Nuclear Hormone Receptor Regulation of MicroRNAs Controls Innate Immune Responses in C. elegans

Feng Liu1,2, Chen-Xi He3, Li-Jun Luo1, Quan-Li Zou1, Yong-Xu Zhao1, Ratni Saini4, San-Feng Han5, Hans-Joachim Knölker4, Li-Shun Wang5, Bao-Xue Ge1,3*

1 Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences/Shanghai Jiao Tong University School of Medicine, Shanghai, China, 2 Graduate School of Chinese Academy of Sciences, Beijing, China, 3 Shanghai Key Laboratory of Tuberculosis, Clinical and Translational Research Center, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, China, 4 Department of Chemistry, Technical University of Dresden, Dresden, Germany, 5 Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Abstract

Nuclear hormone receptors respond to small molecules such as retinoids or steroids and regulate development. Signaling in the conserved p38/PMK-1 MAP kinase pathway regulates innate immunity. In this study, we show that the Caenorhabditis elegans nuclear receptor DAF-12 negatively regulates the defense against pathogens via the downstream let-7 family of microRNAs, which directly target SKN-1, a gene downstream of PMK-1. These findings identify nuclear hormone receptors as components of innate immunity that crosstalk with the p38/PMK-1 MAP kinase pathway.

Introduction

Innate immunity is an evolutionarily conserved response to pathogens and forms the first line of defense for most organisms. When infected by pathogens, the nematode Caenorhabditis elegans mounts a rapid innate immune response and produces an array of anti-microbial genes, similar to other organisms throughout the animal kingdom [1], [2]. Several conserved signaling pathways that function in the perception of and defense against bacterial pathogens have been identified in C. elegans. These pathways include the NSY-1/PMK-1 MAP kinase signaling pathway, the DAF-2/DAF-16 insulin/insulin-like growth factor (IGF)-1 like signaling pathway, the DBL-1/transforming growth factor-β (TGF-β) signaling pathway and the BAR-1 β-catenin signaling pathway [1]–[4]. Although many conserved innate immune components have been identified in C. elegans using genetic and biochemical approaches, extensive characterization of the signaling networks that regulate the host response and outcome of infections is warranted.

Nuclear hormone receptors (NRs) are a class of transcription factors that are regulated by small lipophilic hormones. In all, 284 NRs have been identified in C. elegans, and approximately 20 of them have been genetically analyzed [5]. The dauer formation abnormal gene daf-12, a well-characterized nuclear hormone receptor, and the orphan receptors nhr-6 and nhr-48 are the conserved homologs of the mammalian vitamin D receptor and liver-X receptor [6], [7]. DAF-12 regulates developmental progression and arrest in response to environmental cues [6], [8]. In favorable conditions, the activation of TGF-β and insulin/IGF-1 signaling cascades results in the production of the DAF-12 steroidal ligands, dafachronic acids (DAs). DAs are synthesized from cholesterol via a multi-step pathway involving the daf-36 Rieske-like oxygenase and the daf-9 cytochrome P450 enzyme, which promote a rapid progression through four larval stages (L1 to L4) to reproductive adults [9]–[14]. In unfavorable environments, DAs expression is suppressed, and DAF-12, without its ligand, binds to the co-repressor DIN-1, resulting in an arrest at a stress-resistant, long-lived alternative third larval stage, called the dauer diapauses (L3d) [15]. In addition, DAF-12 regulates the normal lifespan of worms and the longevity of germ-line-ablated animals [16]–[21]. However, the role of DAF-12 in the immune regulation of C. elegans remains unknown.

MicroRNAs (miRNAs) are small non-coding RNA molecules that repress target gene expression by base-pairing with partially complementary sequences in the 3’-untranslated regions (3’-UTR) of target miRNAs [22], [23]. MiRNAs influence molecular signaling pathways and regulate many biological processes, including immune function [24]. Originally discovered in C. elegans, lethal-7 (let-7) miRNA is conserved across species in both sequence and temporal expression [25], [26]. In C. elegans, the let-7 miRNA homologs mir-48, mir-84 and mir-241 (together referred to as let-7s) regulate developmental timing and promote cellular differentiation pathways [27], [28]. The human let-7-related miRNAs also have anti-proliferative functions, and the downregulation of let-7 levels is associated with a variety of cancers, such as lung, breast and colon cancer [27], [28]. DAF-12 and its steroidal ligands activate the expression of let-7s, which downregulate the heterochronic gene hbl-1, thus integrating environmental signals and developmental progression [29], [30]. However, the functional role of let-7 family of miRNAs in the innate immune response to pathogens is unknown. Therefore, it remains to be determined whether miRNAs contribute to dauer formation and the immune response in C. elegans.

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* E-mail: gebaoxue@sibs.ac.cn
**Author Summary**

When infected by the *Pseudomonas aeruginosa*, the nematode *Caenorhabditis elegans* invokes an innate immune response that protects the worm from pathogenic attack. The appropriate level of immune response in *C. elegans* requires the accurate regulation of multiple signal pathways, especially signals of repression, which attenuate the expression of pathogen-responsive genes. In the current study, we identified the nuclear hormone receptor DAF-12 and its downstream let-7 family of microRNAs, mir-84 and mir-241, are required for the regulation of *C. elegans* innate immunity against *P. aeruginosa* infection. Dafachronic acids, as DAF-12 ligands, can dramatically suppress the resistance of *C. elegans* to *P. aeruginosa* infection. Inhibition of the conserved PMK-1/p38 MAP kinase pathway can markedly attenuate the promoted resistance of daf-12 and let-7 family of microRNAs mutants to *P. aeruginosa* infection. However, neither daf-12 nor let-7 family of microRNAs affect the activation of PMK-1/p38. Moreover, our data also reveals the role of SKN-1 in integrating the signals from the PMK-1/p38 MAPK and DAF-12-let-7 pathways to mediate the *C. elegans* innate immune response.

Responses to pathogens is largely unknown. Hence, we sought to investigate whether DAF-12 and the let-7 family of miRNAs play a role in the regulation of the innate immune responses to bacterial infection in *C. elegans*.

**Results**

**DAF-12 regulates pathogenic defense**

We used an RNAi feeding method to search for the host components that influence the response of *C. elegans* to infection with *Pseudomonas aeruginosa* strain PA14, which is a human opportunistic pathogen that can also infect and kill *C. elegans*. Using 399 RNAi clones targeting transcription factors, we identified 17 transcriptional factors that affect the survival of worms on the *P. aeruginosa* lawns (Table S1). Among these candidates, treatment with daf-12 RNAi improved either the resistance of *C. elegans* to *P. aeruginosa* infection or its survival on an avirulent *E. coli* lawn (Fig. 1A, Fig. S1A). Transgenic daf-12(dhbl26) worms containing daf-12::GFP were more susceptible to *P. aeruginosa* (Fig. S1B). DAF-12, along with NHR-8 and NHR-48, is a conserved homolog of the mammalian vitamin D/liver X receptor (LXR) in *C. elegans* [6], [7]. However, inhibition of nhr-8 and nhr-48 increased pathogenic susceptibility to *P. aeruginosa* infection (Fig. S2A), suggesting that nhr-8 and nhr-48 have roles opposite to that of daf-12 in innate immune regulation.

To further investigate the role of DAF-12 in the immune response to bacterial infection, we examined the survival rate and lifespan of daf-12 alleles that have been previously identified on the basis of development and aging [6], [8]. A daf-12 null mutant daf-12(kh61rh411) that contained two nonsense mutations affecting both DNA binding domain (DBD) and ligand binding domain (LBD) [6] and was more resistant to *P. aeruginosa*, had a shortened lifespan compared to wild-type N2 animals (Fig. 1B and 1C). The daf-12(sa156) mutant containing a C121Y mutation in the zinc finger of DBD [6], which may interrupt the DNA binding activity of DAF-12, displayed a normal lifespan but increased resistance to *P. aeruginosa* infection (Fig. 1B and 1C). In contrast, the two other two mutants, daf-12(m20), which has a nonsense mutation affecting DBD [6], and daf-12(m25), containing a M562I mutation in LBD [6], exhibited extended lifespans and normal pathogenic resistance to *P. aeruginosa* infection (Fig. S3A and 3B).

These results not only identify DAF-12 as a negative regulator of innate immune responses to the infection of *P. aeruginosa* but also suggest a cross-talk between developmental progression and host defense.

We have also examined whether DAF-12 is involved in *C. elegans* host defense to other different pathogens, and found that inhibition of daf-12 greatly increased the resistance of daf-12(sa156) mutants to *Staphylococcus aureus* infection (Fig. S2B). We next performed transmission electron microscopy analysis to examine gut cells of wild-type worms or daf-12(sa156) worms fed *P. aeruginosa* or *E. coli*. When fed *E. coli*, both of the wild-type N2 and daf-12(sa156) worms displayed normal intestinal ultrastructure, whereas when infected by *P. aeruginosa*, the daf-12(sa156) worms exhibited less severely damaged gut cells and more intact microvilli than in the wild-type worms (Fig. 1D). To determine the cellular localization of DAF-12, we utilized previously generated transgenic daf-12(dhbl26) worms containing daf-12::GFP [7] and showed a significant accumulation of DAF-12 in the nuclei of neurons and intestinal cells when worms were fed *E. coli*. However, when infected with *P. aeruginosa*, DAF-12 expression was not affected (Fig. S4C), but the associated GFP signal was diffusely distributed throughout both neuronal and intestinal cells (Fig. S4A and S4B), suggesting that the *P. aeruginosa* infection suppresses nuclear localization of DAF-12 and promotes its translocation to the cytoplasm.

**DAF-12 regulates antimicrobial genes expression**

We then examined the effect of *P. aeruginosa* infection on the expression of eight selected anti-microbial genes that are regulated by the NSY-1/PMK-1 pathway or the insulin/IGF-1-like pathway [31]. We found that in the daf-12(kh61rh411) and daf-12 RNAi-treated worms infected with *P. aeruginosa*, expression levels of five of the eight anti-microbial genes were significantly higher compared to the wild-type control (Fig. 2A). To further confirm the quantitative RT-PCR results, we treated the dod-22::gfp or F53G11.7::gfp transgenic worms with daf-12 RNAi, fed them *E. coli* or *P. aeruginosa*, and then subjected them to confocal image analysis. Treatment with daf-12 RNAi greatly increased the expression of dod-22::GFP and F53G11.7::GFP at both basal *E. coli* levels and in *P. aeruginosa*-induced levels (Fig. 2B, Fig. S5A and S5B).

**Dafachronic acids (DAs) regulate pathogenic defense via DAF-12**

DAF-12 is known to control *C. elegans* response to its environment. Under favorable conditions, the stimulation of the insulin/IGF-1 and TGF-β pathways leads to the production of sterol-derived dafachronic acids (DAs). Δ5-DA and Δ5-DA bind to DAF-12, leading to developmental progression [10], [11]. The substitution of dietary cholesterol with Δ5-DA reduced the resistance of wild-type worms, but not daf-12(sa156) worms, to *P. aeruginosa* infection (Fig. 3A). An increased dose of Δ5-DA did not lead to further increases in pathogenic susceptibility of the wild-type N2 worm to *P. aeruginosa* infection (Fig. S6). DAs are derivatives of dietary cholesterol that are synthesized via several pathways involving the cytochrome P-450 DAF-9 and the SAM-dependent methyltransferase STRM-1 [12], [13], [32]. Inhibition of DAF-9 expression by RNAi feeding increased the resistance of the worm to *P. aeruginosa* infection (Fig. 3B). In unfavorable environments, the downregulation of the insulin/IGF-1 and TGF-β pathways suppresses DA production, and without its ligand, DAF-12 associates with the co-repressor DIN-1 to promote dauer.
Figure 1. DAF-12 regulates pathogenic defense. (A) Survival curve of daf-12 RNAi-treated worms exposed to P. aeruginosa (PA) relative to control RNAi-treated worms (P<0.0001). (B) P. aeruginosa killing assay of wild-type N2, daf-12(sa156) (P<0.001) and daf-12 (rh61rh411) (P<0.001) worms. (C) Lifespan curve of N2, daf-12(sa156) (P = 0.4118) and daf-12(rh61rh411) (P = 0.0019) worms. (D) Transmission electron micrographs (TEM) of a gut section from wild-type N2 and daf-12(sa156) worms fed E. coli or P. aeruginosa for 48 hours. Red arrows indicate microvilli. (Scale bars 1000 nm) All data shown above are representative of at least three independent experiments (n≥50 adult nematodes per strain).

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Figure 2. DAF-12 regulates antimicrobial gene expression. (A) qRT-PCR analysis of anti-microbial peptide (AMP) gene expression in N2, daf-12(RNAi) and daf-12(rh61rh411) worms infected with P. aeruginosa for 24 hours. The data shown are the mean ± SEM of three independent experiments, each of which was performed in triplicate, *P < 0.05. (B) Confocal microscopy of daf-12 RNAi-treated or control RNAi-treated dod-22::GFP and F55G11.7::GFP worms on E. coli or P. aeruginosa for 24 hours. The data are representative of three independent experiments (n=50 adult nematodes per strain).

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Dafachronic acids (DAs) regulate pathogenic defense through DAF-12. (A) P. aeruginosa killing of N2 and daf-12(sa156) worms grown with cholesterol (400 nM), Δ⁴-DAs (400 nM) (P = 0.2845 and 0.4552, respectively) and Δ⁷-DAs (400 nM) (P = 0.0426 and 0.5508, respectively). (B) P. aeruginosa killing assay of daf-9 RNAi (P < 0.001) animals. All data shown are representative of at least three independent experiments (n≥50 adult nematodes per strain).

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NHR Regulates Innate Immunity of C. elegans

A

P. aeruginosa

Percent survival

Time (hrs)

wild type
nsy-1(RNAi)
daf-12(sa156)
nsy-1(RNAi);
daf-12(sa156)

B

P. aeruginosa

Percent survival

Time (hrs)

wt
pmk-1(km25)
daf-12(RNAi)
pmk-1(km25);
daf-12(RNAi)

C

L4440
dafl-2(RNAi)
nsy-1(RNAi)
L4440
dafl-2(RNAi)
nsy-1(RNAi)

p-PMK1

Fold

Tubulin

0.15 0.13 0.13 1.00 0.85 0.34
programs [15]. Thus, we next examined the role of DYN-1, a corepressor of DAF-12, in the immune response of *C. elegans*. The inhibition of *dn-1* did not affect the survival of wild-type worms on a *P. aeruginosa* lawn, but moderately attenuated the extended pathogenic resistance of *daf-12* RNAi worms (Fig. 7A), suggesting that DAF-12 regulating the immune response of *C. elegans* may be partially dependent on *dn-1*.

**Inhibition of DAF-12-mediated immunity by NSY-1/PMK-1**

Several conserved signaling pathways, including the NSY-1/PMK-1 pathway and the insulin/IGF-1-like pathway, are involved in the pathogenic defense of *C. elegans* [2]. The loss of function of the insulin receptor DAF-2 activates the downstream target DAF-16, which triggers the expression of anti-microbial genes in response to pathogenic infection [3], [33]. However, *daf-16* RNAi had no effect on the prolonged survival of *daf-12(sa156)* worms infected with *P. aeruginosa* (Fig. 7B). We then tested whether the NSY-1/PMK-1 pathway is involved in the enhanced resistance of *daf-12* mutants to *P. aeruginosa*. Either inhibition of *nsy-1* by RNAi or mutation of *pmk-1* attenuated the enhanced pathogenic resistance of *daf-12(sa156)* worms or *daf-12* RNAi-treated worms, respectively (Fig. 4A and 4B), suggesting that DAF-12 may target the PMK-1 pathway to regulate the *C. elegans* immune response against *P. aeruginosa* infection. However, *daf-12* RNAi did not markedly change the *P. aeruginosa*-stimulated phosphorylation of PMK-1 (Fig. 4C), suggesting that DAF-12 might act upstream or parallel to PMK-1 to suppress the PMK-1/p38 MAPK pathway.

**MicroRNAs let-7s regulate both DAF-12 and NSY-1/PMK-1-mediated pathogenic defense**

MicroRNAs are approximately 20- to 22-nucleotide-long RNA molecules that bind to the 3‘ untranslated region (3’UTR) of target messenger RNAs (mRNAs) and that decrease their expression [34], [35]. DAF-12 activates the expression of the let-7 miRNA homologs *mir-84* and *mir-241* (referred to as let-7s) to control developmental progression [29], [30]. To test whether *mir-84* or *mir-241* play a role in pathogenic defense, we infected the strains *mir-84(n4037)* and *mir-241(n4316)* with *P. aeruginosa*. Both *mir-84(n4037)* and *mir-241(n4316)* worms were more resistant to *P. aeruginosa* infection than the wild type (Fig. 5A). Likewise, both *mir-84(n4037)* and *mir-241(n4316)* worms had slightly longer lifespans than wild-type animals (Fig. 5B). We then employed the quantitative real-time PCR method to detect miRNA expression and found that *P. aeruginosa* infection of wild-type worms induced higher levels of *mir-84* and *mir-241* compared to *E. coli*. However, the *daf-12* mutation markedly reduced the expression of both *mir-84* and *mir-241* (Fig. 5C). Confocal microscopic imaging of *mir-84*::gfp also indicated that the *mir-84* expression was highly upregulated in *P. aeruginosa*-infected wild-type worms, but not in the *daf-12(hbl10411l)* worms (Fig. 5D, Fig. S5C). To further determine the role of let-7s miRNAs in *C. elegans* innate immunity, we tested the function of *mir-48*, another let-7 relative, in *P. aeruginosa* infection and found that the *mir-48(n4097)* mutant exhibited decreased resistance to *P. aeruginosa* (Fig. 5B), suggesting that the let-7s miRNAs may target different regulators of *C. elegans* innate immunity. Quantitative real-time PCR results showed that the expression of *daf-12*-targeted antimicrobial genes was also upregulated in let-7s miRNAs mutants (Fig. 5A), suggesting that these genes are also targeted by let-7s miRNAs. We then fed the *daf-12* and let-7s mutants a GFP-tagged *P. aeruginosa* PA-14 strain and examined the bacterial burdens in worm intestines by confocal microscopy. We found that the inhibition of *daf-12* and *mir-241* may suppress bacterial accumulation through antimicrobial gene expression. The *nsy-1* RNAi also counteracted the pathogenic defense of *mir-84* mutant worms, and mutations of *mir-84* or *mir-241* did not affect the phosphorylation of PMK-1, suggesting that the let-7s miRNAs function downstream of DAF-12 to suppress the PMK-1/p38 MAPK signaling pathway (Fig. 5E, Fig. S8B).

**SKN-1 is a direct functional target of let-7s**

The finger protein hbl-1 is one target of miRNAs let-7s, and the expression of *hbl-1* is regulated by *daf-12* and *let-7s* [29]. The inhibition of *hbl-1* by RNAi reduced the pathogenic resistance, but not the lifespan of *C. elegans* (Fig. S11A and S11B). To identify other target genes of *let-7* family of miRNAs, we performed a bioinformatics analysis, determining that *skn-1* is a potential target (Fig. 6A). To determine whether let-7s miRNAs could bind to the 3’-UTR of the *skn-1* mRNA and suppress it, we fused the 3’-UTR region of the *C. elegans* *skn-1* mRNA to the 3’-end of a luciferase reporter gene and co-transfected it with synthesized dsRNAs mimicking let-7s miRNAs (let-7s mimics) into HEK293T cells. In contrast to the luciferase activity in the 3’-UTR seed region mutants (*skn-1 3’-UTR (mut)*) that could not bind with and respond to let-7s miRNAs, the luciferase activity of the *skn-1* 3’-UTR decreased by approximately 30% in response to *mir-48* mimics or *mir-84* mimics and by approximately 10% in response to *mir-241* mimics (Fig. 6B). Western blot results also showed that the SKN-1 protein expression could be upregulated by inhibition of *daf-12*, *mir-84* and *mir-241* (Fig. 6C, Fig. S11C). These results suggested that *skn-1* is a target of *mir-84* and *mir-241*.

**DAF-12 regulates pathogenic resistance through SKN-1**

**SKN-1 is a kinase substrate of PMK-1 and regulates *C. elegans* resistance to oxidative stress** [36], [37]. The inhibition of *skn-1* by RNAi markedly attenuated the pathogenic resistance of *C. elegans* (Fig. S12A) [38] but did not affect the pathogenic resistance of *pmk-1* (km25) mutants (Fig. S12B). In worms infected with *P. aeruginosa* but not *E. coli*, SKN-1 accumulated in the nuclei of intestinal cells (Fig. S12C and S12D) [38], [39]. However, *nsy-1* RNAi attenuated the nuclear accumulation of SKN-1 (Fig. S12E), suggesting that *skn-1* may also act downstream of NSY-1/PMK-1 to regulate the immune response. Conversely, *skn-1* RNAi markedly reversed the enhanced pathogenic resistance of the *daf-12(sa156)* mutant as well as that of the *mir-84* or *mir-241* mutants (Fig. 7A, 7B and 7C). Quantitative real-time RT-PCR results showed that the inhibition of *daf-12* and of let-7s miRNAs significantly increase the expression of *gcu-1*, a SKN-1 downstream gene (Fig. S13A), suggesting that the DAF-12 and let-7s miRNAs may suppress SKN-1 activity. We treated the *skn-1::gfp* transgenic worms [37] with *daf-12* RNAi and confocal imaging analysis.
NHR Regulates Innate Immunity of C. elegans

A

P. aeruginosa

B

E. coli

C

**mir-84**

**mir-241**

D

**E. coli**

**P. aeruginosa**

E

**E. coli**

**P. aeruginosa**

WT nsy-1 daf-12 mir-84 mir-241 WT nsy-1 daf-12 mir-84 mir-241

p-PMK1

Fold

Tubulin
Figure 5. MicroRNAs let-7s regulate pathogenic defense. (A) Survival curve of N2, mir-84(n4307) (P<0.001) and mir-241(n4316) (P<0.001) on P. aeruginosa. (B) Lifespan assay of N2, mir-84(n4037) (P<0.001) and mir-241(n4316) (P<0.001) worms on E. coli. (C) qRT-PCR analysis of mir-84 and mir-241 expression in N2 and daf-12(n4316) worms infected with P. aeruginosa for 24 hours. The data shown are the mean ± SEM of three independent experiments, each of which was performed in triplicate, *P<0.05 (D) Confocal microscopy of intestinal mir-84::GFP expression in daf-12(n4316) mutants on E. coli or P. aeruginosa. (E) Immunoblot analysis of the lysates from N2, nhr-1 RNAi, daf-12(n4316) mutants, mir-84(n4037) and mir-241(n4316) worms on E. coli or P. aeruginosa using anti-phospho-p38 antibody and anti-tubulin antibody (loading control). All data shown are representative of at least three independent experiments (n=50 adult nematodes per strain).

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Furthermore, we have demonstrated that SKN-1 is a direct target of let-7s miRNAs. SKN-1 accumulate in the nuclei of intestinal cells of worms infected with P. aeruginosa, but not E. coli, which is consistent with Papp et al. and Haeven et al.’s reports that exposure to P. aeruginosa leads to SKN-1 accumulation in intestinal nuclei [38], [39]. Their data have also shown that PA14 infection triggers the transcriptional activation of gcs-1 and gsp-4, two downstream target gene of SKN-1. However, data in our experiments suggested that infection of P. aeruginosa may induce more let-7s miRNAs and thus downregulate the production of SKN-1. One possible explanation is that even if the SKN-1 production is downregulated during the infection, the activity of SKN-1 is more dependent on its protein modification rather than its quantity. Regulation of SKN-1 at both the level of its activity and quantity precisely modulate the innate immune response to microbial infection. Furthermore, we found that the inhibition of skn-1 by RNAi markedly reduced DAF-12/let-7s-mediated pathogenic defense. These findings provide evidence that nuclear hormone receptors control let-7s miRNAs regulation of the C. elegans innate immunity, suggesting that DAF-12 may couple developmental progression and the response to pathogenic infection in order to coordinate appropriate immune responses.

The oxidative stress response is an evolutionally conserved response to reactive oxygen species (ROS), which are produced by mitochondrial respiration, toxins and pathogen virulence factors [44]. SKN-1 is required for the proper response of C. elegans to oxidative stress, which is mediated by the NSY-1/PMK-1 and DAF-2 insulin-like signaling pathways [36], [37]. Our present findings demonstrate an essential role of SKN-1 in the pathogenic resistance of C. elegans, an observation that is consistent with two other independent studies [38], [39]. Thus, SKN-1 appears to integrate longevity, stress resistance and pathogenic resistance. Although the molecular pathway by which SKN-1 regulates the innate response to pathogens remains unclear, an SKN-1-mediated oxidative stress response could potentially protect the worms from the peroxidation damage caused by ROS during pathogenic infection. Further investigation of the common downstream target of various SKN-1 actions is required to elucidate the role of SKN-1 in the pathogenic resistance of C. elegans.

In summary, our data demonstrate that DAF-12 and its steroidal ligands, DAs, negatively regulate the innate immune responses of C. elegans to pathogenic infection. DAF-12 appears to activate let-7s miRNAs to directly target SKN-1, a component of the NSY-1/PMK-1 immune signaling pathway, thus regulating the pathogenic resistance of C. elegans [Fig. S14]. These findings not only reveal a novel signaling pathway in the C. elegans defense against pathogens but also provide a link between endocrine signaling and innate immune responses, thus integrating developmental progression and pathogenic resistance.
Figure 6. SKN-1 is a direct functional target of let-7s miRNAs. (A) Bioinformatics alignment of let-7s miRNAs and the 3’UTR of SKN-1. (B) Luciferase assays using the skn-1 3’UTR or the skn-1 3’UTR (mut) with let-7s mimics in HEK293T cells. The data shown are the mean ± SEM of three independent experiments, each of which was performed in triplicate, *P<0.05. (C) Immunoblot analysis of the lysates from N2, daf-12(RNAi), mir-84(n4037) and mir-241(n4316) worms on E. coli or P. aeruginosa using anti-SKN-1 antibody and anti-tubulin antibody (loading control).

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Materials and Methods

Materials

(25S-) Δ1- and Δ2-DAs were produced in the Knölker laboratory [45]. All C. elegans strains were obtained from Caenorhabditis Genetics Center (CGC) unless otherwise noted.

Nematode methods

The C. elegans strains used in this study are listed in Table S2. All of the strains were maintained at 20°C using standard methods unless otherwise noted.

Lifespan and P. aeruginosa killing assay

Lifespan and P. aeruginosa killing assays were conducted at least three times, as previously described [46]. A P value less than or equal to 0.05 was considered statistically significant. Statistical analysis of lifespan and P. aeruginosa killing assay is shown in Table S3, S4, S6 and S7.

Screening of a transcription factor RNAi library

RNAi of candidate transcription factors in N2 worms was carried out using standard bacterial feeding methods. For all feeding assays, worms were exposed to RNAI bacteria from the time of hatching. Synchronized young adult animals were transferred to M9 containing 0.1% NaN3, fixed in the 2% soft agar and subjected to confocal imaging assay. Images were captured using Leica TCS SP5.

Confocal microscopy assay

Worms were washed from their plates with M9, anaesthetized with M9 containing 0.1% Na3S, fixed in the 2% soft agar and subjected to confocal imaging assay. Images were captured using Leica TCS SP5.

Transmission electron microscopy assay

Wild-type N2 and daf-12(sa156) young adults were fed P. aeruginosa or E. coli for 48 hours. Worms were rinsed from plates with M9 buffer, and anaesthetized in 8% alcohol in M9. Fixation and sectioning was performed with a conventional two-steps method as described in Worm Method. Photographs were captured using HITACHI H-7650.

Dafachronic acids assay

Wild-type N2 and daf-12(sa156) young adults were removed from plates and bathed with Δ4-DAs, Δ7-DAs or cholesterol (400 nM) in M9 for 3 hours before killing assay. The killing assay was performed on P. aeruginosa plates supplemented with Δ4-DAs, Δ7-DAs or cholesterol (400 nM).

Luciferase assay

A 0.5-kb region of the skn-1 3’ UTR containing the predicted miRNA let-7s binding sites was cloned into the psi-CHECK2 to obtain the skn-1 3’ UTR-luc construct. The skn-1 3’ UTR (mut)-luc construct was obtained from skn-1 3’ UTR construct by mutating the complementary sequence of let-7s seed region (TAGCTCA to TAGGGTA). Constructs were co-transfected with synthesized dsRNAs mimicking the let-7s miRNAs to HEK293T cells and the luciferase assay was performed using the dual-luciferase reporter assay system (Promega).

Quantitative real-time PCR of antimicrobial peptide expression

Synchronized C. elegans animals were treated essentially as described above for the killing assays except for the omission of FUDR. Infected samples were compared to control samples fed on the same medium with E. coli OP50-1. Total RNA was extracted as described [31] and reverse transcribed using the ReverTra Ace Q-PCR RT kit (Toyobo). cDNA was subjected to qRT-PCR analysis as described [31]. The primer sequences are listed in Table S5. All values were normalized to act-1. One-tailed t-tests were performed with GraphPad Prism4. A P value less than or equal to 0.05 was considered significant.

Quantitative real-time PCR for microRNA let-7s

Synchronized worms were collected in TRIzol (Invitrogen) and treated as described [47]. The miRNasy Mini kit (QIAGEN) and TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) were used for total RNA and cDNA preparation, respectively. qRT-PCR was performed with Power SYBR Green master mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). SnoRNA U18 was used as an internal control. The primer sequences were gifted from Prof. Adam Antebi from the Max Planck Institute for Biology of Ageing. One-tailed t-tests were performed with GraphPad Prism4. A P value less than or equal to 0.05 was considered significant.

Western blot analysis of PMK-1 activation and SKN-1 expression

Synchronized L4 populations of wild-type N2, daf-12(RX4D), daf-12(hd154h1411), mir-84(n4037) and mir-241(n4316) animals were infected with P. aeruginosa as described [1]. Western blot analyses of activated p38 MAPK were performed as described [46]. Western blot analyses of SKN-1 expression were performed using anti-SKN-1 (Santa Cruz).

Supporting Information

Figure S1 RNAi treatment of DAF-12 increased C. elegans lifespan. (A) Lifespan of N2 worms treated with daf-12 RNAi (P<0.0001) and control vector. (B) Survival curve of wild-type N2 and daf-12(dls-26) (P<0.0001) worms on P. aeruginosa.

Figure S2 NHR-8 and NHR-48 regulate C. elegans innate immunity. (A) Survival curve of wild-type N2, nhr-8(ok186) (P<0.0001) and nhr-48(ok178) (P<0.0001) worms on P. aeruginosa. (B) Survival curve of wild-type N2 and daf-12(sa156) (P<0.0001) worms on S. aureus.

Figure S3 DAF-12 mutations have different roles in regulation of aging process and innate immunity. (A) Survival curve of wild-type N2, daf-12(m20) (P<0.0001), daf-

Figure 7. DAF-12 regulates pathogenic resistance through SKN-1. (A) Survival curve of N2, daf-12(sa156) (P<0.0001), skn-1(RNAi) (P<0.0001) and daf-12(sa156), skn-1(RNAi) worms (P=0.745 compared to skn-1 (RNAi)) on P. aeruginosa. (B) Survival curve of wild-type N2, mir-241(n4315) (P<0.0001), skn-1(RNAi) (P<0.0001) and mir-241(n4315) skn-1(RNAi) worms (P=0.008 compared to skn-1 RNAi) on P. aeruginosa. (C) Survival curve of wild-type N2, mir-84(n4037) (P<0.0001), skn-1(RNAi) (P<0.0001) and mir-84(n4037); skn-1(RNAi) worms (P<0.0001 compared to skn-1 RNAi) on P. aeruginosa. All data shown are representative of at least three independent experiments (n=50 adult nematodes per strain). doi:10.1371/journal.ppat.1003545.g007
Figure S4 P. aeruginosa infection induces DAF-12 translocation. (A) Confocal imaging of DAF-12::gfp transgenic worm fed E. coli or P. aeruginosa for 24 hours. (B) Quantification of daf-12::gfp translocation observed in worms fed E. coli (n = 9) or P. aeruginosa (n = 13) in Fig. S4A. (C) Western blot assay of daf-12-GFP of DAF-12::gfp transgenic worm fed E. coli or P. aeruginosa for 24 hours using anti-GFP antibody. (TIF)

Figure S5 Quantification of GFP signals. (A) Quantification of F55G11.7::GFP signals in Fig. 2B. (B) Quantification of DOD-22::GFP signals in Fig. 2B. (C) Quantification of mir-84p::GFP signals in Fig. 5D. (TIF)

Figure S6 An increased dose of L-DA does not lead to further increases in pathogenic susceptibility. Survival curve of wild-type N2 worm with cholesterol (400 nM), L-DA (400 nM) (P = 0.0007) and L-DA (1 μM) (P = 0.5995) compared to 400 nM on P. aeruginosa. (TIF)

Figure S7 DAF-16 RNAi has no effect on increased resistance to P. aeruginosa of DAF-12 mutants. (A) P. aeruginosa killing assay of N2, din-1(dh127) (P = 0.0823), daf-12(RNAi) (P = 0.0001) and din-1(dh127);daf-12(RNAi) (P = 0.002 compared to daf-12 RNAi) animals. (B) Survival curve of wild-type N2, daf-16(RNAi) (P = 0.0485), daf-12(sa156) (P < 0.0001) and daf-12(sa156);daf-16(RNAi) (P = 0.9012 compared to daf-12(sa156)) worms on P. aeruginosa. (TIF)

Figure S8 Mir-84 regulates innate immunity through PMK-1 pathway. (A) Survival curve of wild-type N2 and mir-40(n4097) (P < 0.0001) worms on P. aeruginosa. (B) Survival curve of wild-type N2, nys-1(RNAi) (P < 0.001), mir-84(n4037) (P < 0.0001) and mir-84(n4037);nys-1(RNAi) (P = 0.1114 compared to mir-84(n4037)) upon P. aeruginosa infection. (TIF)

Figure S9 MiRNAs let-7s and SKN-1 regulate DAF-12-mediated AMPs expression. (A) Quantitative real-time PCR assay of antimicrobial gene expression of wild-type N2, mir-84(n4037) and mir-241(n4316) young adults fed E. coli or P. aeruginosa for 24 hours. (B) Quantitative real-time PCR assay of antimicrobial gene expression of wild-type N2 and skn-1(RNAi) young adults fed E. coli or P. aeruginosa for 24 hours. (TIF)

Figure S10 DAF-12 and let-7s miRNAs regulate bacterial accumulation in worm intestine. (A) Confocal imaging of wild-type N2, daf-12(sa156), mir-84(n4037) and mir-241(n4316) animals fed GFP-tagged P. aeruginosa for 24 hours. (B) Quantification of GFP signals in Supp. Fig. 10A. (TIF)

Figure S11 HBL-1 regulates innate immunity. (A) Survival curve of N2 and hbl-1(RNAi) (P < 0.0001) worms on P. aeruginosa. (B) Lifespan assay of N2 and hbl-1(RNAi) (P = 0.8882) worms on E. coli. (C) Immunoblot analysis of the lysates from N2, skn-1(RNAi) and daf-12(h631h411) young adults fed E. coli or P. aeruginosa using anti-SKN-1 antibody and anti-tubulin antibody (loading control). (TIF)

Figure S12 SKN-1 acts at downstream of PMK-1 to regulate innate immunity. (A) Survival curve of N2 and skn-1(RNAi) (P < 0.0001) worms on P. aeruginosa. (B) Survival curve of wild-type N2, pmk-1(km25) (P < 0.0001) and pmk-1(km25);skn-1(RNAi) (P = 0.0067) worms on P. aeruginosa. (C–E) Confocal microscopy of nys-1 RNAi-treated or control RNAi-treated skn-1::GFP worms on P. aeruginosa or E. coli. Arrows shows the nuclear skn-1::GFP. (TIF)

Figure S13 DAF-12 regulates SKN-1 activity. (A) Quantitative real-time PCR assay of gcs-1 expression of wild-type N2, daf-12(RNAi), mir-84(n4037) and mir-241(n4316) young adults fed E. coli or P. aeruginosa for 24 hours. (B) Confocal imaging of daf-12 RNAi treated or control treated young adults of skn-1::gfp transgenic worms. Arrows shows the nuclear skn-1::GFP. (C) Quantification of skn-1::gfp observed in worms treated with daf-12 RNAi (n = 23) or control (n = 19) in Fig. S13B. (TIF)

Figure S14 The hypothesized diagram. Caenorhabditis elegans nuclear receptor DAF-12 negatively regulates the pathogenic defense via its downstream microRNAs, let-7s, which may directly target SKN-1, thus counteract the activation of SKN-1 by NSY-1/PMK-1 pathway. (TIF)

Table S1 List of alleles isolated from the RNAi screening. (TIF)

Table S2 List of all the C. elegans strains used in this study. (TIF)

Table S3 The statistical analysis of all P. aeruginosa killing assays shown in figures. (TIF)

Table S4 The statistical analysis of all lifespan assays shown in figures. (TIF)

Table S5 The combination of primers used in quantitative real-time RT-PCR assay. (TIF)

Table S6 The statistical analysis of all PMK-1 killing assays shown in figures. (TIF)

Table S7 The statistical analysis of all lifespan assays shown in supplementary figures. (TIF)

Author Contributions
Conceived and designed the experiments: FL BXG. Performed the experiments: FL CXH LJJ QLZ YYX SFH LSW. Analyzed the data: FL BXG. Contributed reagents/materials/analysis tools: RS HJK. Wrote the paper: FL BXG.

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