**mir-355 Functions as An Important Link between p38 MAPK Signaling and Insulin Signaling in the Regulation of Innate Immunity**

**Lingtong Zhi, Yonglin Yu, Zhixia Jiang & Dayong Wang**

We performed a systematic identification of microRNAs (miRNAs) involved in the control of innate immunity. We identified 7 novel miRNA mutants with altered survival, colony forming in the body, and expression pattern of putative antimicrobial genes after *Pseudomonas aeruginosa* infection. Loss-of-function mutation of *mir-45, mir-75, mir-246, mir-256*, or *mir-355* induced resistance to *P. aeruginosa* infection, whereas loss-of-function mutation of *mir-63* or *mir-360* induced susceptibility to *P. aeruginosa* infection. DAF-2 in the insulin signaling pathway acted as a target for intestinal *mir-355* to regulate innate immunity. *mir-355* functioned as an important link between p38 MAPK signaling pathway and insulin signaling pathway in the regulation of innate immunity. Our results provide an important molecular basis for further elucidation of the functions of various miRNAs in the regulation of innate immunity.

microRNAs (miRNAs), a class of non-coding RNAs with 19–22 nucleotides, are encoded within the genome in organisms. miRNAs are initially transcribed as primary transcripts (pri-miRs). The pri-miRs are further cleaved to produce 70 nucleotide-long precursor miRNAs (pre-miRs) and then mature miRNAs, respectively. The mature miRNAs regulate various fundamental biological processes by imperfectly binding their multiple targeted mRNAs and suppressing the expression of their targeted genes post-transcriptionally. Bioinformatic or functional analyses has suggested that miRNAs can directly target multiple proteins, implying the property of multiple functions for miRNAs.

*Caenorhabditis elegans* is a powerful model animal to determine the functions and mechanisms of miRNAs in regulating certain biological processes, such as transition of developmental timing and longevity. For example, *lin-4* and *let-7* have been proven to be involved in the control of transition of developmental timing. *lin-4* and *let-7*, two important founding members of miRNAs, were first identified in *C. elegans* via forward genetic screens.

*Pseudomonas aeruginosa* is considered to be toxic, and can cause a lethal intestinal infection on nematode host. Upon early *P. aeruginosa* infection, *C. elegans* can upregulate mRNA expression of some defense genes, including genes encoding anti-microbial peptides. In the present study, we performed a systematic identification...
of the possible miRNAs involved in the control of innate immune response to *P. aeruginosa* PA14 infection in *C. elegans*. Moreover, we focused on *mir-355* to examine its molecular basis in the regulation of innate immunity. Our results provide an important basis for further understanding and systematically elucidating the functions of miRNAs in the regulation of innate immunity.

Results

Mutations of some miRNAs altered the survival of nematodes infected with *P. aeruginosa* PA14. Using deletion mutants, we performed a systematic identification of miRNAs involved in the control of *P. aeruginosa* PA14 infection and the corresponding innate immune response in nematodes. Based on phenotypic analysis of survival in miRNA mutants infected with *P. aeruginosa* PA14, we identified 11 miRNA mutants out of the examined 82 miRNA mutants with the abnormal survival compared with wild-type nematodes (Fig. 1, Table S1). These miRNA mutants were let-7 (mg279), mir-45 (n4280), mir-63 (n4568), mir-75 (n4472), mir-84 (n4307), mir-233 (n4761), mir-241 (n4316), mir-246 (n4636), mir-256 (n4471), mir-355 (n4618), and mir-360 (n4635) (Fig. 1). Loss-of-function mutation of let-7, mir-45, mir-75, mir-84, mir-241, mir-246, or mir-256 caused the resistance to the adverse effect of *P. aeruginosa* PA14 infection on survival in nematodes (Fig. 1). In contrast, loss-of-function mutation of mir-63, mir-233, mir-360, or mir-355 resulted in the susceptibility to the adverse effect of *P. aeruginosa* PA14 infection on survival in nematodes (Fig. 1). Statistical comparisons of the survival plots demonstrated that, after *P. aeruginosa* PA14 infection, the survival of let-7 (mg279), mir-45 (n4280), mir-63 (n4568), mir-75 (n4472), mir-84 (n4307), mir-233 (n4761), mir-241 (n4316), mir-246 (n4636), mir-256 (n4471), mir-355 (n4618), or mir-360 (n4635) was significantly (*P* < 0.001) different from that of wild-type nematodes (Table S1). Among these 11 candidate miRNA mutants, let-7 (mg279), mir-84 (n4307), mir-241 (n4316), and mir-233 (n4761) mutants have been reported in the previous studies. We next examined the *P. aeruginosa* PA14 colony-forming unit (CFU) and the expression pattern of putative antimicrobial genes in the other 7 miRNA mutants infected with *P. aeruginosa* PA14.

*P. aeruginosa* PA14 CFU in the new identified miRNA mutants after infection. We employed the CFU to determine PA14 colony formation in the body of miRNA mutant after *P. aeruginosa* infection. After *P. aeruginosa* PA14 infection, we observed that loss-of-function mutation of mir-63, mir-360, or mir-355 significantly enhanced the PA14 colony formation in the body of nematodes (Fig. 2). Different from these, after *P. aeruginosa* PA14 infection, loss-of-function mutation of mir-45, mir-75, mir-246, or mir-256 significantly suppressed the PA14 colony formation in the body of nematodes (Fig. 2).

Expression patterns of putative antimicrobial genes in the new identified miRNA mutants after *P. aeruginosa* infection. We selected some putative antimicrobial genes (lys-1, lys-8, clec-85, dod-22, K08D8.5, F55G11.7, and F55G11.4) to further determine the innate immune response in *P. aeruginosa* PA14 infected miRNA mutants. *P. aeruginosa* PA14 infection significantly increases the transcriptional expression of these antimicrobial genes. In *C. elegans*, lys-1 and lys-8 encode lysozymes, clec-85 encodes a C-type lectin protein, dod-22 and F55G11.7 encode orthologs of human epoxide hydrolase 1, and K08D8.5 and F55G11.4 encode CUB-like domain-containing proteins. After *P. aeruginosa* PA14 infection, mutation of mir-45 increased the expression levels of lys-8, clec-85, dod-22, F55G11.7, and F55G11.4, mutation of mir-75 increased the expression levels of lys-1, lys-8, dod-22, F55G11.7, and F55G11.4, and mutation of mir-246 increased the expression levels of lys-8, clec-85, dod-22, K08D8.5, and F55G11.7, and mutation of mir-256 increased the expression levels of lys-1, lys-8, clec-85, dod-22, and K08D8.5 (Fig. 3). In contrast, mutation of mir-63 decreased the expression levels of lys-1, dod-22, F55G11.7, and F55G11.4, mutation of mir-355 decreased the expression levels of lys-1, lys-8, K08D8.5, F55G11.7, and F55G11.4, and mutation of mir-360 decreased the expression levels of lys-8, dod-22, K08D8.5, and F55G11.7 (Fig. 3). Therefore, loss-of-function mutation of these 7 miRNAs may alter the innate immune response of nematodes to *P. aeruginosa* PA14 infection.

Prediction of targets for new identified miRNAs during the control of innate immune response to *P. aeruginosa* PA14 infection. We further used TargetScan software (http://www.targetscan.org/worm_52/) with preferentially conserved targeting (PCT) between 0 and 1 and miRBase (http://www.mirbase.org) with a score threshold of ~0.1 to predict potential targets for new identified miRNAs in regulating the innate immune response by searching for the presence of conserved sites that match the seed region of new identified miRNAs. In *C. elegans*, insulin and TGF-β signaling pathways are two important signaling pathways in the control of innate immune response to *P. aeruginosa* PA14 infection. In the insulin signaling pathway, *daf-2* gene encodes an insulin receptor. In the TGF-β signaling pathway, *sma-3* gene encodes a Smad protein. Among the predicted targets, we found that *sma-3* in the TGF-β signaling pathway might function as the potential target for *mir-246* in the regulation of innate immunity, and *daf-2* in the insulin signaling pathway might function as the potential target for *mir-355* in the regulation of innate immunity. We next focused on the *mir-355* to examine its molecular basis in the regulation of innate immune response to *P. aeruginosa* PA14 infection. In *C. elegans*, after *P. aeruginosa* PA14 infection, we observed the significant increase in the *mir-355* expression (Fig. S1).

Genetic interaction between *mir-355* and *daf-2* in the regulation of innate immune response to *P. aeruginosa* PA14 infection. We assumed that the *daf-2* mutation would suppress the phenotypes in nematodes with *mir-355* mutation, if *daf-2* is the target of *mir-355*. After *P. aeruginosa* PA14 infection, mutation of *daf-2* significantly increased the survival, decreased the *P. aeruginosa* PA14 CFU, and enhanced the expression levels of putative antimicrobial genes (K08D8.5 and F55G11.7) in *mir-355* (n4618) mutant (Fig. 4). Therefore, *daf-2* may be the target for *mir-355* in the regulation of innate immune response to *P. aeruginosa* PA14 infection.
Effects of intestinal overexpression of daf-2 lacking 3′ UTR or containing 3′ UTR on innate immune response of nematodes overexpressing intestinal mir-355 to P. aeruginosa PA14 infection. 

In C. elegans, mir-355 is expressed in the intestine. Meanwhile, the insulin signaling pathway can function in the intestine to regulate the innate immunity in nematodes. To further confirm the role of DAF-2 as a molecular target of intestinal mir-355 in the regulation of innate immunity, we introduced the intestinal daf-2 lacking 3′ UTR (Ex(Pges-1-daf-2-3′UTR)) into the transgenic nematodes overexpressing intestinal mir-355. After P. aeruginosa PA14 infection, the transgenic strain Is(Pges-1-mir-355);Ex(Pges-1-daf-2-3′UTR) exhibited the similar survival to that in the transgenic strain Ex(Pges-1-daf-2-3′UTR) (Fig. 5a). The P. aeruginosa PA14 CFU in the transgenic strain Is(Pges-1-mir-355);Ex(Pges-1-daf-2-3′UTR) was similar to that in the transgenic strain

Figure 1. Survival in miRNA mutants infected with P. aeruginosa PA14. Bars represent mean ± SD.
**P. aeruginosa** increased the strain overexpressing intestinal MAPM signaling pathway to regulate the innate immune response to **P. aeruginosa** (Fig. S3). These results suggest that expression of **mir-355** may act downstream of PMK-1 in the p38 MAPK signaling pathway. Moreover, we observed that the expression patterns of antimicrobial genes (K08D8.5 and F55G11.7) in the transgenic strain (**Is(Pges-1-mir-355);Ex(Pges-1-daf-2-3’UTR)**) were similar to those in the transgenic strain (**Ex(Pges-1-daf-2-3’UTR)**) (Fig. 5c). Therefore, intestinal overexpression of **daf-2** lacking 3’ UTR may effectively suppress the resistance of nematodes overexpressing intestinal **mir-355** to **P. aeruginosa** PA14 infection.

We also introduced the intestinal **daf-2** containing the 3’ UTR (**Ex(Pges-1-daf-2 + 3’UTR)**) into the transgenic nematodes overexpressing intestinal **mir-355**. After **P. aeruginosa** PA14 infection, the transgenic strain (**Is(Pges-1-mir-355);Ex(Pges-1-daf-2 + 3’UTR)**) exhibited the similar survival to that in the transgenic strain (**Is(Pges-1-mir-355)**) (Fig. 6a). The **P. aeruginosa** PA14 CFU in the transgenic strain (**Is(Pges-1-mir-355);Ex(Pges-1+daf-2 + 3’UTR)**) was also similar to that in the transgenic strain (**Is(Pges-1-mir-355)**) (Fig. 6b). Moreover, we observed that the expression patterns of antimicrobial genes (K08D8.5 and F55G11.7) in the transgenic strain (**Is(Pges-1-mir-355);Ex(Pges-1-daf-2 + 3’UTR)**) were similar to those in the transgenic strain (**Is(Pges-1-mir-355)**) (Fig. 6c). These results suggest that intestinal overexpression of **mir-355** can inhibit the susceptibility of nematodes overexpressing intestinal **daf-2** containing 3’ UTR.

**In vivo 3’-UTR binding assay of **daf-2**.** To further confirm whether **mir-355** regulated the protein levels of DAF-2 through 3’-UTR, we generated a ges-1 promoter driven GFP vector containing 3’-UTR of **daf-2(Pges-1::GFP-3’-UTR)** (**daf-2 wt**) or **ges-1::GFP-3’-UTR (daf-2 mut)**. A **daf-2 3’-UTR mutant** reporter construct was generated by replacing the putative **mir-355** binding site with an oligonucleotide containing the exact identical sequence of **mir-355**. A **ges-1::mCherry3’-UTR** construct that drives the mCherry expression was employed as an internal control. After **P. aeruginosa** PA14 infection, the GFP expression was suppressed in wild-type nematodes (Fig. S2). In contrast, mutagenesis of putative binding site for **mir-355** (**ges-1-daf-2-3’UTR**) abolished this suppression of GFP expression in wild-type nematodes (Fig. S2). After **P. aeruginosa** PA14 infection, we observed the higher GFP expression in **ges-1-daf-2-3’UTR** mutant than that in wild-type nematodes (Fig. S2). These results demonstrate that **mir-355** may inhibit the DAF-2 function through binding to its 3’-UTR and suppressing its translation in **P. aeruginosa** PA14 infected nematodes.

**mir-355** acted downstream of **PMK-1** to regulate the innate immune response to **P. aeruginosa** PA14 infection. In **C. elegans**, p38 MAPK signaling pathway is a conserved signaling pathway required for the pathogen resistance15,16. In the p38 MAPK signaling pathway,** pmk-1** encodes a p38 MAPK. Overexpression of intestinal **pmk-1** induced a resistance to **P. aeruginosa** PA14 infection, decreased **P. aeruginosa** PA14 CFU, and enhanced the expressions of putative antimicrobial genes (K08D8.5 and F55G11.7) (Fig. 7). In the transgenic strain overexpressing intestinal **pmk-1**, we found that mutation of **mir-355** significantly suppressed the survival, increased the **P. aeruginosa** PA14 CFU, and inhibited the expressions of putative antimicrobial genes (K08D8.5 and F55G11.7) (Fig. 7). Moreover, after **P. aeruginosa** PA14 infection,** pmk-1** mutation significantly decreased the expression of **mir-355** (Fig. S3). These results suggest that **mir-355** may act downstream of **PMK-1** in the p38 MAPM signaling pathway to regulate the innate immune response to **P. aeruginosa** PA14 infection.

**Genetic interaction between **mir-355** and **DAF-16** or **SKN-1** in the regulation of innate immune response to **P. aeruginosa** PA14 infection.** In **C. elegans**, DAF-16, a FOXO transcriptional factor, acts downstream of DAF-2 in the insulin signaling pathway to regulate the innate immune response to pathogen infection17,18. **SKN-1**, a bZip transcriptional factor, functions in the p38 MAPK signaling pathway to regulate diverse biological processes, such as stress response18. Meanwhile, **SKN-1** can be directly phosphorylated by some kinases downstream of DAF-2 in the insulin signaling pathway19. Additionally, the activation of **SKN-1** in response to pathogens is dependent on p38 MAPK signaling18. We found that RNA interference (RNAi) knockdown of **daf-16** or **skn-1** suppressed the survival, increased the **P. aeruginosa** PA14 CFU, and decreased the expressions of putative antimicrobial genes (K08D8.5 and F55G11.7) in **P. aeruginosa** PA14 infected transgenic strain of **Ex(Pges-1-mir-355)** (Fig. 8a–c). After **P. aeruginosa** PA14 infection, we further found that the stain...
of *daf-16(mu86):Is(Pges-1-mir-355):skn-1(RNAi)* showed more severely suppressed survival compared with the strain of *daf-16(mu86):Is(Pges-1-mir-355)* or the strain of *Is(Pges-1-mir-355):skn-1(RNAi)* (Fig. S4).

After *P. aeruginosa* PA14 infection, *mir-355* mutation induced a significant decrease in *daf-16* expression (Fig. S5). In *C. elegans*, *skn-1* has three different isoforms. *skn-1a* and *skn-1c* are expressed in the intestine, and *skn-1b* is expressed in the neurons. After *P. aeruginosa* PA14 infection, *mir-355* mutation induced a significant decrease in *daf-16* expression.
decrease in skn-1a or skn-1c expression, whereas mir-355 mutation did not significantly affect the skn-1b expression (Fig. S5).

Discussion
In C. elegans, with the exception of lin-4, let-7, lsy-6, and mir-1, individual deletion of most of the miRNAs did not cause the overt phenotypes31, and the majority of miRNA may be not essential for the developmental control32. In contrast to these, a large amount of miRNAs were differentially expressed during the aging, and some miRNAs have been shown to be involved in the control of aging on the level of organism lifespan, tissue aging or cellular senescence in C. elegans33. In this study, we further performed the systematic identification of possible miRNAs involved in the control of innate immune response to P. aeruginosa PA14 infection. Based on the phenotypic analysis of survival, we identified 11 miRNAs (let-7, mir-45, mir-63, mir-75, mir-84, mir-241, mir-246, mir-256, mir-355, mir-233, and mir-360) having the function in the control of innate immunity to P. aeruginosa PA14 infection (Fig. 1). Among these 11 miRNAs, mir-45, mir-63, mir-75, mir-246, mir-256, and mir-355 are new identified miRNAs with the function in the control of innate immunity. Among these new identified miRNA mutants, mir-45(n4280), mir-63(n4472), mir-75(n4472), mir-246(n4636), and mir-256(n4471) mutants were resistant to P. aeruginosa PA14 infection, whereas mir-63(n4568), mir-355(n4618), and mir-360(n4635) mutants were susceptible to P. aeruginosa PA14 infection (Fig. 1). Under normal conditions, loss-of-function mutation of mir-45, mir-63, mir-75, mir-246, mir-256, or mir-355 did not obviously affect the longevity31. Under normal conditions, loss-of-function mutation of mir-360 also does not affect the longevity31.

In this study, the CFU assay demonstrated that the P. aeruginosa PA14 infected mir-63(n4568), mir-355(n4618), and mir-360(n4635) mutants had the enhanced P. aeruginosa PA14 colony formation in the body.
compared with \textit{P. aeruginosa} PA14 infected wild-type nematodes; however, the \textit{P. aeruginosa} PA14 infected \textit{mir-45(n4280)}, \textit{mir-75(n4472)}, \textit{mir-246(n4636)}, and \textit{mir-256(n4471)} mutants had the decreased \textit{P. aeruginosa} PA14 colony formation in the body compared with \textit{P. aeruginosa} PA14 infected wild-type nematodes (Fig. 2). These results suggest that the observed susceptibility to \textit{P. aeruginosa} PA14 infection in \textit{mir-63(n4568)}, \textit{mir-355(n4618)}, or \textit{mir-360(n4635)} mutant may be at least partially due to the decreased \textit{P. aeruginosa} PA14 colony formation in the body of nematodes, and the observed resistance to \textit{P. aeruginosa} PA14 infection in \textit{mir-45(n4280)}, \textit{mir-75(n4472)}, \textit{mir-246(n4636)}, or \textit{mir-256(n4471)} mutant may be at least partially due to the suppressed \textit{P. aeruginosa} PA14 colony formation in the body of nematodes. Moreover, the analysis on expression patterns of putative antimicrobial genes further suggested that the observed susceptibility to \textit{P. aeruginosa} PA14 infection in \textit{mir-63(n4568)}, \textit{mir-355(n4618)}, or \textit{mir-360(n4635)} mutant may be largely due to the decreased expression of the examined antimicrobial genes, and the observed resistance to \textit{P. aeruginosa} PA14 infection in \textit{mir-45(n4280)}, \textit{mir-75(n4472)}, \textit{mir-246(n4636)}, or \textit{mir-256(n4471)} mutant may be largely due to the increased expression of the examined putative antimicrobial genes (Fig. 3). Interestingly, mutations of these miRNAs induced different expression patterns of the putative antimicrobial genes in \textit{P. aeruginosa} PA14 infected nematodes (Fig. 3), implying that the new identified 7 miRNAs may regulate the innate immune response to \textit{P. aeruginosa} PA14 infection through different molecular mechanisms.

In \textit{C. elegans}, \textit{mir-45} has been shown to be involved in the control of toxicity formation of multi-walled carbon nanotubes\textsuperscript{34}. \textit{mir-63} was involved in the control of embryonic hypoxic response\textsuperscript{35}, \textit{mir-246} regulates both the longevity and the embryonic hypoxic response\textsuperscript{35,36}. It was reported that \textit{mir-355} could regulate the toxicity of multi-walled carbon nanotubes\textsuperscript{34}. Besides the innate immune response to fungal infection\textsuperscript{37}, \textit{mir-360} has also been shown to be involved in the control of reproductive toxicity of graphene oxide and the beneficial effects of

**Figure 5.** Effects of intestinal overexpression of \textit{daf-2} lacking 3′ UTR on innate immune response to \textit{P. aeruginosa} PA14 infection in nematodes overexpressing intestinal \textit{mir-355}. (a) Effects of intestinal overexpression of \textit{daf-2} lacking 3′ UTR on survival of nematodes overexpressing intestinal \textit{mir-355} after \textit{P. aeruginosa} PA14 infection. Statistical comparisons of the survival plots indicate that, after the \textit{P. aeruginosa} PA14 infection, the survival of transgenic strain \textit{Is(Pges-1-mir-355);Ex(Pges-1-daf-2-3′UTR)} was significantly different from that of transgenic strain \textit{Is(Pges-1-mir-355)} (\(P < 0.001\)). Bars represent mean ± SD. (b) Effects of intestinal overexpression of \textit{daf-2} lacking 3′ UTR on \textit{P. aeruginosa} PA14 CFU in the body of nematodes overexpressing intestinal \textit{mir-355}. Bars represent mean ± SD. **\(P < 0.01\) vs wild-type (if not specially indicated). (c) Effects of intestinal overexpression of \textit{daf-2} lacking 3′ UTR on expression patterns of putative antimicrobial genes of nematodes overexpressing intestinal \textit{mir-355} after \textit{P. aeruginosa} PA14 infection. Normalized expression is presented relative to wild-type expression. Bars represent mean ± SD. **\(P < 0.01\).
glycyrrhizic acid against the toxicity of graphene oxide\textsuperscript{37,38}. In contrast, the biological functions of mir-75 and mir-256 are still unclear. In this study, our results further indicate the novel function of these 7 miRNAs in the regulation of innate immunity. After \textit{P. aeruginosa} infection, we observed the significant increase in mir-355 expression (Fig. S1), which implies that the mir-355 expression may be activated to mediate a protection mechanism for nematodes against the \textit{P. aeruginosa} infection in nematodes.

Previous studies have identified the potential target(s) for some miRNAs involved in the control of innate immunity in nematodes. For example, mir-233 is directly targeted to \textit{SCA-1}, a homologue of the sarco/endo-plasmic reticulum Ca\textsuperscript{2+}-ATPase, to regulate the innate immune response to \textit{P. aeruginosa} infection\textsuperscript{18}. \textit{let-7} might be directly target to \textit{LIN-41} or to \textit{HBL-1} to regulate the innate immunity in \textit{P. aeruginosa} infected nematodes\textsuperscript{20}. \textit{SKN-1/Nrf} could act the direct target for both mir-84 and mir-241, another two members in the \textit{let-7} family, in the control of innate immune response to \textit{P. aeruginosa} infection\textsuperscript{19}. With the aid of TargetScan and miRBase, we found that some of the new identified 7 miRNAs may regulate the innate immune response to \textit{P. aeruginosa} infection by at least suppressing the functions of insulin or TGF-\textbeta signaling pathway. This information further reflects the crucial roles of insulin and TGF-\textbeta signaling pathways in the regulation of innate immune response to \textit{P. aeruginosa} infection. Moreover, the predicted targets in insulin and TGF-\textbeta signaling pathways provide important clues for further elucidating the underlying mechanisms of new identified miRNAs in the regulation of innate immunity.

Importantly, some of the candidate miRNAs are conserved in human\textsuperscript{39}. Among the new identified miRNAs involved in the control of innate immunity, mir-45 is the homologue of human miR-134 and mir-708, mir-63 is the homologue of human miR-96, mir-183, mir-200a, and mir-514, mir-75 is the homologue of human miR-9, miR-320, and miR-548a, and mir-256 is the homologue of human miR-1, miR-122, miR-206, and miR-519\textsuperscript{39}. The
data obtained in *C. elegans* imply that the homologues of these *C. elegans* miRNAs in human might be also very important for the innate immunity regulation.

In this study, based on the genetic interaction assay between *mir-355* and DAF-2 (Fig. 4), we confirmed that DAF-2 in the insulin signaling pathway may act as the potential target for *mir-355* in the regulation of innate immune response to *P. aeruginosa* PA14 infection. More importantly, the investigations on the effects of intestinal overexpression of *daf-2* lacking 3′ UTR or containing 3′ UTR on innate immunity in nematodes overexpressing intestinal *mir-355* suggested the 3′ UTR binding property of *mir-355* to DAF-2 during the control of innate immune response to *P. aeruginosa* PA14 infection (Figs 5 and 6). Our results further imply the crucial function of *mir-355*-DAF-2 signaling cascade in the intestinal cells in the regulation of innate immune response to *P. aeruginosa* PA14 infection.

Moreover, in this study, we found that *mir-355* mutation could suppress the resistance of *Ex(Pges-1-pmk-1)* to *P. aeruginosa* PA14 infection (Fig. 7), and RNAi knockdown of *daf-16* or *skn-1* could suppress the resistance of *Ex(Pges-1-mir-355)* to *P. aeruginosa* PA14 infection (Fig. 8a–c). Therefore, *mir-355* may act downstream of *mir-355*-DAF-2 signaling cascade in the intestinal cells in the regulation of innate immune response to *P. aeruginosa* PA14 infection.

Moreover, in this study, we performed the large scale genetic screen of miRNAs involved in the control of innate immune response to *P. aeruginosa* PA14 infection using deletion miRNA mutants. Based on this large scale deletion studies, we identified 7 novel miRNAs involved in the control of innate immune response to *P. aeruginosa* PA14 infection. Among these 7 novel miRNAs, loss-of-function mutant of *mir-45*, *mir-75*, *mir-246*, or *mir-256* was resistant to *P. aeruginosa* PA14 infection, whereas loss-of-function mutant of *mir-63*, *mir-355*, or *mir-360* was susceptible to *P. aeruginosa* PA14 infection. Our results proved the novel functions of these 7 miRNAs in the regulation of innate immunity. Some proteins in the insulin or TGF-β signaling pathway might act as the potential targets for these 7 miRNAs in the regulation of innate immunity. Moreover, we found that DAF-2 in the insulin signaling pathway can act as the target for *mir-355* in the intestine to regulate the innate immunity.
control of innate immunity, mir-355 may function as an important molecular link between the p38 MAPK signaling pathway and the insulin signaling pathway.

Methods

C. elegans strains. Nematodes strains used in the present study were wild-type N2, mutants of let-7(mg279) X, lys-6(a71) V, lin-4(e912) II, mir-1(n4101) I, mir-2(n4108) I, mir-34(n4276) X, mir-35(gk262) II, mir-35-41(nDf50) II, mir-42-44(nDf49) II, mir-45(n2480) II, mir-46-48(n4747) III, mir-47(gk167) X, mir-51(n4473) IV, mir-52(n4100) IV, mir-53(n4113) IV, mir-54-55(nDf58) X, mir-57(gk175) II, mir-58(n4640) IV, mir-59(n4604) IV, mir-60(n4947) II, mir-61I&250(nDf59) V, mir-62(n4539) X, mir-63(n4568) X, mir-64-66&229(nDf63) III,
mir-67(n4899) III, mir-70(n4109) V, mir-71(n4115) I, mir-72(n4130) II, mir-73-74(nDf47) X, mir-75(n4472) X, mir-76(n4474) III, mir-77(n4286) II, mir-78(n4637) IV, mir-79(n4126) I, mir-80(nDf53) III, mir-81-82(nDf54) X, mir-83(n4630) IV, mir-84(n4307) III, mir-85(n4117) II, mir-86(n4607) III, mir-87(nrd104) V, mir-124(n4255) IV, mir-228(n4382) IV, mir-230(nDf53) X, mir-231(n4571) III, mir-232(nDf56) IV, mir-233(n4761) X, mir-234(n4520) II, mir-235(n4504) I, mir-237(n4296) X, mir-238(n4112) III, mir-239a(n239b(nDf62) X, mir-240-246(n4541) X, mir-241(n4316) V, mir-242(n4605) IV, mir-243(n4759) IV, mir-244(n4367) I, mir-245(n4798) I, mir-246(n4636) IV, mir-247-249(n4408) X, mir-250(nDf64) II, mir-251(n4607) X, mir-252-255(n4570) V, mir-253(n4670) I, mir-254(n4470) X, mir-256(n4471) V, mir-257(n4548) V, mir-258-259(n4797) X, mir-259(n4106) V, mir-260(n4601) II, mir-261(n4594) II, mir-262-265(n4534) V, mir-266(n4639) V, mir-269(n4641) IV, mir-270(n4595) IV, mir-273(n4438) I, mir-355(n4618) II, mir-357-358(nDf60) V, mir-359(n4540) X, mir-360(n4635) X, pmk-1(n2025) IV, df-2(n1370) III, and mir-355(n4618);df-2(n1370), and transgenic strains of Ex(Pges-1-mpk-1), mir-355(n4618);Ex(Pges-1-mpk-1), Ex(Pges-1-daf-2-3′UTR), Ex(Pges-1-daf-2 + 3′UTR), Is(Pges-1-mir-355), df-16(RNAi);Is(Pges-1-mir-355), df-16(mu86);Is(Pges-1-mir-355), Is(Pges-1-mir-355);skn-1(RNAi), df-16(mu86);Is(Pges-1-mir-355);skn-1(RNAi), Is(Pges-1-mir-355);Ex(Pges-1-daf-2-3′UTR), and Is(Pges-1-mir-355);Ex(Pges-1-daf-2 + 3′UTR). Is(Pges-1-mir-355) is a transgenic strain with multi-copy mir-355 insertion. All the used miRNA deletion mutants are deletion mutants89.101. The mutants were backcrossed with wild-type for at least four times. In nDf64, mir-253 and part of F44E7.5 are deleted. Some of the used strains were from Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Nematodes were normally maintained on nematode growth medium (NGM) plates seeded with Escherichia coli OP50 as a food source at 20 °C as described89.

**P. aeruginosa PA14 pathogenesis assay.** Age synchronous populations of young adults were prepared, and infected with *P. aeruginosa* PA14 as described41. *P. aeruginosa* PA14 cultured in Luria broth was seeded on the killing plates containing a modified NGM (0.35% instead of 0.25% peptone). *P. aeruginosa* PA14 was incubated first at 37 °C for 24-h, and then at 25 °C for 24-h. *P. aeruginosa* PA14 infection was started by adding 60 young adult nematodes to the killing plates at 25 °C. Full-lawn PA14 killing plates were prepared for the *P. aeruginosa* PA14 infection.

**Survival assay.** Survival assay was performed basically as described42. During the *P. aeruginosa* PA14 infection, nematodes were scored for dead or live every 12-h. Nematodes were counted as dead, if no response was detected after prodding with a platinum wire. Nematodes were transferred daily at 25 °C (if not specially indicated) for the first 5 days of adulthood. For the survival assay, graphs are representative of three trials. The survival curves were considered to be significantly different from the control, when the p-values were less than 0.001.

**Bacterial CFU assay.** The CFU of *P. aeruginosa* PA14 was analyzed as described previously43. Young adult nematodes were infected with *P. aeruginosa* PA14 infection for 24-h. After *P. aeruginosa* infection, the examined nematodes were transferred into a M9 buffer containing 25 mM levamisole to stop pharyngeal pumping. The nematodes were placed onto a NGM plate containing ampicillin (1 mg/mL) and gentamicin (1 mg/mL) for 15-min to eliminate *P. aeruginosa* PA14 stuck onto the body surface of animals. The nematodes were transferred onto a new NGM plate containing ampicillin (1 mg/mL) and gentamicin (1 mg/mL) for 30-min to further eliminate the external *P. aeruginosa* PA14. The nematodes were lysed with a motorized pestle, and the lysates were serially diluted with M9 buffer. The diluted lysates were plated onto Luria-Bertani plates containing rifampicin (100 μg/mL) for the selection of *P. aeruginosa* PA14. After incubation at 37 °C overnight, colonies of *P. aeruginosa* PA14 were counted for the determination of CFU per nematode. Six replicates of ten nematodes each were performed.

**Quantitative real-time polymerase chain reaction (qRT-PCR).** The young adult nematodes were infected with *P. aeruginosa* PA14 for 24-h. Total RNA (~1 μg) of nematode was extracted using an RNeasy Mini kit (Qiagen), and reverse-transcribed using a cDNA Synthesis kit (Bio-Rad Laboratories). qRT-PCR was performed at an optimized annealing temperature of 58 °C. The examined putative antimicrobial genes were lys-1, lys-8, dec-85, dod-22, K08D8.5, F55G11.7, and F55G11.4. Relative quantification of targeted genes in comparison to the reference *tba-1* gene encoding a tubulin was determined. The expression of mir-355 is presented as the relative expression ratio between mir-355 and F55G11.9, which encodes a small nuclear RNA U6. The primer used for the transcription of mir-355 was GTGTTATACCGTGCAGGTTCCAGGGATTTCTCGCATGACT. The primer for qRT-PCR of mir-355 was TGCTAC TTTGTTTAGCCTGAG, and the common reward primer was GTGCAGGGTCCGAGGT. The primers for qRT-PCR of F55G11.9 were GAAGATTACGATGACCC and TTGGAACGTTATGAAT. The designed primers for targeted genes and reference *tba-1* gene were shown in Table S2. Three replicates were performed.

**RNAi assay.** RNAi was basically performed by feeding nematodes with *E. coli* strain HT115 (DE3) expressing double-stranded RNA that is homologous to a targeted gene45. *E. coli* HT115 (DE3) grown in LB broth containing ampicillin (100 μg/mL) was plated onto NGM plants containing ampicillin (100 μg/mL) and isopropyl-1-thio-β-D-galactopyranoside (IPTG, 5 mM). L1 larvae nematodes were transferred onto RNAi plates for 2 days at 20 °C until they developed into the gravid. The gravid adults were transferred onto a fresh RNAi-expressing bacterial lawn to let them lay eggs so as to obtain the second generation of RNAi population. The eggs were allowed to develop into young adults for the subsequent assays of lifespan, CFU, and gene expression pattern.

**DNA constructs and germline transformation.** To generate entry vector carrying promoter sequence, the *ges-1* promoter used for intestine-specific expression was amplified by PCR from *C. elegans* genomic DNA. The *ges-1* promoter was inserted into pPD95_77 vector in the sense orientation. The *mir-355, pmk-1*, and
**3′-UTR reporters and microscopy.** The 3′-UTR (wt) of *daf-2* was amplified by PCR from the genomic DNA. This fragment was ligated to the NcoI/XbaI site of pC20 (a gift from Dr. V. Ambros) and integrated into the *Pges-1::mCherry-3′-UTR (tag-196)* plasmid to generate the transgenic strain *Pges-1::mCherry-3′-UTR (daf-2 mut)*. The expression of GFP and mCherry was observed and analyzed under a fluorescence microscope (Olympus BX41, Olympus Corporation, Japan). The designed primers for DNA construct generation were shown in Table S3.

**Statistical analysis.** All data in this article were expressed as means ± standard deviation (SD). Graphs were generated using Microsoft Excel (Microsoft Corporation, Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). Probability levels of 0.05 and 0.01 were considered statistically significant. Lifespan was analyzed using the log-rank test.

**References**

1. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
2. Guo, H., Ingolia, N. T., Weissman, J. S. & Bartel, D. P. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* **466**, 835–840 (2011).
3. Yarborough, M. L., et al. Primate-specific miR-576-3p sets host defense signaling threshold. *Nat. Commun.* **5**, 4963 (2015).
4. Lewis, B. P., Shih, J. H., Jones-Rhoades, M. W., Bartel, D. P. & Burge, C. B. Prediction of mammalian microRNA targets. *Cell* **115**, 787–798 (2003).
5. Lee, R. C. & Ambros, V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862–864 (2001).
6. Lim, L. P., et al. The microRNAs of *Caenorhabditis elegans*. *Genes Dev.* **17**, 991–1008 (2003).
7. Imukai, S. & Slack, F. MicroRNAs and the genetic network in aging. *J. Mol. Biol.* **425**, 3601–3608 (2013).
8. Lee, R. C., Feinbaum, R. L. & Ambros, V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854 (1993).
9. Reinhart, B. et al. The 21 nucleotide let-7 RNA regulates *C. elegans* developmental timing. *Nature* **403**, 901–906 (2000).
10. Kurz, C. L. & Ewbank, J. J. *Caenorhabditis elegans*: an emerging genetic model for the study of innate immunity. *Nat. Rev. Genet.* **4**, 380–390 (2003).
11. Felix, M. A. & Duveau, F. Population dynamics and habitat sharing of natural populations of *Caenorhabditis elegans* and *C. briggsae*. *BMC Biol.* **10**, 59 (2012).
12. Sifri, C. D., Begun, J. & Ausubel, F. M. The worm has turned-microbial virulence modeled in *Caenorhabditis elegans*. Trends *Microbiol.* **13**, 119–127 (2005).
13. Iraizzoqi, J. E., Urbach, J. M. & Ausubel, F. M. Evolution of host innate defense: insights from *C. elegans* and primitive invertebrates. *Nat. Rev. Immunol.* **10**, 47–58 (2010).
14. Alper, S., McBride, S. J., Lackford, B., Freedman, J. H. & Schwartz, D. A. Specificity and complexity of the *Caenorhabditis elegans* innate immune response. *Mol. Cell. Biol.* **27**, 5544–5553 (2007).
15. Kim, D. H. et al. A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. *Science* **297**, 623–626 (2002).
16. Evans, E. A., Kawhi, T. & Tan, M. *Pseudomonas aeruginosa* suppresses host immunity by activating the DAF-2 insulin-like signaling pathway in *Caenorhabditis elegans*. *PLoS Pathog.* **9**, e1000175 (2008).
17. Roberts, A. F., Guimennet, T. L., Gleason, R. J., Wang, H. & Padgett, R. W. Regulation of genes affecting body size and innate immunity by the DAF-1/MBP-like pathway in *Caenorhabditis elegans*. *BMC Dev. Biol.* **10**, 61 (2010).
18. Dai, L., Gao, J., Zou, C., Ma, Y. & Zhang, K. *mir-233* modulates the unfolded protein response in *C. elegans* during *Pseudomonas aeruginosa* infection. *PLoS Pathog.* **11**, e1004606 (2015).
19. Liu, F. et al. Nuclear hormone receptor regulation of microRNAs controls innate immune responses in *C. elegans*. *PLoS Pathog.* **9**, e1003545 (2013).
20. Ren, Z. & Ambros, V. *Caenorhabditis elegans* microRNAs of the let-7 family act in innate immune response circuits and confer robust developmental timing against pathogen stress. *Proc. Natl. Acad. Sci. USA* **112**, E2366–E2375 (2015).
21. Sun, L.-M., Zhi, L.-T., Shakoor, S., Liao, K. & Wang, D.-Y. MicroRNAs involved in the control of innate immunity in Candida infected *Caenorhabditis elegans*. *Sci. Rep.* **6**, 36036 (2016).
22. Zhi, L.-T., Yu, Y.-L., Li, Y.-Y., Wang, D.-Y. & Wang, D.-Y. Molecular control of innate immune response to *Pseudomonas aeruginosa* infection by intestinal let-7 *In Caenorhabditis elegans*. *PLoS Pathog.* **13**, e1006152 (2017).
23. Yu, Y. -L., Zhi, L. -T., Guan, X. -M., Wang, D. -Y. & Wang, D. -Y. FLP-4 neuropeptide and its receptor in a neuronal circuit regulate preference choice through functions of ASH-2 trithorax complex in *Caenorhabditis elegans*. *Sci. Rep.* **6**, 21485 (2016).
24. Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. Most mammalian microRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
25. Betel, D., Koppal, A., Agius, P., Sander, C. & Leslie, C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* **11**, R90 (2010).
26. Martinez, N. J. et al. Genome-scale spatiotemporal analysis of *Caenorhabditis elegans* microRNA promoter activity. *Genome Res.* **18**, 2095–2015 (2008).
27. Partridge, F. A., Gravato-Nobre, M. J. & Hodgkin, J. Signal transduction pathways that function in both development and innate immunity. *Dev. Dyn.* **239**, 1330–1336 (2010).
28. Zhao, Y.-L. & Zhi, L.-T. et al. p38 MAPK SKN-1/Nrf signaling cascade is required for intestinal barrier against graphene oxide toxicity in Caenorhabditis elegans. Nanotoxicology 10, 1469–1479 (2016).
29. Tullet, J. M. et al. Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. Cell 132, 1025–1038 (2008).
30. Hoeven, R. V., McCallum, K. C., Cruz, M. R. & Garson, D. A. Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in C. elegans. PLoS Pathog. 7, e1002453 (2011).
31. Miska, E. A. et al. Most Caenorhabditis elegans microRNAs are individually not essential for development or viability. PLoS Genet. 3, e215 (2007).
32. Alvarez-Saavedra, E. & Horvitz, H. R. Many families of C. elegans microRNAs are not essential for development or viability. Curr Biol. 20, 367–373 (2010).
33. Smith-Vikos, T. & Slack, F. J. MicroRNAs and their roles in aging. J. Cell. Sci. 125, 7–17 (2012).
34. Zhao, Y.-L., Jia, R.-H., Qiao, Y. & Wang, D.-Y. Glycyrrhizic acid, active component from Glycyrrhiza radix, prevents toxicity of graphene oxide by influencing functions of microRNAs in nematode Caenorhabditis elegans. Nanomedicine: Nanotechnol. Biol. Med. 12, 735–744 (2016).
35. Kagias, K. & Pocock, R. microRNA regulation of the embryonic hypoxic response in Caenorhabditis elegans. Sci. Rep. 5, 11284 (2015).
36. Brenner, S. The genetics of Caenorhabditis elegans. Genetics 77, 71–94 (1974).
37. Zhao, Y.-L., Wu, Q.-L. & Wang, D.-Y. An epigenetic signal encoded protection mechanism is activated by graphene oxide to inhibit its induced reproductive toxicity in Caenorhabditis elegans. Biomaterials 79, 15–24 (2016).
38. Zhao, Y.-L., Jia, R.-H., Qiao, Y. & Wang, D.-Y. Glycyrrhizic acid, active component from Glycyrrhiza radix, prevents toxicity of graphene oxide by influencing functions of microRNAs in nematode Caenorhabditis elegans. Nanomedicine: Nanotechnol. Biol. Med. 12, 735–744 (2016).
39. Ibañez-Ventoso, C., Vora, M. & Driscoll, M. Sequence relationships among C. elegans, D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology. PLoS ONE 3, e2818 (2008).
40. Brenner, S. The genetics of Caenorhabditis elegans. Genetics 77, 71–94 (1974).
41. Wang, D.-Y., Cao, M., Dinh, J. & Dong, Y.-Q. Methods for creating mutations in C. elegans that extend lifespan. Methods Mol. Biol. 1048, 65–75 (2013).
42. Yu, Y.-L., Zhi, L.-T., Wu, Q.-L., Jing, L.-N. & Wang, D.-Y. NPR-9 regulates innate immune response in Caenorhabditis elegans by antagonizing activity of AIB interneurons. Cell. Mol. Immunol. https://doi.org/10.1038/cmi.2016.8 (2016).
43. Kamath, R. X., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. & Ahringer, J. Effectiveness of specific RNA-mediated interference through ingested double stranded RNA in C. elegans. Genome Biol. 2, 1–10 (2001).
44. Mello, C. & Fire, A. DNA transformation. Methods Cell. Biol. 48, 451–482 (1995).
45. Evans, T. C. Transformation and microinjection. WormBook, https://doi.org/10.1895/wormbook.1.108.1.

Acknowledgements
This work was supported by the grants from Fundamental Research Funds for the Central Universities of China (KYLX15_0172), and Scientific Research Foundation of Graduate School of Southeast University.

Author Contributions
Conceived and designed the experiments: D.W. Performed the experiments and analyzed the data: L.Z., Y.Y. and Z.J. Wrote the paper: D.W.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-15271-2.

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017