Supporting Information

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SI Materials and Methods

RNAi Knockdown. Gene knockdown by RNAi was carried out using bacterial-feeding RNAi (1). RNAi clones were obtained from Ahringer RNAi feeding library (2). Gravid adult animals were placed on the RNAi plates, and their progeny were scored for their heterochronic phenotypes.

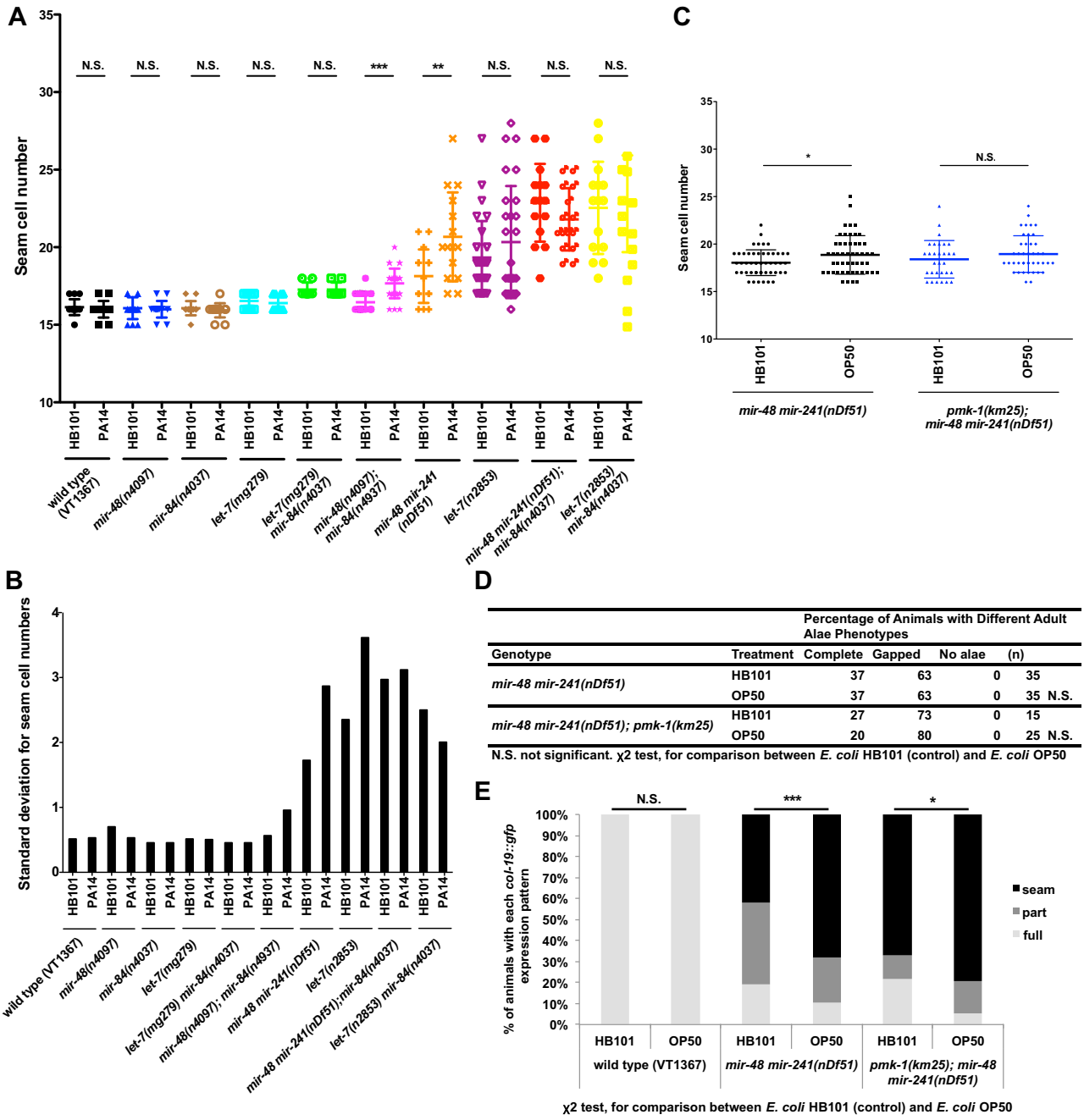
Epifluorescence Microscopy. Animals carrying transgene *agIs219* [*T24B8.5p::gfp::unc-54-3′UTR*] were used as an indication of the p38 MAPK pathway activity in the RNAi experiments for *vhp-1* (3). Images were acquired using a Zeiss SteREO Discovery.V12 microscope with a Zeiss AxioCam MRc camera and Zeiss ZEN 2012 (blue edition) software.

Transgenic Constructs. The pZR001 plasmid was made by cloning the 2,459 bp of the *let-7* genomic rescue region (4) into the mos1-mediated single copy insertion (mosSCI) destination vector pCFJ150. An integration *maIs380* at the Chromosome II *ttTi5605* site was obtained using the mosSCI protocol (5).

Western Blot Analysis. For p38 Western blots, synchronized L4-stage animals grown on *E. coli* HB101 at 20 °C were transferred to *P. aeruginosa* slow-killing assay plates (6) at 25 °C. At each indicated time point, animals were washed off plates with M9 buffer and flash-frozen in liquid nitrogen. Lysates were prepared by resuspending samples in lysis buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 2 mM DTT, PhosSTOP (Roche), and protease inhibitor (Roche)] and homogenized using a Branson SLPe sonicator (EMERSON). Lysates were centrifuged at 164,000 rpm for 20 min at 4 °C with Eppendorf centrifuge 5417R, and the supernatants were collected. Protein concentration was measured by BioRad Protein Assay Dye Reagent Concentrate (catalog no. 500-0006). Sixty micrograms of proteins was used for the immunoblot analysis. The activated p38 was recognized by anti-ACTIVE p38 pAb (Cell Signaling) at a 1:1,000 dilution. The total PMK-1 polyclonal antibody was applied at a 1:1,000 dilution (a gift from Kunihiro Matsumoto, Nagoya University, Nagoya, Japan), and anti–α-tubulin antibody (catalog no. T6074; Sigma–Aldrich) was used at a 1:20,000 dilution.

***P. aeruginosa* Avoidance Behavior Assay.** L4-stage animals were put on *P. aeruginosa* slow-killing plates (6), and numbers of animals on and off the bacterial lawn were counted 2, 4, 6, 8, 12, and 24 h later.

1. Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263(1-2):103–112.
2. Fraser AG, et al. (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 408(6810):325–330.
3. Shivers RP, et al. (2010) Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in *Caenorhabditis elegans*. *PLoS Genet* 6(4):e1000892.
4. Reinhart BJ, et al. (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403(6772):901–906.
5. Frøkjaer-Jensen C, et al. (2008) Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat Genet* 40(11):1375–1383.
6. Powell JR, Ausubel FM (2008) Models of *Caenorhabditis elegans* infection by bacterial and fungal pathogens. *Methods Mol Biol* 415:403–427.



Genotype	Treatment	Percentage of Animals with Different Adult Alae Phenotypes: Complete	Gapped	No alae	(n)	
mir-48 mir-241(nDf51)	HB101	37	63	0	35	
	OP50	37	63	0	35	N.S.
mir-48 mir-241(nDf51); pmk-1(km25)	HB101	27	73	0	15	
	OP50	20	80	0	25	N.S.

N.S. not significant. χ2 test, for comparison between *E. coli* HB101 (control) and *E. coli* OP50

χ2 test, for comparison between *E. coli* HB101 (control) and *E. coli* OP50

Fig. S1. Effects of *P. aeruginosa* and *E. coli* OP50 on heterochronic phenotypes of *let-7-Fam* miRNA mutants. (*A*) Seam cell number of several *let-7-Fam* miRNA mutants grown on *E. coli* HB101 and *P. aeruginosa* PA14. (*B*) SD for all of the seam cell number data in *A*. Seam cell phenotype (*C*), adult alae phenotype (*D*), and *col-19::gfp* expression pattern phenotype (*E*) of *mir-48 mir-241*(*nDf51*) and *pmk-1*(*km25*); *mir-48 mir-241*(*nDf51*) animals on *E. coli* HB101 and *E. coli* OP50. *col-19* is an adult-specific collagen whose expression pattern is regulated by *let-7-Fam* miRNAs (1–3). full, *col-19::gfp* is expressed in all of the seam cells and hyp7 nuclei; part, *col-19::gfp* is expressed in the seam cells and part of the hyp7 nuclei; seam, *col-19::gfp* is only expressed in the seam cells. All of the WT animals have a full *col-19::gfp* expression pattern, and *let-7-Fam* miRNA mutants lose a degree of *col-19::gfp* expression in hyp7 nuclei depending on the level of *let-7-Fam* miRNA activity in the mutants. Hence, the *col-19::gfp* expression pattern can be used as an indication of *let-7-Fam* miRNA activity: The less *let-7-Fam* miRNA activity there is, the less *col-19::gfp* expression there is in the hyp7 nuclei. The *col-19::gfp* expression pattern phenotype was scored using a Zeiss SteREO Discovery.V12 microscope. N.S., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; two-tailed t test ($n \geq 15$).

1. Rougvie AE, Ambros V (1995) The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. *Development* 121(8): 2491–2500.
2. Slack FJ, et al. (2000) The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the LIN-29 transcription factor. *Mol Cell* 5(4):659–669.
3. Hada K, et al. (2010) The nuclear receptor gene *nhr-25* plays multiple roles in the *Caenorhabditis elegans* heterochronic gene network to control the larva-to-adult transition. *Dev Biol* 344(2):1100–1109.

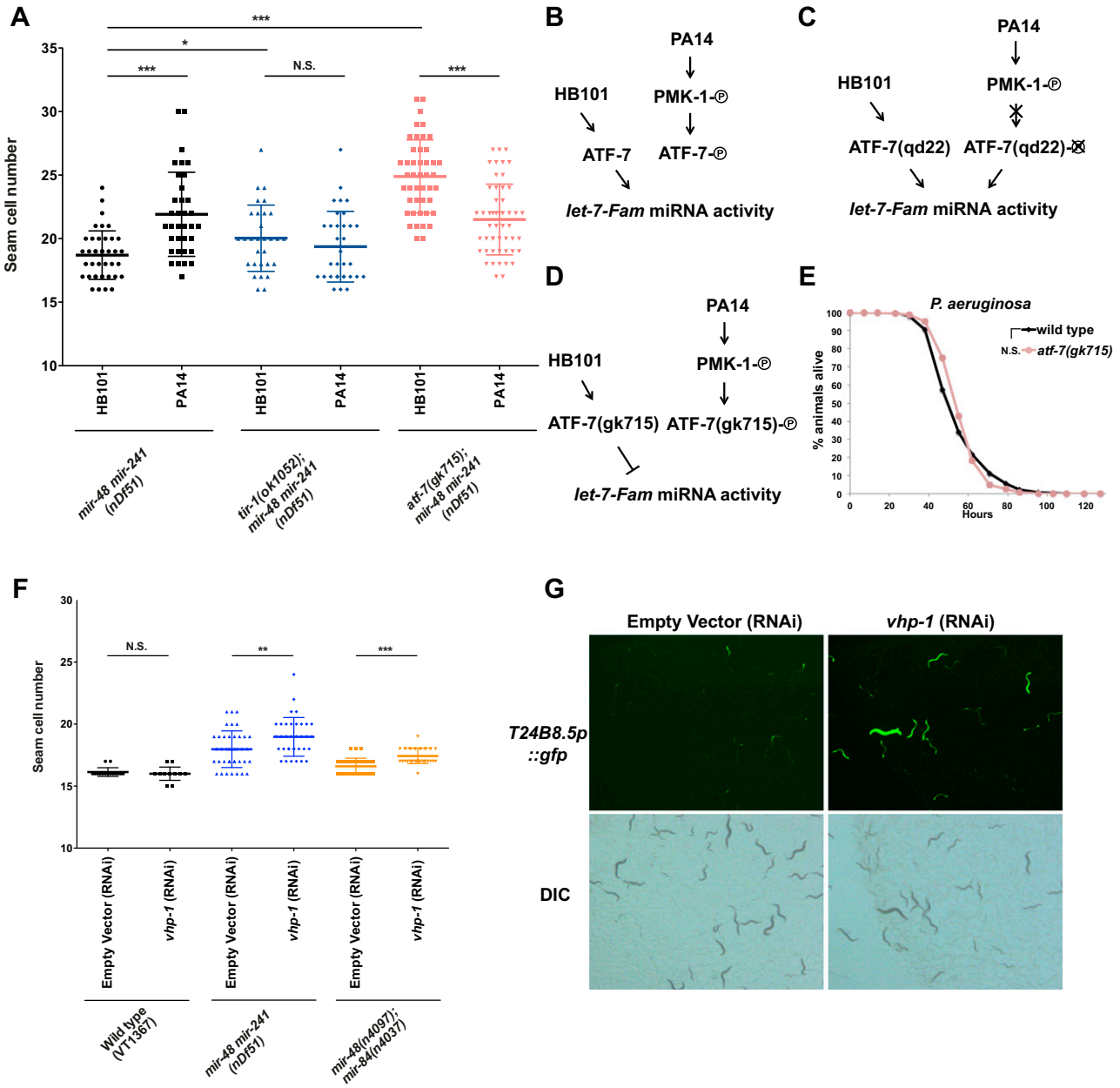
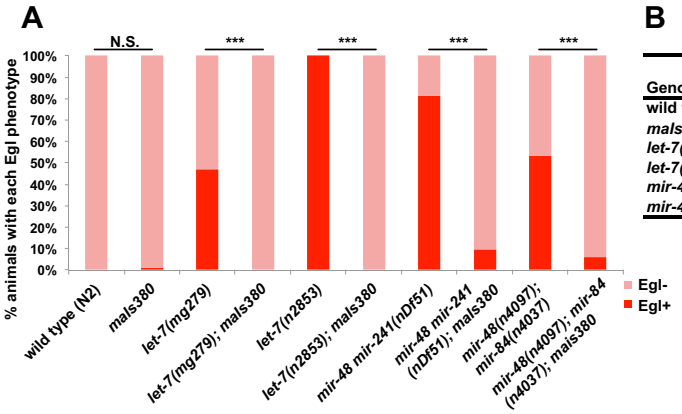


Fig. S2. Effects of the p38 MAPK pathway on *let-7-Fam* miRNA activity. (*A*) Effects of *tir-1*(*ok1052*) and *atf-7*(*gk715*) alleles on the seam cell phenotype of *mir-48 mir-241*(*nDf51*) animals on *E. coli* HB101 and *P. aeruginosa* PA14. (*B–D*) Models for how different *atf-7* alleles affect *let-7-Fam* miRNA activity. (*B*) On *E. coli* HB101, the unphosphorylated ATF-7 positively regulates *let-7-Fam* miRNA activity. After being phosphorylated by PMK-1 upon *P. aeruginosa* PA14 infection, ATF-7 loses its positive regulation on *let-7-Fam* miRNAs, which results in the heterochronic phenotype enhancement of *let-7-Fam* miRNA mutants. (*C*) Regulation of ATF-7 on *let-7-Fam* miRNAs depends on the phosphorylation potential of ATF-7. Therefore, the phosphorylation-inactive form of ATF-7 (*qd22* allele) does not affect *let-7-Fam* mutant heterochronic phenotypes with or without *P. aeruginosa* infection. (*D*) ATF-7 (gk715) negatively regulates *let-7-Fam* miRNA activity on HB101. (*E*) Because *atf-7*(*gk715*) animals have no enhanced susceptibility to *P. aeruginosa* infection, the phosphorylation activity of ATF-7(gk715) is likely still intact. After being infected with *P. aeruginosa*, ATF-7 is phosphorylated, and this releases its negative regulation on *let-7-Fam* miRNAs; therefore, the heterochronic phenotypes are suppressed compared with the ones from the control HB101 group. (*F*) *vhp-1* positively regulates *let-7-Fam* miRNA activity. (*G*) p38 MAPK is activated by *vhp-1* RNAi as indicated by transgene reporter *agIs219*[*T24B8.5p::gfp::unc-54-3′UTR*]. DIC, Differential Interference Contrast. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; two-tailed *t* test ($n \geq 15$).



B

Genotype	Percentage of Animals with Different Adult Alae				
	Complete	Gapped	No alae	(n)	
wild type (N2)	100	0	0	15	
mals380	100	0	0	30	N.S.
let-7(n2853)	0	44	56	25	
let-7(n2853); mals380	100	0	0	30	***
mir-48 mir-241(nDf51)	33	67	0	30	
mir-48 mir-241(nDf51); mals380	27	73	0	30	N.S.

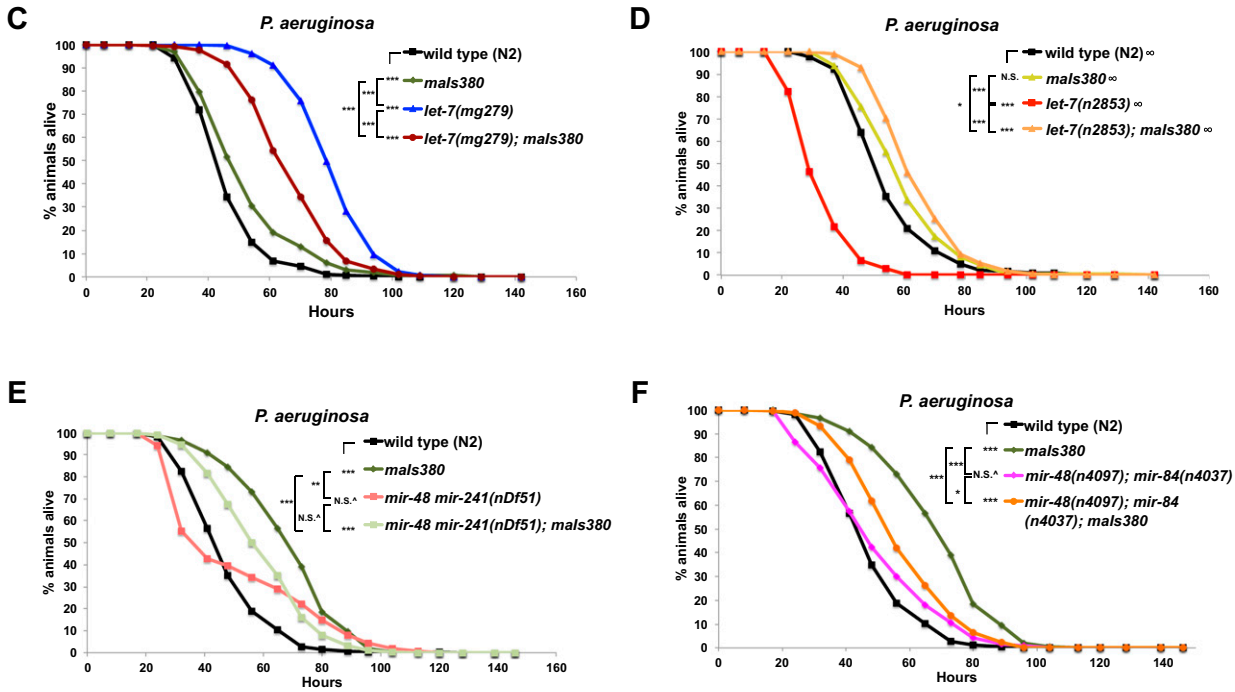


Fig. S3. Effects of a *let-7*-rescuing transgene on the phenotypes of *let-7-Fam* miRNA mutants. (*A*) Egg-laying defective phenotype of *let-7-Fam* miRNA mutants in combination with the *let-7* rescuing transgene, *mals380*. Egl–, WT for egg laying, Egl+, egg-laying defective. ***$P < 0.001$; χ^2 test for comparison in the same genetic background with and without transgene [$n \geq 100$; except *let-7*(*n2853*), $n = 36$]. Interestingly, the *let-7* transgene rescued the egg-laying defective phenotype of *mir-48 mir-241*(*nDf51*) and *mir-48*(*n4097*); *mir-84(n4037)* animals, suggesting a redundancy within the family in the regulation of vulva development. (*B*) Adult alae phenotype of *let-7-Fam* miRNA mutants with the transgene *mals380*. Because 100% of *let-7*(*mg279*) and *mir-48*(*n4097*); *mir-84* (*n4037*) animals have complete adult alae, this phenotype was not used to confirm the transgene's rescue of these strains. ***$P < 0.001$; χ^2 test for comparison in the same genetic background with and without transgene. The *mals380 let-7*–expressing transgene did not rescue the adult alae phenotype of *mir-48 mir-241*(*nDf51*) animals, indicating that although *let-7* seems to function together with the other *let-7-Fam* miRNAs in several processes, the roles of *let-7-Fam* miRNAs in regulating developmental timing are more distinct. Survival phenotypes of *let-7*(*mg279*) (*C*), *let-7*(*n2853*) (*D*), *mir-48 mir-241*(*nDf51*) (*E*), and *mir-48* (*n4097*); *mir-84*(*n4037*) (*F*) animals with *let-7* rescuing transgenes *mals380* on *P. aeruginosa*. The *mals380* strain used in *E* and *F* was not backcrossed. The *unc-119(ed3)* mutation and/or other possible mutations were suspected to cause the further enhanced survival than the same transgene in *C* and *D*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; log-rank test. Fisher's exact test was also performed for *mir-48 mir-241*(*nDf51*) and *mir-48*(*n4097*); *mir-84(n4037)* groups. ^Statistically significant outcome in Fisher's exact test (Table S2).

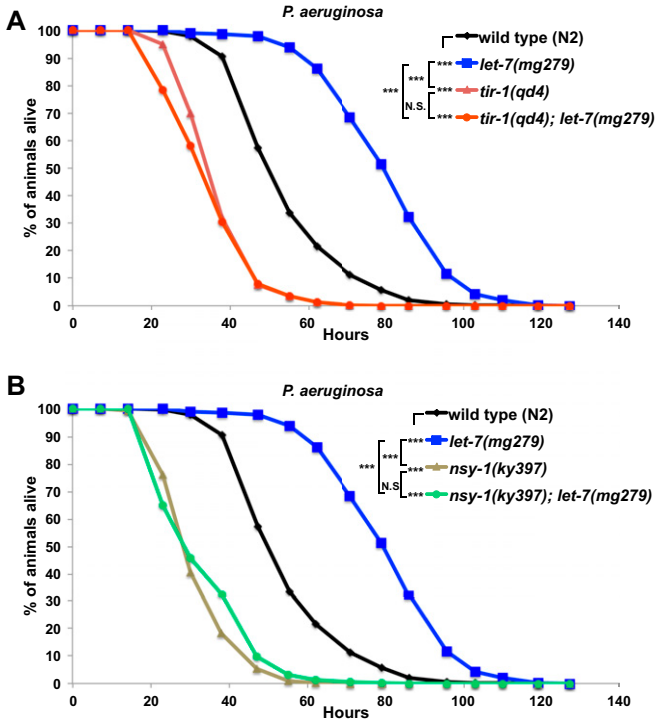


Fig. S4. Survival on PA14 of adults doubly mutant for *let-7*(*mg279*) and *tir-1*(*qd4*) (*A*) or *nsy-1*(*ky379*) (*B*). ***$P < 0.001$; log-rank test.

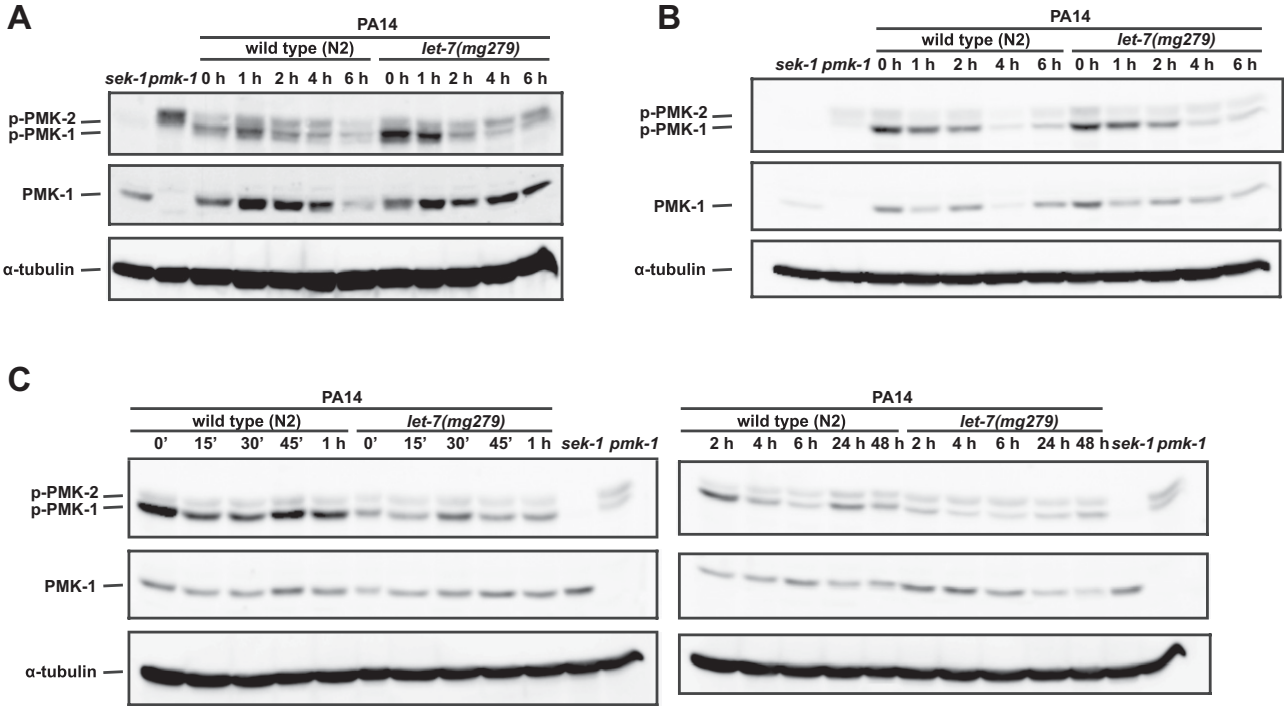


Fig. S5. Western blots of whole-animal lysates of WT, *let-7*(*mg279*), *sek-1*(*km4*), and *pmk-1*(*km25*) animals. All of the animals were grown on HB101 before being treated with PA14. The *sek-1*(*km4*) and *pmk-1*(*km25*) animals were collected at the L4 stage. L4-stage (0 h) WT and *let-7*(*mg279*) animals were treated with PA14 and collected at the time points, accordingly. (*A*–*C*) Three independent experiments.

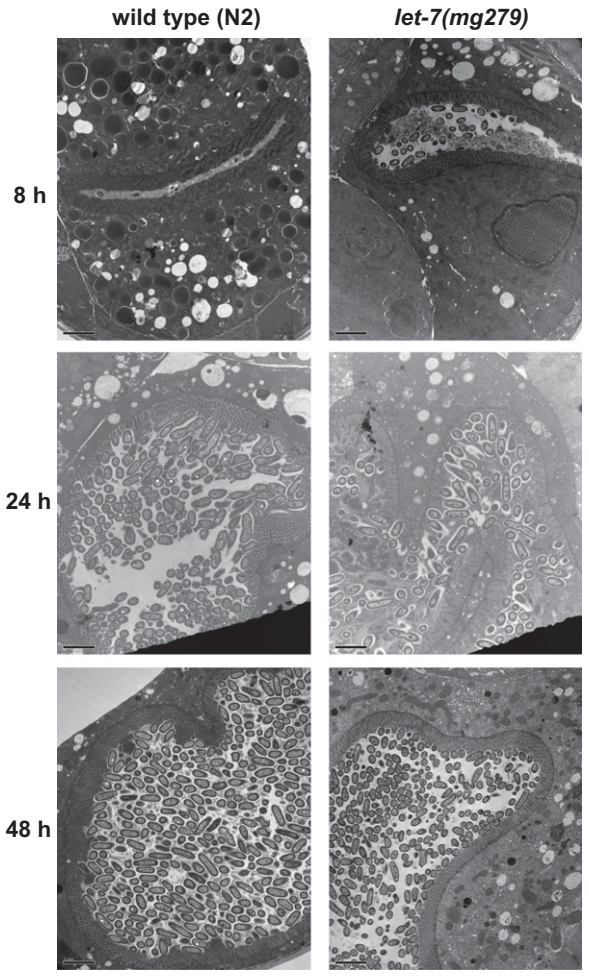


Fig. S6. Transmission EM images of WT (N2) and *let-7*(*mg279*) animals upon *P. aeruginosa* infection for 8, 24, and 48 h. (Scale bars: 2 μm.)

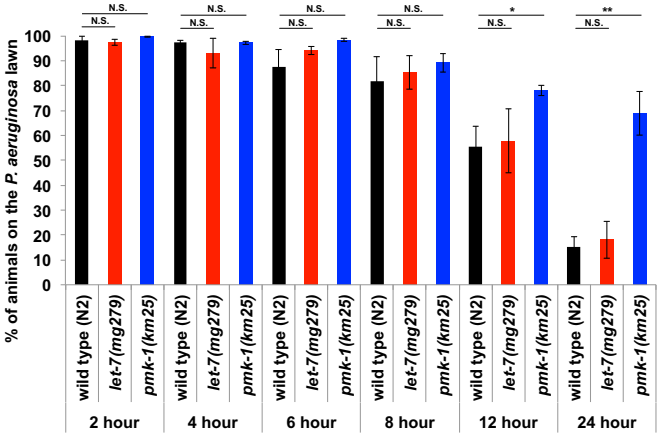


Fig. S7. *P. aeruginosa* avoidance behavior for WT (N2), *let-7*(*mg279*), and *pmk-1*(*km25*) animals at different time points. *$P < 0.05$, **$P < 0.01$; two-tailed *t* test. Error bars represent SD ($n \geq 30$).

Table S1. *C. elegans* strains used in this study

Name	Genotype
AU78	*agIs219* [*T24B8.5p::gfp::unc-54-3′UTR* + *ttx-3::gfp::unc-54-3′UTR*] III
CT11	*hbl-1*(*mg285*) X
KU25	*pmk-1*(*km25*) IV
KU4	*sek-1*(*km4*) X
MT7626	*let-7*(*n2853*) X
N2	WT
VC1518	*atf-7*(*gk715*) III
VT1064	*mir-48*(*n4097*) *maIs105* V; *mir-84*(*n4037*) X
VT1153	*maIs137* [*Plet-7*(*2kb*)*::gfp* + *unc-119*(+)]
VT1159	*maIs138* [*Pmir-84*(*2kb*)*::gfp* + *unc-119*(+)]
VT1259	*maIs150* [*Pmir-48*(*1.5kb*)*::gfp* + *unc-119*(+)]
VT1296	*mir-48 mir-241*(*nDf51*) *maIs105* V
VT1307	*mir-48*(*n4097*) *maIs105* V
VT1313	*mir-84*(*n4037*) X; *maIs105* V
VT1365	*let-7*(*mg279*) X; *maIs105* V
VT1367	*maIs105* [*col-19::gfp*] V (WT*)
VT1423	*mir-48 mir-241*(*nDf51*) *maIs105* V; *mir-84*(*n4037*) X
VT1718	*mir-48*(*n4097*) V; *mir-84*(*n4037*) X
VT2692	*let-7*(*n2853*) X; *maIs105* V
VT2731	*nsy-1*(*ky397*) II
VT2769	*nsy-1*(*ky397*) II; *mir-48 mir-241*(*nDf51*) *maIs105 V*
VT2770	*sek-1*(*km4*) X; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT2771	*pmk-1*(*km25*) IV; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT2788	*let-7*(*n2853*) *mir-84*(*n4037*) X; *maIs105* V
VT2816	*pmk-1*(*km25*) IV; *maIs138*
VT2817	*let-7*(*mg279*) *mir-84*(*n4037*) X; *maIs105* V
VT2855	*let-7*(*mg279*) X
VT2890	*tir-1*(*qd4*) III; *let-7*(*mg279*) X
VT2891	*nsy-1*(*ky397*) II; *let-7*(*mg279*) X
VT2892	*sek-1*(*km4*) *let-7*(*mg279*) X
VT2893	*pmk-1*(*km25*) IV; *let-7*(*mg279*) X
VT2909	*atf-7*(*gk715*) III; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT2923	*pmk-1*(*km25*) IV; *maIs137*
VT2924	*pmk-1*(*km25*) IV; *maIs150* V
VT2933	*lin-41*(*ma104*) I; *let-7*(*mg279*) X
VT2934	*hbl-1*(*mg285*) *let-7*(*mg279*) X
VT2936	*tir-1*(*tm3036*) III; *mir-48 mir-241*(*nDf51*) m*aIs105* V
VT2937	*tir-1*(*ok1052*) III; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT2955	*atf-7*(*qd22*) *agIs219* III; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT2956	*atf-7*(*qd22qd130*) *agIs219* III; *mir-48 mir-241*(*nDf51*) *maIs105* V
VT3037	*maIs380* [*let-7*(+) + *unc-119*(+)] II; *unc-119*(*ed3*) III
VT3047	*let-7*(*n2853*) X; *maIs380* II
VT3049	*let-7*(*mg279*) X; *maIs380* II
VT3051	*mir-48*(*n4097*) V; *mir-84*(*n4037*) X; *maIs380* II
VT3053	*mir-48 mir-241*(*nDf51*); *maIs380* II
VT3086	*maIs380* II
VT432	*lin-41*(*ma104*) I
ZD101	*tir-1*(*qd4*) III

*This strain was used as the WT in all of the heterochronic phenotype assays.

Table S2. Fisher's exact test results for comparing survival curves of various *C. elegans* strains on *P. aeruginosa*

Comparison	Fisher's exact test				Log-rank test
	P value at 25%	*P* value at 50%	*P* value at 75%	*P* value at 90%	*P* value
WT (N2) vs. *maIs380*	9.10E-09	1.20E-12	1.20E-11	3.60E-05	<1.0E-10
WT (N2) vs. *mir-48 mir-241*(*nDf51*)	0.0001	0.0657	0.0011	0.0003	0.0509
WT (N2) vs. *mir-48 mir-241*(*nDf51*); *maIs380*	0.0001	2.60E-06	3.30E-05	0.0008	2.30E-08
maIs380 vs. *mir-48 mir-241*(*nDf51*)	9.90E-13	0.0002	0.7037	1	0.0011
maIs380 vs. *mir-48 mir-241*(*nDf51*); *maIs380*	0.0013	0.0044	0.0568	0.1338	0.0004
mir-48 mir-241(nDf51) vs. *mir-48 mir-241*(*nDf51*); *maIs380*	2.20E-11	0.0218	0.3676	0.2134	0.2488
WT (N2) vs. *mir-48*(*n4097*); *mir-84*(*n4037*)	0.7753	0.1947	0.0486	0.0185	0.0752
WT (N2) vs. *mir-48*(*n4097*); *mir-84*(*n4037*); *maIs380*	5.00E-04	0.0001	0.0052	0.0055	7.50E-06
maIs380 vs. *mir-48*(*n4097*); *mir-84*(*n4037*)	9.70E-08	4.80E-08	3.70E-06	0.0331	<1.0E-10
maIs380 vs. *mir-48*(*n4097*); *mir-84*(*n4037*); *maIs380*	0.0007	2.60E-05	0.0153	0.0582	1.20E-06
mir-48(*n4097*); *mir-84*(*n4037*) vs. *mir-48*(*n4097*); *mir-84*(*n4037*); *maIs380*	0.0022	0.1079	0.24	0.7674	0.0475