Fig. 2A and C). We interpret this result to indicate that p38 MAPK pathway activation in larvae grown on *P. aeruginosa* is required for the down-regulation of *let-7-Fam* miRNA activity. Furthermore, one of the major downstream transcription factors of the p38 MAPK pathway is ATF-7 (34). We therefore tested whether ATF-7 is involved in the modulation of *let-7-Fam* miRNA mutant phenotypes. We observed that *mir-48 mir-241(nDf51)* animals homozygous for a null mutation of ATF-7 [*atf-7(qd22qd130)*] or for a phosphorylation-defective allele of ATF-7 [*atf-7(qd22)*] (34) did not exhibit enhanced heterochronic phenotypes on *P. aeruginosa* (Fig. 2B 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. 4A, 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. 4C 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.

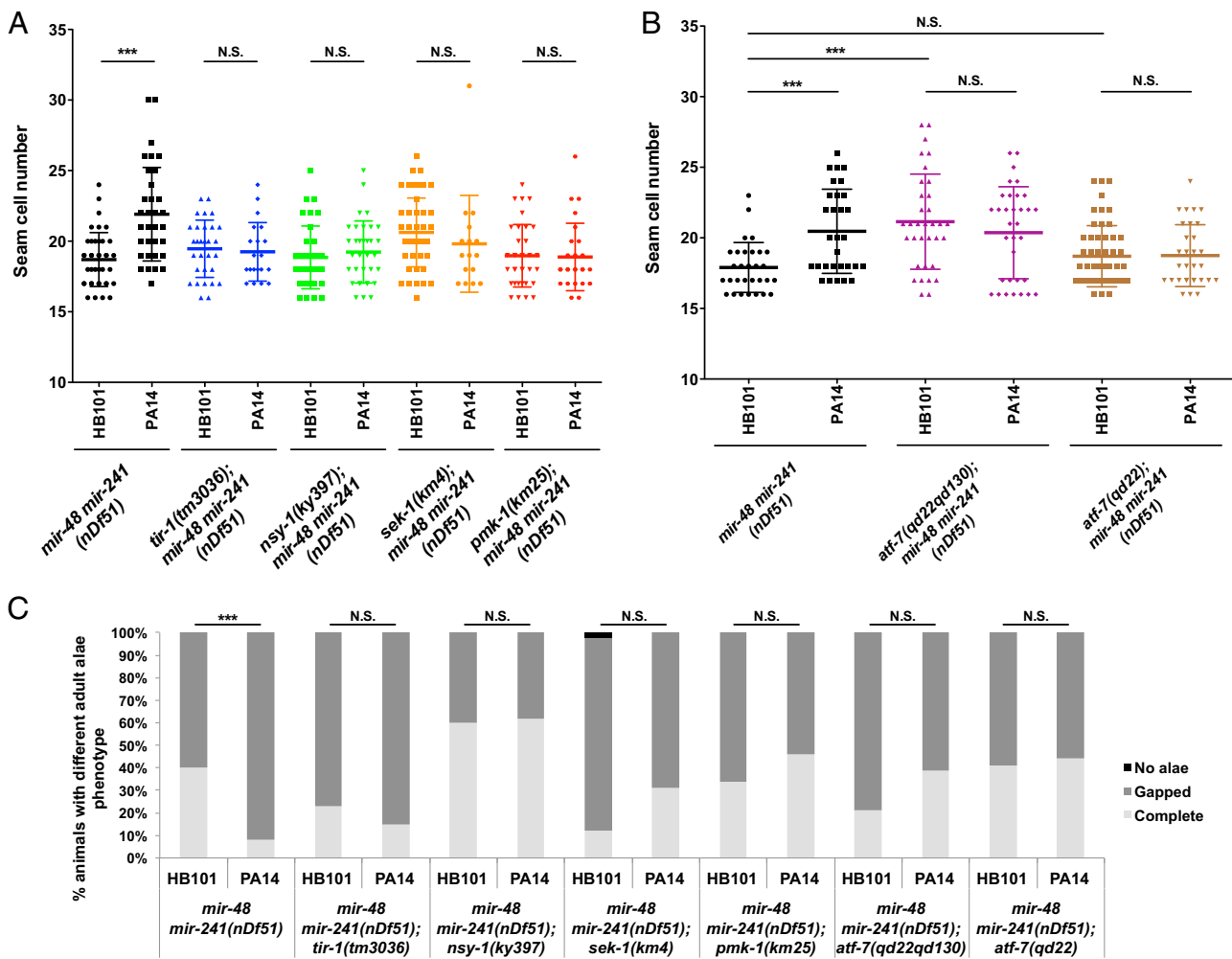


Fig. 2. p38 MAPK pathway and its downstream transcription factor *atf-7* are required for the suppression of *let-7-Fam* miRNA activity on *P. aeruginosa*. (A) Deletion of any p38 MAPK pathway component blocks the enhancement of *mir-48 mir-241(nDf51)* animals' seam cell phenotype on *P. aeruginosa*. (B) Seam cell number of *atf-7(qd22q130); mir-48 mir-241(nDf51)* and *atf-7(qd22); mir-48 mir-241(nDf51)* animals treated with either HB101 (control) or PA14. *qd22q130* is a putative null allele of *atf-7*. *** $P < 0.001$, two-tailed t test ($n \geq 15$). (C) Adult alae phenotype for double mutants of *mir-48 mir-241(nDf51)* and p38 MAPK pathway components. *** $P < 0.001$; χ^2 test ($n > 15$).

***let-7-Fam* miRNAs Exhibit Negative Roles in Resistance to *P. aeruginosa* Infection.** 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. 5A), whereas *let-7(n2853)* animals displayed the opposite, a decreased survival on *P. aeruginosa* (Fig. 5B). In-

terestingly, populations of the double-mutant *mir-48 mir-241(nDf51)* and *mir-48(n4097); mir-84(n4037)* animals exhibited biphasic survival curves (Fig. 5A). 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 (*maIs380*), which fully rescues the developmental phenotypes of both *let-7* alleles (Fig. S3A and B), to also rescue the characteristic survival phenotypes of *let-7(mg279)* and *let-7(n2853)* mutants. *maIs380* 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

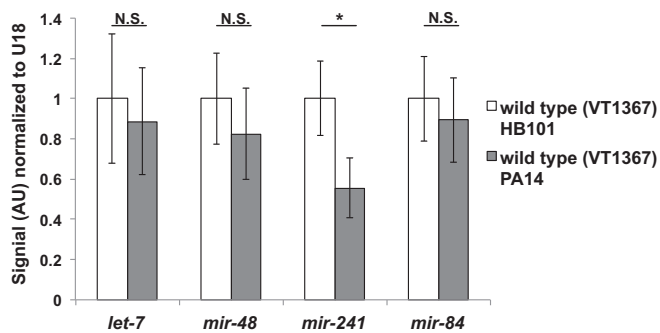


Fig. 3. Mature *let-7-Fam* miRNA levels upon *P. aeruginosa* infection. Total RNA from whole animals was used to perform Firefly miRNA assays. Animals (VT1367) were raised on either HB101 (control) or PA14 from the synchronized L1 stage to young adult stage at 20 °C. Error bars represent SDs. * $P < 0.05$; two-tailed t test. AU, arbitrary unit.

underlying effects of *let-7-Fam* miRNAs on overall fitness of the animals (regardless of diet), we examined the longevity of these mutants on *E. coli* HB101. All of the *let-7-Fam* miRNA mutants we tested displayed a degree of shortened lifespan compared with WT animals (Fig. 5 *C* and *D*), which indicates that *let-7-Fam* miRNAs function positively to regulate longevity in *C. elegans*. Therefore, the decreased survival phenotype of the stronger loss-of-function mutant *let-7(n2853)* animals on *P. aeruginosa* could simply be due to reduced longevity of these animals. However, importantly, the weak loss-of-function mutant *let-7(mg279)* animals exhibit improved survival against *P. aeruginosa* infection despite the fact that these animals have a shortened lifespan. These results suggest that *let-7-Fam* miRNAs negatively modulate pathogen resistance while also positively regulate longevity, such that a mild reduction in *let-7-Fam* miRNA activity [by *let-7(mg279)*] promotes survival on *P. aeruginosa* because the longevity of these animals is not severely compromised. With regard to the biphasic survival phenotype of *mir-48 mir-241(nDf51)* and *mir-48(n4097); mir-84(n4037)* animals on *P. aeruginosa*, we propose that there are variations in the extent of reduced *let-7-Fam* miRNA activity within the population in these mutants, such that animals with mildly reduced *let-7-Fam* miRNA activity exhibit enhanced pathogen resistance, whereas others with greater reduced *let-7-Fam* miRNA activity show decreased survival on *P. aeruginosa* possibly due to reduced longevity in general.

In summary, *let-7-Fam* miRNAs exhibit negative regulation on animals' resistance to *P. aeruginosa* infection, as evidenced by the increased survival on *P. aeruginosa* for *let-7(mg279)* animals. On the other hand, some *let-7-Fam* miRNA mutants exhibited decreased survival on *P. aeruginosa*, which probably results from an epistatic function of *let-7-Fam* miRNAs in promoting longevity.

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.

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. 6*A*). 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.

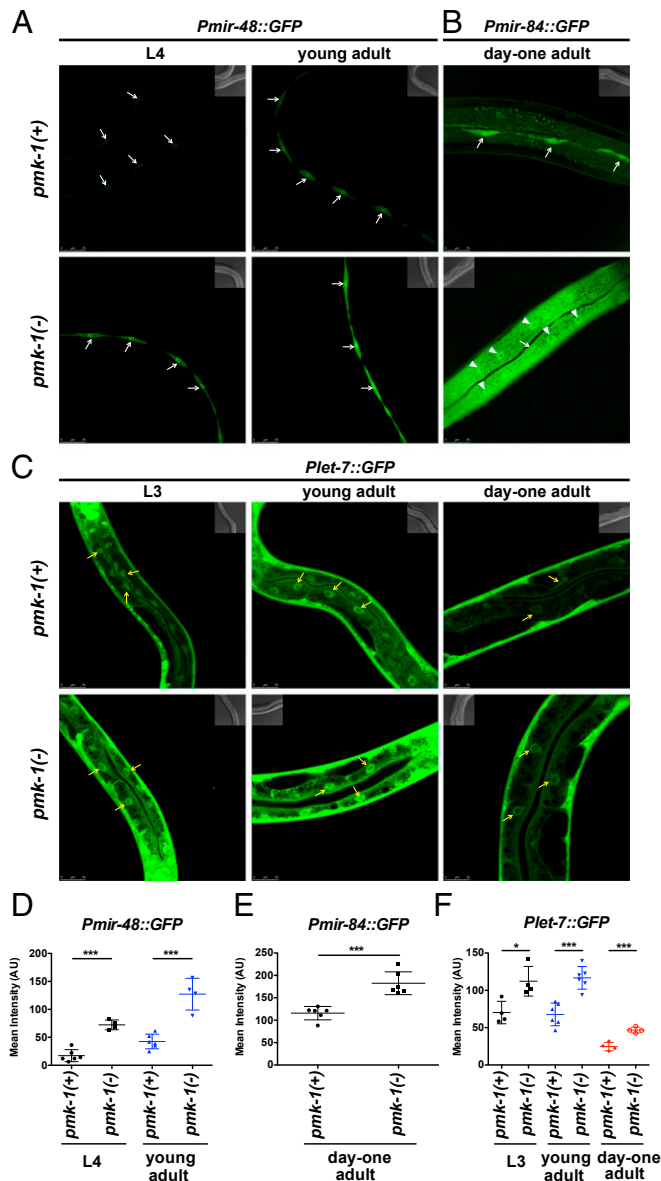


Fig. 4. *pmk-1* regulates the transcriptional activity of *let-7-Fam* miRNA genes. Confocal images show the transcriptional reporters of *mir-48* (A; *mals150*), *mir-84* (B; *mals138*), and *let-7* (C; *mals137*) in WT [*pmk-1(+)*] and *pmk-1(km25)* [*pmk-1(-)*] animals. Animals were raised on *E. coli* HB101 at 20 °C. Stages of the animals are as indicated. White arrows indicate seam cells, white arrowheads indicate hyp7 nuclei, and yellow arrows point to representative intestine nuclei. (D–F) Mean intensities of the intended tissues in A–C. Error bars represent SDs. * $P < 0.05$, *** $P < 0.001$; two-tailed t test.

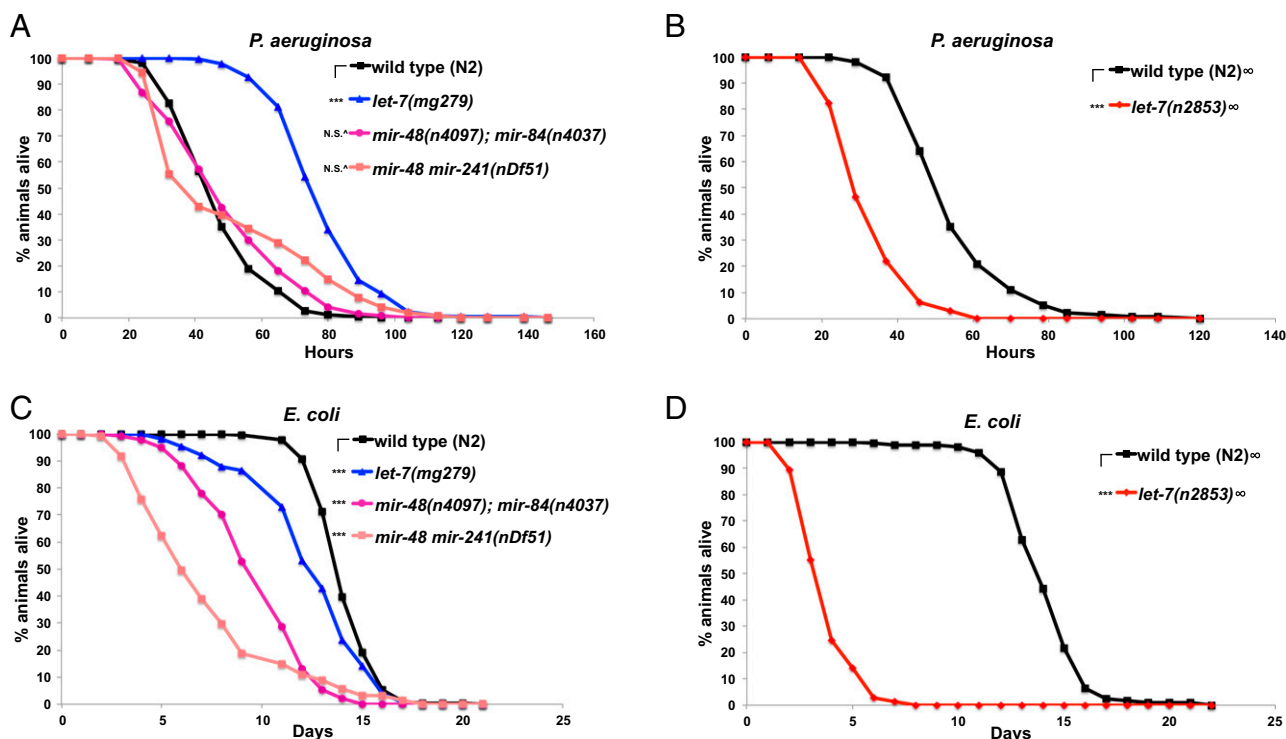


Fig. 5. *let-7-Fam* miRNAs regulate pathogen resistance against *P. aeruginosa* and lifespan on *E. coli*. (A) Survival of WT (N2), *let-7(mg279)*, *mir-48(n4097)*; *mir-84(n4037)*, and *mir-48 mir-241(nDf51)* animals on *P. aeruginosa*. (B) Survival of WT (N2) and *let-7(n2853)* animals on *P. aeruginosa*. (C) Lifespan of WT (N2), *let-7(mg279)*, *mir-48(n4097)*; *mir-84(n4037)*, and *mir-48 mir-241(nDf51)* animals on *E. coli* HB101. (D) Lifespan of WT (N2) and *let-7(n2853)* animals on *E. coli* HB101. Animals were usually raised at 20 °C before being transferred to a *P. aeruginosa* killing assay and lifespan assay at 25 °C, except for experiments with *let-7(n2853)* (noted with ∞ next to the strain names) in which strains were raised at 15 °C (to reduce the number of bursting adults) before being transferred to 25 °C for the *P. aeruginosa* killing assay and lifespan assay. *** $P < 0.001$; log-rank test. For the *let-7-Fam* miRNA mutants that exhibit different survival kinetics on *P. aeruginosa* compared with WT, Fisher's exact tests were applied (data in Table S2). \wedge Statistically significant outcome in Fisher's exact test.

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 F 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 phosphorylated 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. S5). 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*.

***let-7(mg279)* Animals Exhibit Reduced Accumulation of Outer Membrane Vesicles in the Intestine During *P. aeruginosa* Infection.** To explore further the basis for why *let-7(mg279)* animals survive better than WT animals on *P. aeruginosa*, we examined the intestines of infected worms using transmission EM. At 8 or 24 h after infection, we did not observe any difference in the intestinal cytopathology

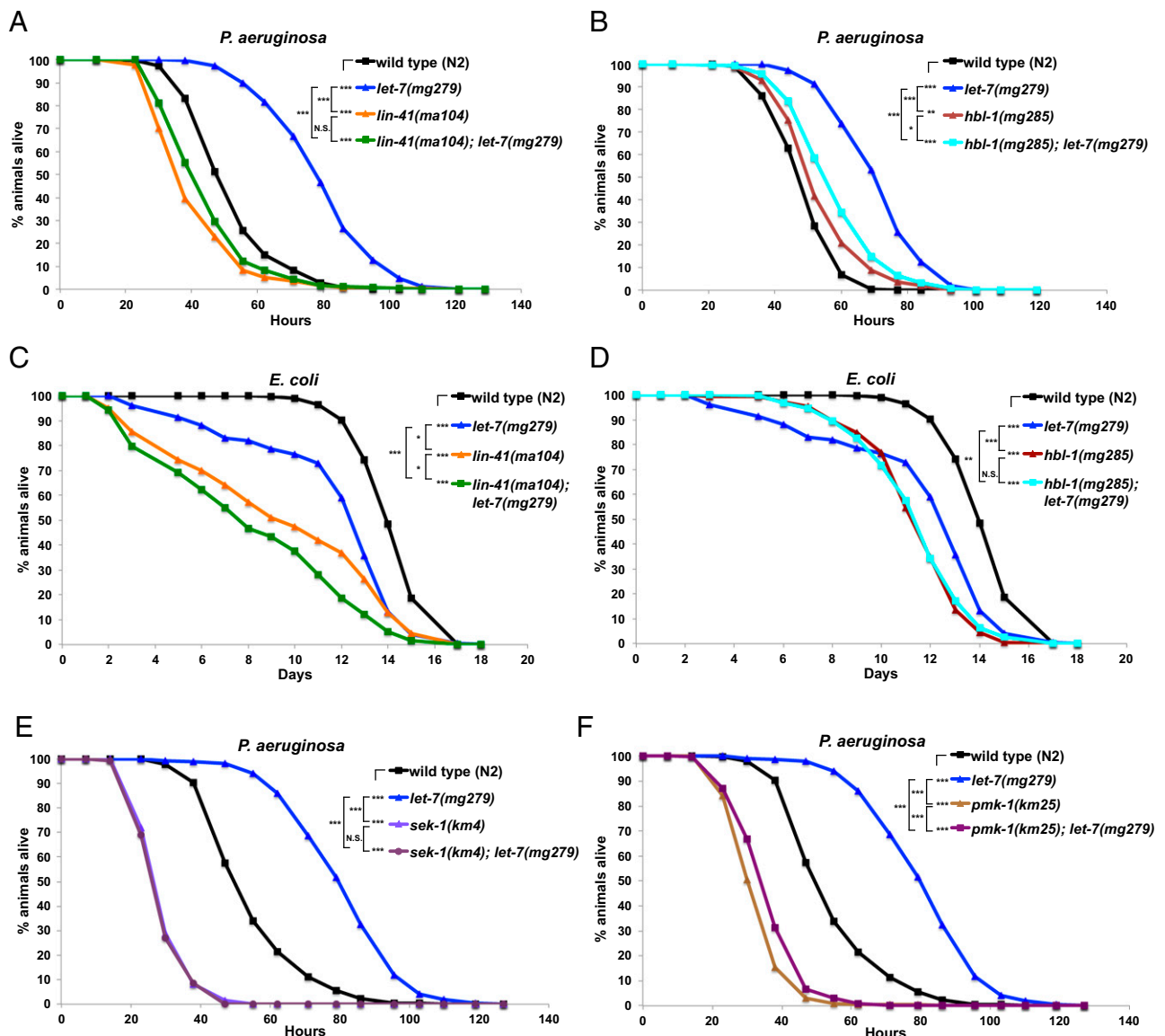


Fig. 6. *lin-1*, *hbl-1*, and p38 MAPK pathway modulate the enhanced pathogen resistance of *let-7(mg279)* animals. *lin-1(ma104)* (A) and *hbl-1(mg285)* (B) suppress the enhanced survival phenotype of *let-7(mg279)* animals on PA14. (C) Lifespan of strains used in A on *E. coli* HB101. (D) Lifespan of strains used in B on *E. coli* HB101. *sek-1* (E) and *pmk-1* (F) are also required for the enhanced survival phenotype of *let-7(mg279)* animals on PA14. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; log-rank test.

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

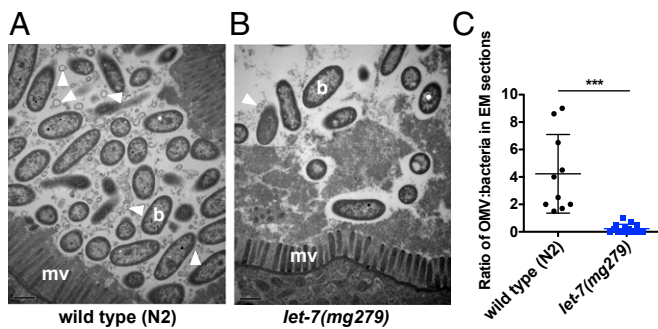


Fig. 7. *let-7(mg279)* animals exhibit reduced abundance of bacterial OMVs in their intestinal lumen during *P. aeruginosa* infection. Transmission electron micrographs of transversal midbody sections of a WT (N2) animal (A) and *let-7(mg279)* animal (B) infected with *P. aeruginosa* PA14 for 48 h. b, bacterial cell; mv, microvilli. Arrowheads point to representative OMVs. (Scale bars: 0.5 μ m.) (C) Ratio of OMVs to bacteria in electron micrographs. The electron micrographs were randomly sampled with a 1- μ m 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.

mutants, 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 declines, 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 phenotype 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 function 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(km25)* partially suppresses the enhanced heterochronic phenotypes of *mir-48 mir-241(nDf51)* animals on OP50 (Fig. S1C–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 pathogenicity of the bacteria. However, other properties of OP50 could contribute to the phenotype as well because *pmk-1(km25)* 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 developmental timing regardless of the bacterial diets tested here, supporting our conclusion that the full *let-7-Fam* miRNA genotype 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 connecting the p38 MAPK innate immunity pathway and the heterochronic gene developmental timing pathway via *let-7-Fam* miRNAs. The role of the p38 pathway in this process is supported by our observation that loss-of-function mutations that disable the p38 MAPK pathway block the phenotypic enhancement of *let-7-Fam* miRNA mutants on *P. aeruginosa*. Also in support of this conclusion is our result that constitutive activation 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. S2F and G).

Although our findings show that the p38 MAPK pathway is involved in regulation of *let-7-Fam* miRNA activity, certain aspects 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 suggests 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 phosphorylation potential (Fig. S2B–E). This finding is consistent with previously published observations that upon phosphorylation by PMK-1 in the face of *P. aeruginosa* infection, ATF-7 switches its mode of

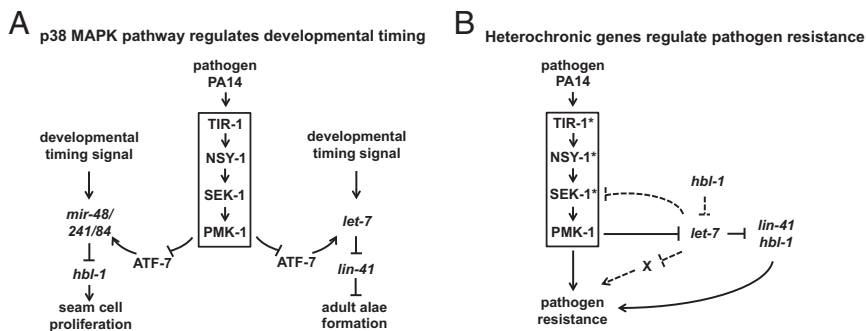


Fig. 8. Model. (A) Model for the regulation of developmental timing by *P. aeruginosa* and the p38 MAPK pathway in *C. elegans*. (B) Model for the roles of heterochronic genes in pathogen resistance on *P. aeruginosa*. *Predicated targets of *let-7-Fam* miRNAs in the p38 MAPK pathway. X represents other 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 *atf-7(gk715)* allele enhances the phenotypes of the *let-7-Fam* miRNA mutant even more than the *atf-7* null allele does, and the phenotype is suppressed when animals were exposed to *P. aeruginosa* (Fig. S24). This *atf-7(gk715)* allele removes the first exon from two *atf-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, unstressed 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 site(s) 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 NF κ B 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 NF κ B 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 NF κ B 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 *mals105 [col-19::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). A standard protocol provided by the manufacturer (Firefly BioWorks) was followed. Guava easyCyte 8HT (Millipore) was used for analysis. Signals (arbitrary units) were normalized to small nucleolar 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 microsystem 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 μ M 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

each time point so as to derive a total population size of 100 for each technical replicate. Then, the normalized data for three technical replicates were averaged, and survival curves were compared using the log-rank test and Fisher's exact test in Online Application for Survival Analysis of Lifespan Assays (OASIS) (57). All experiments were independently performed at least twice.

EM. WT (N2) and *let-7(mg279)* animals were synchronized and grown on NGM plates seeded with *E. coli* HB101. L4-stage animals were transferred onto *P. aeruginosa* slow-killing plates and incubated at 25 °C for 8, 24, and 48 h. Worms were harvested and prepared for transmission EM as described (39).

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