Phosphorylation of the Conserved Transcription Factor ATF-7 by PMK-1 p38 MAPK Regulates Innate Immunity in Caenorhabditis elegans

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.


As Published: http://dx.doi.org/10.1371/journal.pgen.1000892
Publisher: Public Library of Science
Version: Final published version
Citable link: http://hdl.handle.net/1721.1/57502
Terms of Use: Creative Commons Attribution
Detailed Terms: http://creativecommons.org/licenses/by/2.5/
Phosphorylation of the Conserved Transcription Factor ATF-7 by PMK-1 p38 MAPK Regulates Innate Immunity in Caenorhabditis elegans

Robert P. Shivers1,*, Daniel J. Pagano1, Tristan Kooistra1, Claire E. Richardson1, Kirthi C. Reddy1, Janelle K. Whitney1, Odile Kamanzi1, Kunihiro Matsumoto2, Naoki Hisamoto2, Dennis H. Kim1*

1 Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, 2 Department of Molecular Biology, Nagoya University, Nagoya, Japan

Abstract

Innate immunity in Caenorhabditis elegans requires a conserved PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway that regulates the basal and pathogen-induced expression of immune effectors. The mechanisms by which PMK-1 p38 MAPK regulates the transcriptional activation of the C. elegans immune response have not been identified. Furthermore, in mammalian systems the genetic analysis of physiological targets of p38 MAPK in immunity has been limited. Here, we show that C. elegans ATF-7, a member of the conserved cyclic AMP-responsive element binding (CREB)/activating transcription factor (ATF) family of basic-region leucine zipper (bZIP) transcription factors and an ortholog of mammalian ATF2/ATF7, has a pivotal role in the regulation of PMK-1-mediated innate immunity. Genetic analysis of loss-of-function alleles and a gain-of-function allele of atf-7, combined with expression analysis of PMK-1-regulated genes and biochemical characterization of the interaction between ATF-7 and PMK-1, suggest that ATF-7 functions as a repressor of PMK-1–regulated genes that undergoes a switch to an activator upon phosphorylation by PMK-1. Whereas loss-of-function mutations in atf-7 can restore basal expression of PMK-1–regulated genes observed in the pmk-1 null mutant, the induction of PMK-1–regulated genes by pathogenic Pseudomonas aeruginosa PA14 is abrogated. The switching modes of ATF-7 activity, from repressor to activator in response to activated PMK-1 p38 MAPK, are reminiscent of the mechanism of regulation mediated by the corresponding ancestral Sko1p and Hog1p proteins in the yeast response to osmotic stress. Our data point to the regulation of the ATF2/ATF7/CREB5 family of transcriptional regulators by p38 MAPK as an ancient conserved mechanism for the control of innate immunity in metazoans, and suggest that ATF2/ATF7 may function in a similar manner in the regulation of mammalian innate immunity.


Editor: Kaveh Ashrafi, University of California San Francisco, United States of America

Received July 28, 2009; Accepted March 2, 2010; Published April 1, 2010

Copyright: © 2010 Shivers et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by NIH Grant GM084477, a Burroughs Wellcome Fund Career Award in the Biomedical Sciences, Ellison Medical Foundation New Scholar Award, and a Swanson Family Career Development Professorship to DHK. TK was supported by a summer undergraduate research fellowship from the Howard Hughes Medical Institute. KCR was supported by postdoctoral fellowships from the Jane Coffin Childs Cancer Research Foundation and the Charles A. King Trust Foundation. Work in the Matsumoto laboratory is supported by grants from the Ministry of Education, Culture, and Science of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dhkim@mit.edu
† These authors contributed equally to this work.
¤ Current address: The Commonwealth Medical College, Scranton, Pennsylvania, United States of America

Introduction

Studies of innate immunity in phylogenetically diverse organisms have revealed the conservation of key signaling pathways mediating pathogen defense [1,2]. In mammals, the initial encounter between cells of the immune system and pathogenic bacteria triggers the activation of the innate immune response to infection, which is under the control of the transcription factor NF-kB and stress-activated mitogen-activated protein kinases (MAPKs) p38 and JNK [3]. Multiple phosphorylation targets for p38 and JNK MAPKs have been identified in mammalian systems, including members of the cyclic AMP-responsive element binding (CREB)/activating transcription factor (ATF) family such as ATF2 [4], activating protein 1 (AP-1), transcription factors Fos and Jun [3], and multiple kinases including the MAPK-activated protein kinase MK2 [6]. Genetic analysis of MK2 knockout mice is suggestive of a role for p38 MAPK regulation of MK2 in the post-transcriptional regulation of TNF-α production [7]. However, genetic analysis of transcription factor targets of p38 and JNK MAPKs has been limited by lethality of knockouts and possible redundancy [8], and thus the identification and characterization of the physiologically relevant targets of MAPK signaling in innate immunity remains a major challenge [9].

We have focused on the genetic dissection of innate immunity in the nematode Caenorhabditis elegans. Previously, we identified a requirement for a conserved NSY-1-SEK-1-PMK-1 MAPK pathway, orthologous to mammalian ASK1 MAPKKK-MKK3/6 MAPKK-p38 MAPK, in C. elegans innate immunity [10]. Notably, the loss of PMK-1 p38 MAPK activity in C. elegans, unlike...
The loss of mammalian p38 MAPK does not affect growth and development of C. elegans on non-pathogenic bacteria. The ASK1-MKK3/6-p38 MAPK pathway has been shown to be required for innate immune signaling downstream of Toll-like Receptor-4 (TLR4) in mice [11], whereas NSY-1-SEK-1-PMK-1 signaling in C. elegans is TLR-independent and functions downstream of a Toll-Interleukin-1 Receptor (TIR) domain protein TIR-1 [12-15], an ortholog of mammalian SARM [16-18]. The role of SARM in mammalian innate immunity is somewhat unclear [16,17], with some studies suggestive of a role for SARM in the inhibition of TIRF-dependent TLR signaling [16]. Recent studies of the PMK-1 pathway are suggestive of a role for protein kinase C-dependent signaling upstream of TIR-1 [19,20].

The TIR-1-NSY-1-SEK-1-PMK-1 pathway acts cell autonomously in the intestine to regulate innate immunity in C. elegans [21], paralleling the role of this pathway in the epidermal response to Drechmeria [15]. The transcriptional profiling of C. elegans mutants deficient in PMK-1 pathway activity has identified a number of PMK-1-dependent candidate effector genes, including C-type lectins and putative antimicrobial peptides, many of which are induced by pathogen infection [22]. Whereas the GATA family transcription factor ELT-2 has been implicated in the regulation of C. elegans innate immunity in response to intestinal infection [23], in addition to its role in the expression of all intestinally expressed genes [24], the specific targets of PMK-1 p38 MAPK in the regulation of the immune effector response have remained uncertain.

In this paper we report the results of a forward genetic screen for mutants deficient in immune signaling through the PMK-1 p38 MAPK pathway. We report the identification of ATF-7, a putative ortholog of the mammalian ATF2 family of basic-region leucine zipper (bZIP) transcription factors, as a key downstream target of the PMK-1 p38 MAPK pathway in C. elegans. Our data establish a pivotal role for ATF-7 as a transcriptional regulator of the PMK-1-mediated innate immune response in C. elegans.
isolated an intragenic suppressor of genomes for mutants with increased GFP reporter expression and phenotype of the screen for suppressors of the attenuated GFP expression that we would be able to isolate (Figure S12). Interestingly, we observed that the susceptible to loss-of-function allele is described below), which is nearly as of the atf-7(qd22)/atf-7(qd22 qd130)
gene, which was further corroborated by analysis of the phenotype of the atf-7(qd22) single mutant (Figure 3B and Figure S14). The partial suppression of the Esp phenotype of the pmk-1(ken25) mutant on E. coli OP50 was comparable to that observed for WT (Figure S6 and Figure S16).

**Table 1.** List of isolates from a screen for mutants with diminished GFP expression from the agIs219 transgene and enhanced susceptibility to killing by *P. aeruginosa* PA14.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsy-1</td>
<td>qd1</td>
<td>Not identified</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd3</td>
<td>Q256*</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd6</td>
<td>W931*</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd8</td>
<td>G440E</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd23</td>
<td>W514*</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd25</td>
<td>W157*</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd29</td>
<td>Q426*</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd31</td>
<td>P687L</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd38</td>
<td>G793R</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd41</td>
<td>451 bp deletion with 2 bp insertion</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd44</td>
<td>Not identified</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd50</td>
<td>Splice site</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd52</td>
<td>Not identified</td>
</tr>
<tr>
<td>nsy-1</td>
<td>qd55</td>
<td>G673E</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd12</td>
<td>R221K</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd14</td>
<td>S103N</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd24</td>
<td>M217I</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd26</td>
<td>E220K</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd37</td>
<td>G199D</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd39</td>
<td>W123*</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd60</td>
<td>G5R</td>
</tr>
<tr>
<td>sek-1</td>
<td>qd65</td>
<td>G212R</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd9</td>
<td>L296F</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd11</td>
<td>D161N</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd13</td>
<td>M209I</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd15</td>
<td>W353*</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd20</td>
<td>C173Y</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd47</td>
<td>W29*</td>
</tr>
<tr>
<td>pmk-1</td>
<td>qd49</td>
<td>Not identified</td>
</tr>
<tr>
<td>tir-1</td>
<td>qd2</td>
<td>A451V</td>
</tr>
<tr>
<td>tir-1</td>
<td>qd66</td>
<td>Q278*</td>
</tr>
<tr>
<td>tir-1</td>
<td>qd67</td>
<td>P460L</td>
</tr>
<tr>
<td>atf-7</td>
<td>qd22</td>
<td>P58S</td>
</tr>
</tbody>
</table>

Mutations in tir-1 are in reference to gene model F13810.1b. The mutation in atf-7 is in reference to gene model C07G2.2a.

doi:10.1371/journal.pgen.1000892.t001

Suggested that *qd22* is a gain-of-function mutant allele of the *atf-7* gene, which was further corroborated by analysis of the phenotype of the *atf-7(qd22)/atf-7(qd22 qd130)* trans-heterozygote (the *qd130* loss-of-function allele is described below), which is nearly as susceptible to *P. aeruginosa* as the *atf-7(qd22)* mutant (Figure S4 and Figure S12). Interestingly, we observed that the *atf-7(qd22)* allele is recessive with respect to the Esp phenotype (Figure S4).

Based on the evidence that *atf-7(qd22)* was a gain-of-function, and possibly neomorphic, mutant allele of *atf-7*, we anticipated that we would be able to isolate *atf-7* loss-of-function alleles from a screen for suppressors of the attenuated GFP expression phenotype of the *atf-7(qd22)* mutant. We screened 20 000 haploid genomes for mutants with increased GFP reporter expression and isolated an intragenic suppressor of *atf-7(qd22)*, *atf-7(qd22 qd130)*, which carries a nonsense mutation resulting in an early stop codon in the *atf-7* gene (Figure 2A). The *atf-7(qd22 qd130)* mutant allele suppressed the diminished GFP fluorescence phenotype of the *atf-7(qd22)* mutant (Figure 1A), but only partially suppressed the Esp phenotype, demonstrating that the *atf-7* loss-of-function mutant also has an Esp phenotype compared to WT (Figure 1B and Figure S10).

To confirm that the observed Esp phenotype of the *atf-7(qd22 qd130)* mutant was caused by the nonsense mutation in *atf-7*, we also analyzed a second putative null allele of *atf-7*, *atf-7(qd137)* (Figure 2A), which we isolated from a separate screen (described below). The *atf-7(qd137)* mutant exhibited the same Esp phenotype as that observed for the *atf-7(qd22 qd130)* mutant, as well as the trans-heterozygote, *atf-7(qd22 qd130)/atf-7(qd137)* (Figure 1D and Figure S13). We also carried out rescue experiments using a transgene comprised of the genomic *atf-7* locus with GFP fused to the 3’ end between the *atf-7* stop codon and the 3’-untranslated region (UTR). We observed that this transgene partially rescued the Esp phenotype of the *atf-7(qd22 qd130)* mutant (Figure S5). The partial degree of rescue observed for both the *atf-7(qd22)* and *atf-7(qd22 qd130)* mutants may reflect the detrimental effects of overexpression of *atf-7*.

The Esp phenotype of the *atf-7(qd22 qd130)* mutant was consistent with the results of the RNAi experiments (Figure S2B) and suggests that whereas the *atf-7(qd22)* gain-of-function allele confers a strong Esp phenotype, loss of *atf-7* activity also compromises pathogen resistance relative to WT. We observed that the longevity of *atf-7(qd22 qd130)* and *atf-7(qd137)* mutants on *E. coli* OP50 was comparable to that observed for WT (Figure S6 and Figure S16).

**atf-7** loss-of-function alleles suppress the immunodeficiency phenotype of *pmk-1*

Because *atf-7(qd22)* appeared to be a gain-of-function allele of *atf-7* that exhibited the same phenotypes as observed for mutants carrying loss-of-function mutations in PMK-1 pathway components, we hypothesized that ATF-7 might be negatively regulated by the PMK-1 pathway and function as a repressor of the innate immune response. To test this hypothesis, we carried out epistasis analysis using the *atf-7(qd22 qd130)* and *pmk-1(km25)* null alleles. We observed that the *atf-7(qd22 qd130)* loss-of-function allele suppressed the diminished agIs219 GFP reporter gene expression phenotype of the *pmk-1(km25)* mutant (Figure 3A). Also, the *atf-7(qd22 qd130); pmk-1(km25)* double mutant had a reduced pathogen susceptibility compared to *pmk-1(km25)*, comparable to that of the *atf-7(qd22 qd130)* single mutant (Figure 3B and Figure S14). The partial suppression of the Esp phenotype of the *pmk-1(km25)* mutant by *atf-7(qd22 qd130)* was rescued by a transgene carrying wild-type *atf-7* fused with GFP (Figure S7). Furthermore, we found that *atf-7(qd22 qd130)* also suppressed the pathogen susceptibility (Figure 3D and Figure S15) and diminished agIs219 GFP reporter expression (Figure 3C) phenotypes of the *sek-1(km4)* mutant.

The Esp phenotypes of the *atf-7(qd22 qd130); pmk-1(km25)* and *pmk-1(km4)* double mutants are comparable to the Esp phenotype of the *atf-7(qd22 qd130)* single mutant. The lack of effect of the *pmk-1(km25)* or *sek-1(km4)* mutations on the Esp phenotype in the *atf-7(qd22 qd130)* mutant background is particularly noteworthy in view of the strong Esp phenotype conferred by inactivation of the PMK-1 pathway in the WT background.

Based on the genetic interaction between *atf-7(qd22 qd130)* and *pmk-1(km25)*, we anticipated that a screen aimed at isolating suppressors of the diminished agIs219 GFP reporter expression...
and pathogen susceptibility phenotypes of the pmk-1(km25) mutant would yield additional loss-of-function alleles of atf-7. Indeed, we isolated two more putative null alleles of atf-7, the aforementioned atf-7(qd137) allele and atf-7(qd141) (Figure 2A), from a genetic screen of 15,000 haploid genomes. The suppression of the pathogen susceptibility phenotypes of PMK-1 pathway loss-of-function mutants by atf-7 null alleles is consistent with a role for ATF-7 downstream of activated PMK-1 in the negative regulation of innate immunity in C. elegans.

### Regulation of PMK-1-regulated genes by ATF-7

We used quantitative real-time PCR (qRT-PCR) to measure changes in expression of three representative genes previously identified as being regulated by the PMK-1 pathway [22]. We observed that the atf-7(qd22) mutant, consistent with the observed effects on agIs219 GFP reporter expression (Figure 1A), exhibited sharply diminished expression of PMK-1-regulated genes relative to WT on E. coli OP50, comparable to the levels observed in the pmk-1(km25) mutant (Figure 4A). These data confirm that the observed effects of the atf-7(qd22) mutation on agIs219 expression reflect a change in the regulation of PMK-1-regulated genes.

The basal level of expression of PMK-1-regulated genes, as defined by the levels of expression of genes on the relatively non-pathogenic E. coli OP50, was comparable in the atf-7 loss-of-function mutants and WT (Figure 4B). Confirming the observations with the agIs219 transgenic reporter, the atf-7(qd22 qd130) loss-of-function allele suppressed the markedly diminished basal expression of PMK-1-regulated genes in the pmk-1(km25) mutant (Figure 4B). These data are suggestive of a role for ATF-7 in the transcriptional repression of the basal expression of PMK-1-regulated genes, with de-repression of these genes through inhibition of ATF-7 by activated PMK-1. But if ATF-7 functioned solely as a transcriptional repressor of PMK-1-regulated genes, then an increase in basal expression of these genes might be anticipated. However, the basal expression of PMK-1-regulated genes is comparable to the levels observed in WT. This observation, as well as the Esp phenotype of the atf-7 loss-of-function mutants, is suggestive that ATF-7 functions not only as a repressor of the PMK-1-regulated immune response, but as a positive regulator of innate immunity as well, and thus we sought to examine the requirement for ATF-7 in pathogen-induced gene expression.
ATF-7 in C. elegans Innate Immunity

Upon exposure to pathogen infection, a number of genes are up-regulated in a PMK-1-dependent manner [22]. We observe that genes that require PMK-1 for induction by *P. aeruginosa* PA14 also require ATF-7 for pathogen-induced expression (Figure 4B). Although the basal expression of PMK-1-regulated genes on *P. aeruginosa* PA14 (Figure 3B). The ATF-7 expression in *C. elegans* may function to relieve the transcriptional repressor activity of the corresponding mutant ATF-7 protein to PMK-1 activity, further suggesting that the phosphorylation of ATF-7 by PMK-1 may function to relieve the transcriptional repressor activity of ATF-7.

Phosphorylation of ATF-7 by PMK-1

The genetic and gene expression data above are consistent with a role for PMK-1 in the modulation of the transcriptional regulator ATF-7. Because the PMK-1 pathway acts in the intestine in a cell autonomous manner to regulate the innate immune response [21], we anticipated that ATF-7 would also be expressed in the intestine. We observed that a rescuing translational fusion of ATF-7::GFP under the control of the endogenous promoter and 3’ UTR was strongly expressed in the nuclei of intestinal cells (Figure 5).

We next sought to obtain further evidence for a direct interaction between activation of the PMK-1 pathway and ATF-7. We examined whether PMK-1 could phosphorylate ATF-7 by generating activated PMK-1 by co-expressing epitope-tagged *C. elegans* *atf-7*, pmk-1, and sekr-1 cDNAs in Cos7 cells and immunoblotting against T7-ATF-7 to detect changes in gel mobility indicative of phosphorylation. Expression of PMK-1 with SEK-1, which results in activated PMK-1, produced a shift in the T7-ATF-7 protein band indicative of a change in the phosphorylation state (Figure 6A, lane 4). This shift in the ATF-7 band is not seen when either *pmk-1* or *sekr-1* cDNAs are not expressed (Figure 6A, lanes 2 and 3), or when PMK-1 is immunoprecipitated and treated with phosphatase (Figure S8). These data are consistent with PMK-1-dependent phosphorylation of ATF-7.

We used a mutated version of PMK-1 that does not have kinase activity to establish that ATF-7 and PMK-1 physically interact. Immunoprecipitation using the T7 antibody, followed by immunoblotting using anti-HA, revealed an HA-PMK-1 (kinase-dead)-T7-ATF-7 interaction that was dependent on the activated form of PMK-1, as determined by the requirement for co-transfection of *sekr-1* cDNA (Figure 6B).

We introduced the *qd22* mutation into the T7-ATF-7 expressed in Cos7 cells and found that in contrast to the WT ATF-7, the mutant ATF-7 showed no change in gel mobility in the presence of activated PMK-1 (Figure 6A, lane 5), suggestive that the *atf-7(qd22)* allele may encode a form of the protein that can bind PMK-1 (Figure 6B, lane 3), but cannot be phosphorylated by PMK-1. The unusual nature of the *atf-7(qd22)* allele, with respect to the Esp phenotype and effects on PMK-1-regulated gene expression, coupled with the apparent insensitivity of the corresponding mutant ATF-7 protein to PMK-1 activity, further suggests that the phosphorylation of ATF-7 by PMK-1 may function to relieve the transcriptional repressor activity of ATF-7.

**ATF-7 confers immune response specificity to PMK-1 pathway activation**

In order to determine whether our observations were specific to infection by *P. aeruginosa* PA14, we examined the role of ATF-7 in pathogen resistance to two other microbial pathogens that cause lethal infections in *C. elegans*, *Serratia marcescens* and *Enterococcus faecalis*. On *S. marcescens* Db10, the *pmk-1* (km25) mutant had a weak Esp phenotype (Figure 7A) compared to the strong Esp phenotype exhibited on *P. aeruginosa* PA14 (Figure 3B). The *atf-7(qd22) qd130* mutant also had a similarly weak phenotype, as did the *atf-7(qd22) qd130; pmk-1* (km25) double mutant (Figure 7A). These data suggest that the PMK-1 pathway and ATF-7 are required for resistance to *S. marcescens*, but the comparable Esp phenotypes of single mutants and the *atf-7(qd22) qd130; pmk-1* (km25) double mutant are consistent with PMK-1 and ATF-7 functioning as positive regulators of pathogen resistance in the same pathway, or with ATF-7 under negative regulation by PMK-1 with ATF-7 functioning as a transcriptional repressor.

Interestingly, on the Gram-positive pathogen *E. faecalis* MMH594, the *atf-7(qd22) qd130* mutant does not have an appreciable Esp phenotype (Figure 7B), suggestive that ATF-7 may not serve as a positive regulator of resistance to *E. faecalis*. In
addition, the atf-7(qd22 qd130) mutation only partially suppresses the Esp phenotype of the pmk-1(km25) mutant (Figure 7B), suggestive that there are both ATF-7-dependent and ATF-7-independent mechanisms downstream of PMK-1 in response to E. faecalis. Distinct sets of genes have been observed to be induced by exposure to different bacteria, and little is known about how the transcriptional responses to Gram-positive bacteria and Gram-negative bacteria differ [28]. Of note, we observed diminished GFP expression from the agl219 transgene on Gram-positive bacteria relative to expression on E. coli OP50 (D.H.K., unpublished data). Gene expression studies of genes induced in C. elegans infection with Gram-positive bacteria and the identification of such genes that are regulated by the PMK-1 pathway may further illuminate the differences in the role of ATF-7 observed between E. faecalis and P. aeruginosa.

The PMK-1 pathway regulates the response to arsenic and oxidative stress through regulation of the transcription factor SKN-1 [29,30]. We observed that the atf-7(qd22 qd130) mutant did not exhibit enhanced sensitivity to arsenic stress in addition, did not suppress the arsenic sensitivity of the pmk-1(km25) mutant or the sek-1(km4) mutant (Figure 8). In addition, we observed that skn-1 mutants did not exhibit enhanced susceptibility to P. aeruginosa (Figure S9). Whereas PMK-1 mediates multiple responses to environmental stressors, including oxidative stress and pathogen infection, these data suggest that the transcription factor substrates of PMK-1, ATF-7 and SKN-1, confer specificity to PMK-1-mediated responses.

**Discussion**

We have described the identification and characterization of ATF-7, a *C. elegans* ortholog of the mammalian ATF2/ATF7/CREB5 family of bZIP transcription factors, as a transcriptional regulator of PMK-1-mediated innate immunity in *C. elegans*. We isolated four mutant alleles of *C. elegans* atf-7 from three different forward genetic screens. First, the gain-of-function qd22 allele was isolated from a large-scale screen for mutants with diminished PMK-1-dependent GFP reporter gene expression and an Esp phenotype. The isolation of qd22 served as a starting point for the characterization of ATFS-7, and the analysis of this unusual gain-of-function allele provided insights into the mechanism of ATF-7 regulation by PMK-1. The increased levels of PMK-1 activation in the atf-7(qd22) mutant relative to WT (Figure 1C) may reflect feedback loops that serve to counteract the suppression of the PMK-1-mediated transcriptional response by increasing levels of activated PMK-1. Although atf-7(qd22) acts as a gain-of-function allele, we determined that atf-7(qd22) is recessive with regard to pathogen susceptibility (Figure S4). We suggest that whereas the qd22 mutant ATFS-7 protein cannot be phosphorylated by PMK-1 and thus functions as a constitutive repressor (Figure S6A), the resulting protein may undergo changes in structure and folding that compromise the ability of the mutant ATF-7 to compete with WT ATF-7 at corresponding promoter sites in the atf-7(qd22)/atf-7(+) trans-heterozygote.

Based on evidence that atf-7(qd22) was a gain-of-function allele, we isolated the atf-7(qd22 qd130) allele as an intragenic suppressor...
A third genetic screen aimed at isolating suppressors of pmk-1(km25) yielded two additional putative null alleles of atf-7, qd137 and qd141. The genetic analysis of loss-of-function alleles of atf-7 allowed us to begin to address the physiological role of ATF-7 in innate immunity. Genetic interaction analysis of atf-7 mutant alleles suggests that PMK-1 negatively regulates ATF-7, which in turn functions as a negative regulator of C. elegans innate immunity to P. aeruginosa and E. faecalis. At the same time, the Esp phenotype of atf-7 loss-of-function mutants and the analysis of P. aeruginosa-induced gene expression were suggestive of a requirement for ATF-7 in the activation of the inducible innate immune response, as ATF-7 was shown to be required for the increased expression of PMK-1-regulated genes in response to P. aeruginosa infection. Interestingly, the lack of an Esp phenotype of atf-7(qd22 qd130) on E. faecalis may be suggestive of the absence of a PMK-1-regulated inducible response to E. faecalis that is regulated by ATF-7.

We showed that ATF-7 physically interacts with activated PMK-1 and undergoes PMK-1-dependent phosphorylation in mammalian cells in heterologous expression assays. Based on these data, we propose that activation of the PMK-1 pathway in response to pathogen infection results in PMK-1 phosphorylation of ATF-7, leading to a switch in the activity of ATF-7 from transcriptional repressor to an activator that facilitates P. aeruginosa-induced gene expression (Figure 9). In yeast, phosphorylation of the CREB/ATF transcription factor Sko1p downstream of the ancestral Hog1p MAPK pathway in response to osmotic stress converts Sko1p from a transcriptional repressor to an activator [31]. Our data suggest that this mode of transcriptional regulation
by MAPK activation has been conserved in *C. elegans* innate immunity. Further work may define the detailed mechanisms by which ATF-7 transcriptional control is modulated by PMK-1.

In view of the multiple substrates for p38 MAPK that have been established in mammalian cell systems and the multiple activities of p38 MAPK in mammalian innate immunity, it is perhaps surprising that loss of activity of a single transcription factor, the *C. elegans* ortholog of mammalian ATF2, is sufficient to suppress the immunocompromised phenotype caused by loss of p38 MAPK activity in *C. elegans*. Mice homozygous for a deletion of the *ATF2* gene die shortly after birth due to the lack of pulmonary surfactant [8], although analysis of cells from mutant mice expressing low levels of ATF2 are suggestive of a role for ATF2 in the modulation of cytokine activity in both activating and inhibitory activities on the immune response [32]. Interestingly, these studies also indicate a role for ATF2 in the modulation of cytokine-dependent NF-κB immune signaling was lost [33]. The mechanism of regulation of ATF-7 activity by PMK-1 may also provide insights into conserved mechanisms by which p38 MAPK modulates the activity of ATF2/ATF7 in mammalian innate immunity.

**Materials and Methods**

*C. elegans* strains

*C. elegans* was maintained and propagated on *E. coli* OP50 as described [34]. AU78, an N2-derived strain carrying the *agIs219* transgene was used as the wild-type strain [21]. CB4856 was used for single nucleotide polymorphism-based mapping [26].

Previously isolated and characterized mutants used: LG II: *nsy-l (ag3)* [10], *nsy-1(ky397)* [35], LG III: *tir-1(qd4)* [21], LG IV: *pmk-1(km25)* [36], *skn-1(zu135)* [37]. LG X: *sek-1(km4)* [10,38].

Mutants described in this study: ZD442 [*agIs219 atf-7(qd22)* III] was isolated as described below and backcrossed three times to its parental strain, AU78. ZD318 [*agIs219 atf-7(qd22 qq130)* III] was isolated as described below and outcrossed four times to wild-type strain N2. ZD39 [*agIs219 III; pmk-1(km25)* IV] was made by crossing the *agIs219* transgene from strain AU78 into *pmk-1(km25)*. ZD395 [*agIs219 III; sek-1(km4) X*] was made by crossing the *agIs219* transgene from strain AU78 into *sek-1(km4)*. ZD332 [*agIs219 atf-7(qd137) III; pmk-1(km25) IV*] and ZD402 [*agIs219 atf-7(qd141) III; pmk-1(km25) IV*] were isolated as described below. ZD332 was backcrossed three times to ZD39. ZD350 [*agIs219 atf-7(qd137) III*] was made by removing *pmk-1(km25)* from *agIs219 atf-7(qd137); pmk-1(km25)*, which had been previously backcrossed twice to ZD39, by outcrossing to N2. ZD326 [*agIs219 atf-7(qd22 qq130) III; pmk-1(km25) IV*] was made by crossing *pmk-1(km25)* into *agIs219 atf-7(qd22 qq130) and was outcrossed to *pmk-1(km25)* an additional three times. ZD340 [*agIs219 atf-7(qd22 qq130) III; sek-1(km4) X*] was made by crossing *sek-1(km4)* into *agIs219 atf-7(qd22 qq130) and was outcrossed to *sek-1(km4)* an additional three times.

**Pathogenesis assays**

Pathogenesis assays with *P. aeruginosa* PA14 [39], *S. marcescens* Db10 [40] and *E. faecalis* MMH594 [41,42] were performed as described previously with the following modifications. Single colonies of *P. aeruginosa* PA14 and *S. marcescens* Db10 were used to inoculate 3 ml cultures of Luria-Bertani (LB) broth, which were then incubated overnight at 37°C. Five microliters of the *S. marcescens* Db10 culture was used to seed standard 35-mm slow-kil assay plates, whereas five microliters of the *P. aeruginosa* PA14 culture was used to seed 35-mm slow-kil assay plates containing 0.05 mg/ml 5-fluoro-deoxyuridine (FUDR), used to prevent eggs

![Figure 6. Phosphorylation of ATF-7 by PMK-1.](image-url)
from hatching. Seeded plates were incubated at 37°C overnight and then incubated at room temperature overnight. A single colony of *E. faecalis* MMH594 was used to inoculate a 3 ml culture of brain heart infusion (BHI) broth containing 80 μg/ml of kanamycin, which was then incubated at 37°C for 5 hours. Seven microliters of culture was used to seed 35-mm BHI agar plates containing 80 μg/ml of kanamycin, which were incubated at 25°C overnight. In all pathogenesis assays, the size of the bacterial lawn was small, meaning that the culture was seeded in the middle of the plate and was not spread to the edge. For each assay, approximately 20–40 L4-staged worms were picked over to prepared plates, with 3–5 plates per strain. The sample sizes for each assay are provided in Table S1. All pathogenesis assays were conducted at 25°C. Plates were checked at regular intervals for survival and worms that did not respond to gentle prod from a platinum wire were scored as dead. Worms on *S. marcescens* plates were transferred to new plates on days 1, 2, and 3 of the assay. All strains carry the agIs219 transgene. The difference in susceptibility between *atf-7(qd22 qd130)* mutant animals and wild-type worms is significant (p<0.0001). There is no difference in susceptibility between *atf-7(qd22 qd130)* mutant animals and wild-type worms (p>0.9).

### Isolation of mutants with diminished agIs219 reporter expression and enhanced susceptibility to killing by *P. aeruginosa* PA14

Mutagenesis using ethyl methane sulfonate (EMS) was carried out following standard methods [43]. The synchronized F2 generation L1 stage larvae were plated onto NGM plates seeded

---

Figure 7. Requirement for ATF-7 in resistance to other bacterial pathogens. (A) Pathogenesis assay of L4 larval stage wild-type worms; *atf-7(qd22 qd130)* and *pmk-1(km25)* double mutant animals; and *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals, on *E. faecalis* MMH594. All strains carry the agIs219 transgene. The difference in susceptibility between *atf-7(qd22 qd130)* mutant animals and wild-type worms is significant (p<0.0001). (B) Pathogenesis assay of L4 larval stage wild-type worms; *atf-7(qd22 qd130) and pmk-1(km25)* mutant animals; and *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals, on *E. faecalis* MMH594. All strains carry the agIs219 transgene. The difference in susceptibility between *pmk-1(km25)* mutant animals and wild-type worms is significant (p<0.05 for each comparison).

Figure 8. ATF-7 does not contribute to arsenite resistance in *C. elegans*. Arsenite stress assay of L4 larval stage wild-type worms; *atf-7(qd22 qd130), pmk-1(km25), and sek-1(km4)* mutant animals; and *atf-7(qd22 qd130); pmk-1(km25) and *atf-7(qd22 qd130); sek-1(km4)* double mutant animals. Shown is the fraction of worms alive after 18 h. Error bars are standard deviation. The differences in survival between *atf-7(qd22 qd130)* mutant animals and *atf-7(qd22 qd130); pmk-1(km25)* double mutant animals, and *atf-7(qd22 qd130); sek-1(km4) double mutant animals are significant (p<0.05 for each comparison).

Figure 9. Model for the function of ATF-7 in *C. elegans* innate immunity. (A) Repression of PMK-1–regulated immune effector gene expression by ATF-7 in the absence of PMK-1 activation. (B) Activation of basal and pathogen-induced immune effector gene expression by PMK-1 phosphorylation of ATF-7, which switches ATF-7 from a repressor to an activator of transcription.
with *E. coli* OP50 and incubated for 55 hours at 20°C and subsequently sorted with a Union BioMettica COPAS Biossorter. Worms with diminished fluorescence compared to wild-type worms were directly plated onto a plate seeded with *P. aeruginosa* PA14, and incubated at 25°C. The plates were screened at 24 hours for dead worms. Following the rationale of our previously reported screen for Esp mutants [10], carcasses of dead worms were picked to individual NGM plates seeded with *E. coli* OP50, allowing the fertilized eggs inside each carcass to hatch so that the mutant strains could be recovered. In three separate screens a total of 140,000 haploid genomes were mutagenized. We note that the yield of the screen is strongly dependent on the time at which the Esp screening takes place, and that the 24 h time point represented a particularly stringent time such that siblings were rarely isolated among the mutant isolates. The early time of screening also accounts for the relatively low yield of mutants from the number of genomes mutagenized and the high specificity of isolated mutations for the PMK-1 pathway.

Single-nucleotide polymorphism (SNP)-based mapping using the *C. elegans* isolate CB4856 was performed as reported [26] with modifications utilizing SNPs that were analyzed by the DraI restriction enzyme for the rapid rough mapping of mutant isolates [25]. Once chromosomal linkage was determined, complementation testing was performed using previously isolated mutant alleles (atr-1(qd4), nsy-1(ag3) and nsy-1(ky397), sek-1(km4), and pmk-1(km25)). After assignment of the isolated alleles into complementation groups, the open reading frame of the affected gene was sequenced to identify the causative mutation in each allele. Isolates from the screen and the identified mutations are shown in Table 1. Using this approach, a single mutant allele not corresponding to previously identified mutants, qd22, was isolated. Fine mapping of qd22 was carried out using CB4856 SNP-based mapping. The location of qd22 on the left arm of LG III was in the vicinity of the agIs219 integrated transgenic array, and thus a strain carrying qd22 without agIs219 was generated and SNP mapping was carried out using the Esp phenotype of qd22. In order to facilitate interpretation of the pathogen killing assays with recombinants, a strain carrying the qd22 mutation (without the agIs219 transgene) and the CB4856-derived allele of npr-1, 215F, was utilized for crossing with CB4856 because of the enhanced susceptibility conferred by the 215F allele of npr-1 relative to the Bristol N2 background [44,45] in which qd22 was initially isolated.

**Isolation of atf-7(qd22 qd130)**

A forward genetic screen to identify suppressors of the atf-7(qd22) diminished agIs219 GFP fluorescence phenotype was carried out similarly as above. Briefly, *C. elegans* atf-7(qd22) hermaphrodites carrying agIs219 were mutagenized with EMS and synchronized larvae of the F2 generation were plated onto NGM plates seeded with *E. coli* OP50 and incubated for 55 hours at 20°C. The F2 worms were screened for GFP expression from the agIs219 transgene using a Zeiss Stereo V12 Discovery microscope with a GFP wide-band fluorescence cube. Any F2 worm with increased fluorescence compared to atf-7(qd22) was singed to a NGM plate seeded with *E. coli* OP50. Isolates with increased fluorescence were then tested for suppression of the atf-7(qd22) Esp phenotype to *P. aeruginosa* PA14 using the PA14 pathogenesis assay described above. The atf-7 coding region was then sequenced in isolates that had both increased fluorescence and diminished pathogen susceptibility.

**Isolation of atf-7(qd137) and atf-7(qd141)**

A genetic screen for suppressors of the pmk-1(km25) mutant was carried out using ZD39 as the starting strain and following a procedure as outlined for the isolation of atf-7(qd22) suppressor mutants.

**Generation of transgenic animals**

*C. elegans* genomic fosmids (Geneservice) were isolated using Qiagen Miniprep Kits following the standard protocol. Fosmid 24cA04 was injected into ZD342 at a concentration of 20 ng/μl along with 25 ng/μl of *P. aeruginosa* PA14 as a coinjection marker and 55 ng/μl of pBlueScript (Stratagene) as carrier DNA. The fluorescently-tagged atf-7 construct was generated by yeast-mediated ligated of genomic fragments generated by PCR using fosmid 25cA04 as template DNA and Phusion high-fidelity DNA polymerase (New England Biolabs). A 2279 bp genomic region, from 7474 to 30267 with respect to fosmid 25cA04, was covered in the fluorescently-tagged atf-7 construct. This construct was injected into ZD326 at a concentration of 20 ng/μl, along with 32 ng/μl of *P. aeruginosa* PA14 as a coinjection marker and 10 ng/μl of pBlueScript (Stratagene) as carrier DNA. Two independent lines carrying this construct were crossed into strain ZD318 using standard genetic techniques.

**Yeast-mediated ligation construction of atf-7::GFP**

Yeast-mediated ligation of atf-7::GFP was prepared as previously described [46]. Briefly, the 2279 bp operonic region containing the atf-7 gene was amplified in fragments ranging in size from ~1 kb to 4 kb in 8 separate PCR reactions with at least a 50 bp overlap between adjacent fragments. The gene encoding GFP was amplified from the Fire vector pPD95.75 [47]. The 9 PCR products, along with destination vector pRS426 (ATCC) digested with BamHI and XhoI restriction enzymes (New England Biolabs), were transformed into yeast strain FY2 following standard procedures. Phenol-chloroform extraction was used to isolate yeast DNA, which was then transformed into DH5α electrocompetent cells (Invitrogen) and isolated using Qiagen Miniprep Kits following the standard protocol.

**Quantitative real-time PCR**

Synchronized populations of wild-type and indicated mutant strains were grown to the L4 larval stage. For *P. aeruginosa* exposure experiments, L4 stage worms were washed onto plates seeded with *E. coli* OP50 or *P. aeruginosa* PA14 in parallel, dried, and incubated for four hours at 25°C. Samples were collected, frozen in liquid nitrogen, and stored at ~80°C before RNA extraction using TRI reagent (Ambion). cDNA was prepared with the RevertAid kit (Ambion) using oligo dT primers. The reverse transcription reaction was also performed without reverse transcriptase for each sample, and subsequent qRT-PCR on these control reactions showed that no contaminating genomic DNA was present. qRT-PCR was performed with a Mastercycler Realplex (Eppendorf) with SYBR Green detection in triplicate reactions of 20 μl. All primers were previously reported, and relative expression between samples was determined using snb-1 as a reference gene [22]. Fold change was calculated using the Pfaffl method [48]. Standardization between two biological replicates was performed as described [49].

**RNAi of atf-7**

A 771 bp segment of the atf-7 coding region, corresponding to bases 11532 to 12303 with respect to cosmid C07G2, was amplified by PCR and subcloned into the Fire vector L4440. RNAi by bacterial feeding using *E. coli* HT115 bacteria expressing either the L4440-derived atf-7 RNAi vector or the empty L4440 vector (control RNAi) was carried out as reported [50]. L4 animals
were fed on RNAi bacteria plates, and the F1 generation animals were assayed for susceptibility to *P. aeruginosa* PA14 or analyzed for GFP expression from the *agls219* transgene.

**Immunoblotting of *C. elegans* lysates**

Immunoblotting against *C. elegans* PMK-1 and activated PMK-1 (Promega) was carried out as described previously [10].

**Visualization of *agls219* GFP reporter gene expression and fluorescently-tagged atf-7**

To visualize expression of the *agls219* reporter, L4-staged worms, grown at 20°C, were picked over to normal maintenance plates and placed at 20°C overnight. Approximately 18 h, worms were mounted on 2% agarose pads and immobilized in 10 mM sodium azide. Slides were viewed using an AxiosCam HRm camera. To visualize expression of fluorescently-tagged *atf-7*, L4-staged worms, grown on NGM agar plates seeded with *E. coli* OP50 at 20°C, were mounted and imaged as described above with a Plan-Apochromat 20X/0.8 objective (Zeiss). Background intestinal autofluorescence was removed by taking a picture with the DAPI filter and subtracting the resulting picture from the image taken with the GFP filter.

**Expression in Cos7 cells, immunoprecipitation, and immunoblotting**

Cos7 cells were maintained in DMEM supplemented with 10% fetal calf serum, 100 μg/ml penicillin G and 100 μg/ml streptomycin at 37°C and 5% CO₂. Cos7 cells (1×10⁶) were plated in 6-cm dishes and transfected with a total of 6 μg DNA containing various expression vectors by using FuGENE6 (Roche). The ATF-7 expression vector contained the *atf-7c* open reading frame. After 48 h, cells were collected and washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 0.6 ml of extraction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.7 μg/ml aprotinin and 0.5% Triton X-100). Cellular debris was removed by centrifugation at 10 000×g for 5 min. Small aliquots of each cell lysate were boiled with SDS-sample buffer and were used as whole cell extracts. Remaining cell lysates were divided into 200 μl and each cell lysate was incubated with 0.5 μg of various antibodies and 10 μl protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). The immune complexes were washed five times with wash buffer (20 mM HEPES, pH 7.4, 150 mM NaCl) and then boiled with SDS-sample buffer. Phosphatase treatment was performed on immunoprecipitated samples with Lambda protein phosphatase (New England Biolabs) at 30°C for 10 minutes. Immunoblotting was performed as described previously [51].

**Arsenite stress assays**

Sensitivity of mutant strains to oxidative stress was determined using sodium arsenite. Briefly, mutant worms were grown on *E. coli* OP50. L4-staged animals were transferred to standard slow-killing plates supplemented with 5 mM sodium arsenite and 0.05 mg/ml of FUDR, seeded with concentrated *E. coli* OP50. The sample sizes for the arsenite stress assay are provided in Table S1. Stress assays were performed at 20°C. Animals were considered dead when they no longer responded to a gentle prod with a platinum wire. Statistical analysis of data was performed in Prism 5 (GraphPad) using an unpaired, two-tailed, Student’s *t*-test.

**Lifespan assays**

Strains used in the lifespan assays were maintained on *E. coli* OP50 at 20°C. Approximately 40 L4-staged worms (Day 0) were picked over to NGM plates containing 0.03 mg/ml of FUDR, seeded with *E. coli* OP50. Four to five plates for each strain were used in each experiment and plates that had become contaminated or plates in which the worms had borrowed were excluded upon the appearance of contamination/borrowing. Worms that had protruding/exploding vulvas and worms that crawled off the plate were censored. The sample sizes for each assay are provided in Table S1. Lifespan assays were performed at 20°C. Animals were considered dead when they no longer responded to a gentle prod with a platinum wire.

**Supporting Information**

**Figure S1** Lifespan of *atf-7(qd22)* and WT worms on *E. coli* OP50. Lifespan assay of L4 larval stage wild-type worms and *atf-7(qd22)* mutant animals on *E. coli* OP50. Both strains carry the *agls219* transgene. Replicate data can be seen in Figure S16. Found at: doi:10.1371/journal.pgen.1000892.s001 (0.13 MB PDF)

**Figure S2** Rescue of *atf-7(qd22)* Esp phenotype. Pathogenesis assay of L4 larval stage wild-type worms, *atf-7(qd22)* mutant animals, and three independent transgenic lines (*qdEx14*, *qdEx15*, and *qdEx16*) of *atf-7(qd22)* mutant animals carrying *fmd3* 25cA04. All strains carry the *agls219* transgene. The difference in susceptibility between *atf-7(qd22)* mutant animals and each transgenic line carrying *fmd3* 25cA04 is significant (*p<0.0001*). Replicate data can be seen in Figure S11. Found at: doi:10.1371/journal.pgen.1000892.s002 (0.13 MB PDF)

**Figure S3** *atf-7(qd22)* is a gain-of-function allele. (A) Fluorescence microscopy images of GFP expression from the *agls219* transgene in wild-type worms and *atf-7(qd22)* mutant worms each exposed to both control RNAi and RNAi of *atf-7*. (B) Pathogenesis assay comparing the effects of control RNAi and RNAi of *atf-7* on survival of wild-type worms on *P. aeruginosa* PA14. The difference in susceptibility between wild-type worms treated with control RNAi and RNAi of *atf-7* is significant (*p<0.0001*). Replicate data can be seen in Figure S11. (C) Pathogenesis assay comparing the effects of control RNAi and RNAi of *atf-7* on survival of *atf-7(qd22)* mutant animals on *P. aeruginosa* PA14. The difference in susceptibility between *atf-7(qd22)* mutant animals treated with control RNAi and RNAi of *atf-7* is significant (*p<0.0001*). Replicate data can be seen in Figure S11. Found at: doi:10.1371/journal.pgen.1000892.s003 (0.30 MB PDF)

**Figure S4** *atf-7(qd22)* confers a recessive Esp phenotype. Pathogenesis assay of L4 larval stage wild-type worms; *atf-7(qd22)* and *atf-7(qd22 qd130) mutant animals; and *atf-7(qd22)/atf-7(+)* and *atf-7(qd22)/atf-7(qd22 qd130) trans-heterozygotes, on *P. aeruginosa* PA14. All strains carry the *agls219* transgene. The differences in susceptibility between *atf-7(qd22) mutant animals and *atf-7(qd22)/atf-7(+)* trans-heterozygotes, and *atf-7(qd22 qd130) mutant animals and *atf-7(qd22)/atf-7(qd22 qd130) trans-heterozygotes are significant (*p<0.0001 for each comparison). Replicate data can be seen in Figure S12. Found at: doi:10.1371/journal.pgen.1000892.s004 (0.13 MB PDF)

**Figure S5** Expression of *atf-7::GFP* rescues the *atf-7(qd22 qd130)* Esp phenotype. Pathogenesis assay of wild-type worms, *atf-7(qd22 qd130) mutant animals, and two independent transgenic lines (*qdEx17* and *qdEx19*) of *atf-7(qd22 qd130) mutant animals carrying the *atf-7::GFP* construct under the control of the endogenous *atf-7* genomic promoter and 3′ untranslated region, on *P. aeruginosa* PA14. All strains carry the *agls219* transgene. The difference in
susceptibility between atf-7(qd22 qd130) mutant animals and each transgenic line carrying the atf-7::GFP transgene is significant (p<0.0001 for each comparison).

Found at: doi:10.1371/journal.pgen.1000892.s005 (0.13 MB PDF)

**Figure S6** Lifespan of atf-7 loss-of-function mutants and WT worms on *E. coli* OP50. Lifespan assay of L4 larval stage wild-type worms, atf-7(qd22 qd130) and atf-7(qd137) mutant animals on *E. coli* OP50. All strains carry the *agl219* transgene. Replicate data can be seen in Figure S16.

Found at: doi:10.1371/journal.pgen.1000892.s006 (0.13 MB PDF)

**Figure S7** Expression of atf-7::GFP rescues the atf-7(qd22 qd130) suppression of *pmk-1(㎞25)*. Pathogenesis assay of *pmk-1(㎞25)* mutant animals and atf-7(qd22 qd130); pmk-1(㎞25) double mutant animals, along with three independent transgenic lines (*qdEx17, qdEx18*, and *qdEx19*) of atf-7(qd22 qd130); pmk-1(㎞25) double mutant animals carrying the atf-7::GFP construct under the control of the endogenous atf-7 genomic promoter and 3′-untranslated region, on *P. aeruginosa* PA14. All strains carry the *agl219* transgene. The difference in susceptibility between atf-7(qd22 qd130); pmk-1(㎞25) double mutant animals and each transgenic line carrying the atf-7::GFP transgene is significant (p<0.0001 for each comparison).

Found at: doi:10.1371/journal.pgen.1000892.s007 (0.13 MB PDF)

**Figure S8** PMK-1 phosphorylation of ATF-7 is sensitive to phosphatase. Cos7 cells were transfected with T7-ATF-7, HA-PMK-1, and FLAG-SEK-1 as indicated. ATF-7 was immunoprecipitated with anti-T7, treated with phosphatase where indicated, and immunoblotted with anti-T7 (top). Whole cell extracts were immunoblotted with antibodies that recognize HA (middle) and FLAG (bottom).

Found at: doi:10.1371/journal.pgen.1000892.s008 (0.20 MB PDF)

**Figure S9** *skn-1* mutants do not exhibit an Eap phenotype. Pathogenesis assay of L4 larval stage wild-type worms, *skn-1(za67)* and *skn-1(za135)* mutant animals on *P. aeruginosa* PA14.

Found at: doi:10.1371/journal.pgen.1000892.s009 (0.13 MB PDF)

**Figure S10** Replicate of pathogenesis assay shown in Figure 1B. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms, atf-7(qd22) and atf-7(qd22 qd130) mutant animals.

Found at: doi:10.1371/journal.pgen.1000892.s010 (0.18 MB PDF)

**Figure S11** Replicate of pathogenesis assay shown in Figure S3B and S3C. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms treated with control RNAi and atf-7 RNAi, and atf-7(qd22) mutant animals treated with control RNAi and atf-7 RNAi.

Found at: doi:10.1371/journal.pgen.1000892.s011 (0.19 MB PDF)

**Figure S12** Replicate of pathogenesis assay shown in Figure S4. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; atf-7(qd22) and atf-7(qd22 qd130) mutant animals; and atf-7(qd22)/atf-7(+), atf-7(qd22 qd130)/atf-7(+), and atf-7(qd22)/atf-7(qd22 qd130) trans-heterozygotes.

Found at: doi:10.1371/journal.pgen.1000892.s012 (0.20 MB PDF)

**Figure S13** Replicate of pathogenesis assay shown in Figure 1D. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; atf-7(qd22 qd130) and atf-7(qd137) mutant animals; and atf-7(qd22 qd130)/atf-7(+), atf-7(qd137)/atf-7(+), and atf-7(qd22 qd130)/atf-7(qd137) trans-heterozygotes.

Found at: doi:10.1371/journal.pgen.1000892.s013 (0.20 MB PDF)

**Figure S14** Replicate of pathogenesis assay shown in Figure 3B. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; atf-7(qd22 qd130) and pmk-1(㎞25) mutant animals; and atf-7(qd22 qd130); pmk-1(㎞25) double mutant animals.

Found at: doi:10.1371/journal.pgen.1000892.s014 (0.19 MB PDF)

**Figure S15** Replicate of pathogenesis assay shown in Figure 3D. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent *P. aeruginosa* pathogenesis assays with wild-type worms; atf-7(qd22), atf-7(qd22 qd130), and atf-7(qd137) mutant animals.

Found at: doi:10.1371/journal.pgen.1000892.s015 (0.19 MB PDF)

**Figure S16** Replicate of lifespan assay shown in Figure S1 and Figure S6. Chart and bar graphs showing the LT₅₀ means, LT₅₀ standard deviations (S.D.), and sample sizes from two independent lifespan assays with wild-type worms, atf-7(qd22), atf-7(qd22 qd130), and atf-7(qd137) mutant animals.

Found at: doi:10.1371/journal.pgen.1000892.s016 (0.18 MB PDF)

**Table S1** Sample sizes for each pathogenesis, arsenite stress, and lifespan assay. Chart showing the sample sizes for each pathogenesis, arsenite stress, and lifespan assay presented in this work. Sample size does not include censored worms.

**Acknowledgments**

We thank A. W. Reinke for phylogenetic analysis of ATR-7 and discussions. We thank H. Li and M. Meiner for technical assistance. We thank the Caenorhabditis Genetics Center for strains. We thank A. Fire for providing expression vectors from which constructs for transgenic animals were derived. We thank H. R. Horvitz for providing use of the COPAS worm sorter.

**Author Contributions**

Conceived and designed the experiments: RPS DJP TK CER KCR KM NH DHK. Performed the experiments: RPS DJP TK CER KCR JKW OK NH DHK. Analyzed the data: RPS DJP TK CER KCR KM NH DHK. Contributed reagents/materials/analysis tools: KM NH. Wrote the paper: RPS DJP DHK.

**References**